Synthesis, Stability, and Cleavage of Newcastle Disease Virus Glycoproteins in the Absence of Glycosylation

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Polypeptides synthesized in Newcastle disease virus (NDV)-infected CHO cells in the absence of glycosylation were characterized. Incorporation of either [^3H]mannose or [^3H]glucosamine into NDV polypeptides was inhibited to greater than 99% by the antibiotic tunicamycin. Under these conditions, infected cells synthesized proteins which comigrated on polyacrylamide gels with the viral L protein, nucleocapsid protein, membrane protein, and a polypeptide with a molecular weight of 55,000 (P55). These cells did not synthesize polypeptides with the size of the hemagglutinin-neuraminidase (HN) protein or the fusion (F_0) protein. They did, however, synthesize new polypeptides with molecular weights of 75,000 (P75), 67,000 (P67), and 52,000 (P52). Peptide analysis revealed that P75 was a host cell protein whose synthesis is enhanced by tunicamycin. P67 corresponded to the unglycosylated HN protein, and P52 corresponded to a cleaved, unglycosylated form of F_0 . These unglycosylated forms of the glycoproteins were found to be relatively stable in infected cells. P55, previously thought to correspond to the cleaved form of F_0 , was found to be a unique viral protein which is associated with intracellular nucleocapsid structures.

Glycoproteins are polypeptides with one or several covalently linked oligosaccharide chains. The role of the sugar side chains is not clear, although it has been proposed that carbohydrate might play a role in the stability (23, 30, 31, 33) and intracellular maturation (8, 9, 18, 19, 22, 33) of glycoproteins and in the enzymatic or biological activities associated with glycoproteins (30, 31, 33).

Paramyxoviruses such as Newcastle disease virus (NDV) provide an ideal system for studying the role of carbohydrate in viral glycoproteins. NDV is an enveloped RNA virus (4) which assembles itself and buds from its host cell plasma membrane (4). The virions contain two different glycoproteins with three separate biological activities (4, 27-29). The ability to agglutinate avian erythrocytes and a neuraminidase activity reside on one protein, the hemagglutinin-neuraminidase protein (HN; molecular weight, 74,000) (11, 27). Cell fusion is mediated by the other glycoprotein, the fusion protein (11, 28, 29). This protein is synthesized as a precursor (F_0 ; molecular weight, 66,000) which is subsequently cleaved to F_1 and F_2 (molecular weights, 56,000 and 10,000, respectively) (28, 29). The biological activity of the fusion protein requires the proteolytic cleavage (28).

Tunicamycin is an antibiotic which blocks the glycosylation of many glycoproteins without causing other significant changes in cells (8, 9, 18, 19, 36, 37). This agent has been widely used

to explore the role of carbohydrate on glycoproteins as well as to study the structure of glycoprotein in the absence of sugar. To begin to study the contribution of carbohydrate to the NDV life cycle, we have characterized the effect of this glycosylation inhibitor on the synthesis, glycosylation, stability, and processing of the NDV glycoproteins. Characterization of NDV polypeptides synthesized in the absence of glycosylation also led us to discover a previously undescribed NDV protein.

MATERIALS AND METHODS

Cells and virus. CHO cells were grown as described previously (35). NDV, Australia-Victoria strain, propagated and purified as previously described (10), was obtained from M. A. Bratt.

Preparation of cell extracts. Cell extracts were prepared from CHO cells growing either in Spinner cultures or in monolayers. CHO cells (2×10^6) growing at 37°C in Spinner cultures were infected with NDV at a multiplicity of 5 PFU/cell as described previously (6) except that actinomycin D was omitted. When used, tunicamycin (gift to D. J. Tipper from G. Tamura) was added at the time of infection (1 $\mu\text{g}/\text{ml}$). For radioactive labeling with [^{35}S]methionine, cells were harvested by centrifugation 5 to 7 h postinfection, washed twice with 5 ml, and resuspended in 0.5 ml of modified Eagle minimal essential medium (MEM) minus methionine (supplemented with nonessential amino acids). [^{35}S]methionine (0.125 mCi/ml, 500 to 800 Ci/mmol; Amersham Corp) was added to infected cells, and incubation was continued at 37°C. Cells were harvested by centrifugation, resuspended in 5% su-

crose, and disrupted with a tight-fitting Dounce homogenizer.

CHO cells (2×10^6) growing in monolayers in 35-mm plates were infected with NDV at a multiplicity of 10 PFU/cell. When used, tunicamycin was added at the time of infection ($1 \mu\text{g/ml}$). For radioactive labeling with [^{35}S]methionine, monolayers were washed twice with MEM minus methionine. [^{35}S]methionine (0.1 mCi/ml) was added in 0.5 ml of MEM minus methionine, and incubation was continued at 37°C . Cells were disrupted with 1% Triton X-100.

Cells labeled with [^3H]glucosamine were washed and labeled in supplemented MEM containing $500 \mu\text{M}$ D-glucose. Cells labeled with [^3H]mannose were incubated in supplemented MEM containing $2.5 \mu\text{M}$ mannose. [^3H]glucosamine ($25 \mu\text{Ci/ml}$, 20.7 Ci/mmol ; New England Nuclear Corp.) or [^3H]mannose ($25 \mu\text{Ci/ml}$, $200 \text{ to } 400 \text{ Ci/mmol}$; Amersham Corp.) was added 5 h postinfection, and the cells were incubated for 2 h.

Cell-free protein synthesis. NDV RNA prepared as described previously (6) was used to direct protein synthesis in cell extracts derived from wheat germ (24) as has been described previously (6, 24).

Polyacrylamide gels. Polypeptides were resolved on 10, 12.5, or 15% polyacrylamide slab gels ($14 \text{ by } 12 \text{ by } 0.5 \text{ cm}$) prepared and run as outlined by Laemmli (15). The polyacrylamide gels were fixed and stained with Coomassie brilliant blue as described previously (6), dried, and subjected to autoradiography (Kodak X-ray X-Omat film).

Partial proteolysis of polypeptides. Regions of fixed, dried polyacrylamide gel containing proteins were excised, rehydrated (30 min in 0.125 M Tris, $\text{pH } 6.8$ – 0.1% sodium dodecyl sulfate [SDS]– 1 mM EDTA), and placed on top of a 15% polyacrylamide gel. Digestion of the protein (α -chymotrypsin; Boehringer Mannheim) and separation of the resulting polypeptide fragments were done as described by Cleveland et al. (5). After electrophoresis, the polyacrylamide gel was impregnated with 1 M sodium salicylate (2), dried, and exposed to preflashed (17) X-ray film.

Tryptic peptide analysis. Trypsin (Worthington Biochemicals Corp.) digestion of individual polypeptides was done as described previously (21). Tryptic peptides were resolved by paper electrophoresis at $\text{pH } 3.5$ (21).

Antibody precipitation. Cell extracts were incubated with rabbit anti-NDV (Australia-Victoria) serum, and immune complexes were precipitated with *Staphylococcus aureus*-bound protein A as described by Kesler (13), except that precipitation with *S. aureus*-bound protein A was done in the presence of 0.4% SDS, a procedure required to eliminate nonspecific binding of viral proteins to the *S. aureus* cells in the absence of antibody.

RESULTS

Synthesis of NDV proteins in the presence of tunicamycin. Radioactively labeled proteins synthesized by infected cells incubated with or without tunicamycin were resolved in a 10% polyacrylamide slab gel (Fig. 1, lanes a and b). Tunicamycin-treated, infected cells synthe-

sized polypeptides which comigrated with the NDV L protein, the nucleocapsid protein (NP), the 47,000-dalton protein, and the membrane (M) protein. In addition, these cells synthesized a polypeptide with a molecular weight of 55,000 (P55) which comigrated with the polypeptide which has been previously identified as the F_1 protein (7). These cells did not synthesize polypeptides which comigrated with the HN protein or the F_0 protein. They did, however, synthesize three new polypeptides with molecular weights of 67,000 (P67), 52,000 (P52), and 75,000 (P75).

To assess the degree of inhibition of glycosylation by tunicamycin under the conditions used, infected cells, either treated or untreated with tunicamycin, were radioactively labeled with either [^3H]glucosamine or [^3H]mannose. After incubation with either sugar in the absence of tunicamycin, polypeptides which comigrated with the HN, F_0 , F_1 , and F_2 proteins were radioactively labeled (Fig. 1, lanes c and e). Tunicamycin treatment reduced incorporation of label into these polypeptides by greater than 99% (Fig. 1, lanes d and f).

P67 protein. The polypeptide with a molecular weight of 67,000 synthesized in tunicamycin-treated, infected cells was the same size as the unglycosylated HN protein synthesized by a membrane-free, cell-free extract directed by NDV 18 to 22S mRNA (6), a result which suggests that P67 is the unglycosylated HN protein. To verify this conclusion, [^{35}S]methionine-labeled tryptic peptides derived from P67 and HN were compared by high-voltage paper electrophoresis at $\text{pH } 3.5$ (Fig. 2). The [^{35}S]methionine tryptic peptide patterns of the two polypeptides were identical. Thus, P67 must be the unglycosylated form of HN.

P52 protein. Tunicamycin-treated cells also synthesized a new polypeptide with a molecular weight of 52,000, P52. To identify this polypeptide, the [^{35}S]methionine-containing tryptic peptide pattern derived from P52 was compared with those derived from known NDV proteins. The pattern derived from P52 was identical to that obtained from F_0 (Fig. 2). Thus, P52 must be related to the fusion protein.

It should be noted that to clearly resolve P52 from a polypeptide present in infected, untreated cells, proteins must be separated on a 10% polyacrylamide gel (Fig. 1, lanes a and b). On 12.5% polyacrylamide gels, the P52 comigrated with a polypeptide present in infected, untreated cells, although the P52 unique to tunicamycin-treated cells could be detected because material in this region of the gel contained more radioactivity after tunicamycin treatment (Fig. 1, lanes g and h).

FIG. 1. Polypeptides synthesized in the presence of tunicamycin. Monolayers of CHO cells were infected for 5 h and radioactively labeled with [35 S]methionine (a, b, g, and h) for 1 h, [3 H]glucosamine (c and d) for 2 h, and [3 H]mannose (e and f) for 2 h as described in Materials and Methods. Tunicamycin was present from the beginning of infection to the end of the labeling period. Uninfected cells untreated (j) or treated with tunicamycin for 5 h (i) were labeled in parallel. Proteins present in cell extracts were resolved in 10% polyacrylamide gels (a-f) or 12.5% gels (g-j). Extracts derived from 2.0×10^4 infected cells and 1×10^4 uninfected cells were loaded onto each gel slot. Total radioactivity was: (a) 3.2×10^5 cpm; (b) 2.8×10^5 cpm; (c) 3.0×10^4 cpm; (d) 6.4×10^3 cpm; (e) 2.3×10^3 cpm; (f) 7.6×10^2 cpm; (g) 5×10^5 cpm; (h) 3×10^5 cpm; (i) 1.0×10^6 cpm; and (j) 2×10^6 cpm. The portion of the polyacrylamide slab gel containing 3 H was impregnated with 1 M sodium salicylate (4) and exposed to preflashed X-ray film (25) for 3 days. The portions of the slab gels containing 35 S were exposed to X-ray film for 5 h. Autoradiographs of 3 H-labeled material were scanned with an Ortec densitometer to quantitate the reduction in label in the glycoproteins after tunicamycin treatment.

P75 protein. Tunicamycin-treated, infected cells also synthesized a polypeptide with a molecular weight of 75,000. In contrast to P67 and P52, this polypeptide comigrated with a host cell polypeptide (Fig. 1, lanes h to j), the labeling of which increased after tunicamycin treatment of uninfected cells (Fig. 1, lane i). This polypeptide is probably a host cell protein. In support of this conclusion, antibody raised against disrupted NDV virions failed to precipitate this polypeptide (not shown), and peptide analysis of the P75 from tunicamycin-treated, infected and uninfected cells showed that the two proteins were identical (not shown).

P55 protein. On the basis of peptide mapping, P55 had been identified as the glycoprotein F₁ (7, 34). In fact, radioactively labeled sugars were incorporated into material which migrated with a molecular weight of approximately 55,000 (26; Fig. 1). Since presence of carbohydrate on protein is known to slow the migration of polypeptides on polyacrylamide gels (32), it was surprising that tunicamycin-treated cells synthesized a polypeptide which comigrated with the

glycosylated F₁. This result led us to question the identity of P55.

P55 made in the presence of tunicamycin is not a host cell protein: there was no host cell protein which precisely comigrated with P55 (Fig. 1, lanes g to j). To verify that P55 made in the presence of tunicamycin is identical to P55 made in the absence of tunicamycin, the [35 S]methionyl tryptic peptide patterns of P55 derived from both treated and untreated cells were compared and found to be identical (Fig. 2). Furthermore, the tryptic peptide pattern of P55 showed little similarity to the patterns derived from F₀, F₁, NP, or HN (Fig. 2 and 3). These results suggested that P55, made in either the presence or absence of tunicamycin, is not F₁ but is, in fact, a unique, previously undescribed NDV protein.

By analogy to other paramyxoviruses, it seemed possible that P55 might correspond to the Sendai or measles P protein which is associated with the viral nucleocapsid structure (44). To determine whether the NDV P55 is associated with nucleocapsids, infected cell extracts

FIG. 2. [^{35}S]methionyl tryptic peptide analysis of P67, P52, and P55. Cell extracts were prepared and the proteins were separated on 10% polyacrylamide gels as described in legend to Fig. 1. The regions of the polyacrylamide gel containing the polypeptides HN, P67, F_0 , P52, and P55 were excised and digested with trypsin (31). The resulting tryptic peptides were resolved by paper electrophoresis at pH 3.5 (2.5 h at 4,500 V). The paper was dried and exposed to X-ray film for 3 weeks, and the resulting autoradiogram was scanned with an Ortec microdensitometer.

FIG. 3. [^{35}S]methionyl tryptic peptide analysis of F_0 , F_1 , NP, and P55. Cells extracts were prepared and proteins present were separated on 10% polyacrylamide gels as described in legend to Fig. 1. Isolation of F_0 and F_1 is described in the text. The tryptic peptides derived from these polypeptides were resolved by paper electrophoresis at pH 3.5 (3.5 h at 4,5,000 V). The dried paper was exposed to X-ray film for 5 weeks.

radioactively labeled with [^{35}S]methionine were treated with Triton X-100 and layered onto sucrose gradients, which were centrifuged so as to resolve nucleocapsid structures (Fig. 4A). Such a gradient yielded a discrete peak of radioactiv-

ity at approximately 250S. This structure contained the NDV L and NP proteins, small amounts of M protein, but none of the viral glycoproteins, a result consistent with previously characterized nucleocapsid structures (14). In

FIG. 4. Association of P55 with nucleocapsids. A cell extract prepared from suspension cultures of infected CHO cells (5×10^6) were made 1% with respect to Triton X-100 and layered onto a 8 to 40% (wt/vol) sucrose gradient dissolved in phosphate-buffered saline (10), centrifuged at $100,000 \times g$ for 1 h. Nucleocapsids derived from vesicular stomatitis virions (35) were included as a size marker. (A) Pattern of trichloroacetic acid-precipitable material across the gradient. Fractions 8 to 10 were pooled, material present was pelleted by centrifugation, and the proteins present were resolved in 12.5% polyacrylamide gels (B). Proteins present in total cell extracts were resolved in parallel (C). The resulting autoradiograph was scanned with an Ortec microdensitometer.

addition, this structure contained the P55 protein (Fig. 4B).

Cleavage of unglycosylated F_0 . The finding that the methionyl tryptic peptide of P52 was identical to that derived from F_0 suggested that P52 corresponded to the unglycosylated F_0 protein rather than the F_0 cleavage product F_1 . However, it was found that F_0 and F_1 could not be distinguished by [^{35}S]methionine tryptic peptide analysis. Proteins present in infected cells labeled with [^{35}S]methionine for 1 h were resolved on 12.5% polyacrylamide gels in the absence of β -mercaptoethanol. Under these conditions, the disulfide bond between F_1 and F_2 remains intact and the F_1 - F_2 complex migrates with F_0 (29). The region of the fixed dried polyacrylamide gel containing F_0 -sized material was rehydrated in the presence of β -mercaptoethanol, placed on top of a 10% gel, and subjected to electrophoresis. The material in this band resolved into two species (Fig. 5, lane I); one comigrated with F_0 , and the other migrated with a molecular weight of 56,000 and was presumably the glycosylated F_1 . The patterns of [^{35}S]methionyl tryptic peptides derived from these two polypeptides (F_0 and F_1) were found to be identical (Fig. 3). Thus, the cleavage of F_0 to F_1 cannot be detected by this method. How-

ever, the [^{35}S]methionine-containing chymotryptic peptide patterns of F_0 and F_1 did show differences. The pattern derived from F_0 contained two extra peptides (Fig. 6, X and Y) not found in the pattern derived from F_1 . Furthermore, F_1 contained a peptide not found in F_0 (Fig. 6, arrow). The pattern of methionine-containing chymotryptic peptides derived from P52 was identical to that derived from F_1 , suggesting that P52 corresponds to F_1 .

Three other lines of evidence indicate that P52 corresponds to the unglycosylated F_1 rather than F_0 . First, if proteins present in infected cells were resolved on 10% polyacrylamide gels in the absence of β -mercaptoethanol, the P52 polypeptide was not detected (Fig. 5, lane D), whereas the same cell extract if reduced with β -mercaptoethanol could be shown to contain P52 (Fig. 5, lane B). This result is consistent with the idea that, under nonreducing conditions, P52 remains complexed with the unglycosylated F_2 and migrates on polyacrylamide gels with the unglycosylated F_0 .

The location of the unglycosylated F_0 or the unglycosylated F_1 - F_2 complex in the polyacrylamide gel is unclear, but it seems likely that it migrates with a molecular weight similar to that of NP or P55 under nonreducing conditions.

FIG. 5. Localization of P52 in nonreducing polyacrylamide gels. Portions of cell extracts, prepared as described in legend to Fig. 1, were suspended in gel sample buffer containing β -mercaptoethanol (A and B) or in gel sample buffer with no β -mercaptoethanol (C and D). Proteins present in cell extracts derived from untreated (A and C) or tunicamycin-treated (B and D) cells were resolved on 12.5% polyacrylamide gels. The identity of the radioactive material present in slots C and D was determined by peptide analysis (not shown). Unglycosylated HN (P67) did not enter the gel in the absence of β -mercaptoethanol. The top part and the bottom part of the NP/P55 region of the gels shown in slots C and D were excised separately and soaked for 30 min in 0.01 M Tris-hydrochloride, pH 6.9–0.5% SDS–5 M β -mercaptoethanol. The gel slices were then placed on top of a 10% polyacrylamide gel for subsequent electrophoresis. Slots E and G contain polypeptides present in the bottom part of the NP/P55 region of the nonreducing gel; slots F and H are derived from the top portion. Slots E and F contain material derived from untreated cells; slots G and H contain material derived from tunicamycin-treated cells. F_0 isolated from the nonreduced material shown in slot C was similarly reduced and electrophoresed (slot I). Results identical to these shown were obtained from three different sets of cell extracts.

FIG. 6. Chymotryptic peptide analysis of F_0 , F_1 , and P52. Peptides were prepared and resolved by paper electrophoresis as described in legend to Fig. 3 except that chymotrypsin was substituted for trypsin. The dried paper was exposed to X-ray film for 6 weeks.

Depending upon the cell extract and electrophoresis conditions, NP- and P55-sized material resolved into two, three, or four bands (Fig. 5, lanes C and D). Peptide analysis of these bands by the procedure of Cleveland et al. (5) revealed that three of these bands corresponded to NP and one corresponded to P55 (not shown). If a P52-unglycosylated F_2 complex comigrates with the NP/P55 polypeptides, then reduction of the NP/P55-sized material derived from tunicamycin-treated cells should yield the P52 polypeptide. Therefore, the NP/P55 region of a polyacrylamide gel electrophoresed in the absence of β -mercaptoethanol was excised and soaked in β -mercaptoethanol, and the material present was reelectrophoresed on a 10% polyacrylamide gel. Since the NP/P55 region of the nonreduced gel was broad, the top portion of the NP/P55 region and the bottom portion of the NP/P55 regions were rerun separately.

Upon reduction, the NP/P55-sized material derived from tunicamycin-treated cells yielded a polypeptide which comigrated with P52, whereas the NP/P55-sized material derived from untreated cells did not yield a polypeptide in this size range.

Another indication that P52 corresponds to the cleaved form of F_0 comes from cell-free translation of NDV RNA. The products of a wheat germ cell-free protein-synthesizing system directed by NDV mRNA did not contain a discrete band which comigrated with P52 (Fig. 7). It has been reported that wheat germ extracts often do not cleave polypeptides; therefore, failure to detect P52 suggests that the polypeptide is not a primary translation product.

Finally, in infected, tunicamycin-treated cells after a 5-min pulse-label with [35 S]methionine, P52 was present in small amounts, whereas it increased after a chase with nonradioactive methionine (Fig. 8). This result suggests that P52 is not a primary translation product but is cleaved from a precursor.

Stability of unglycosylated glycoproteins. To assess the stability of P67 and P52, the amounts of these proteins present in cells after a 5-min pulse-label and at various times during a chase with nonradioactive methionine were quantitated (Fig. 8). The stability of HN and F_0 was quantitated in parallel. HN decreased by only 10% after a 60-min chase, a result consistent with the finding that intracellular HN is only very inefficiently incorporated into virions (34). P67 similarly decreased by only 10%, indicating that this protein is as stable as HN. The amount of F_0 decreased during the chase period to 20 to 30% of that present after a 5-min pulse, reflecting cleavage of the protein (10). The P52

FIG. 7. Products of cell-free protein synthesis. NDV 18 to 22S mRNA was translated in wheat germ extracts as described in Materials and Methods (A). Cell extracts derived from infected cells treated with tunicamycin were prepared as described in legend to Fig. 1 (B). Proteins were resolved on a 12.5% polyacrylamide slab gel.

protein increased 70 to 80% during a chase, with kinetics of appearance which mirrored the kinetics of disappearance of F_0 . These results suggest that P52 is relatively stable in infected cells.

DISCUSSION

P55, a new NDV protein. P55 had been previously identified as the glycosylated F_1 polypeptide (7, 34). Therefore, in the absence of glycosylation it was quite surprising that the migration of this protein in polyacrylamide gels was unchanged. This result suggested that P55 was not a glycoprotein. However, to establish

FIG. 8. Kinetics of appearance of P52 and stability of P67. Suspension cultures of tunicamycin-treated CHO cells infected for 5 h were radioactively pulse-labeled with [35 S]methionine for 5 min. A chase with nonradioactive methionine was accomplished by adding MEM supplemented with 1 mM methionine. Cell extracts were prepared from portions of the culture removed after the 5-min label and after 10, 20, 40, and 60 min in MEM plus 1 mM methionine. Total trichloroacetic acid-precipitable material present at each time point was monitored to ensure the effectiveness of the chase. Protein present in each cell extract, derived from 2×10^4 cells, was resolved on a 12.5% polyacrylamide slab gel. Autoradiographs of the fixed dried polyacrylamide gel were scanned with an Ortec microdensitometer. The amount of radioactivity present in each polypeptide during the chase with nonradioactive methionine was plotted as the percentage of the amount of radioactivity present after a 5-min pulse-label. Results plotted are derived from three separate experiments.

that this polypeptide was unrelated to the known glycoproteins, it was necessary to identify it.

By [35 S]methionine tryptic peptide analysis, we have shown that P55 is likely to be a unique viral polypeptide. It is not related to F₀, to NP, nor to HN proteins. In addition, P55 is not a glycoprotein: we found that this protein migrated on nonreducing polyacrylamide gels near the NP protein, whereas all the polypeptides which could be labeled with either [3 H]mannose or [3 H]glucosamine migrated with a size of F₀ or larger (T. G. Morrison and D. Simpson, unpublished data).

Furthermore, this protein is not likely to be a membrane protein. This polypeptide is associated with a 250S structure present in detergent-treated, infected cells. This structure has a size similar to that of paramyxovirus ribonucleocapsids (14) and contains the NDV L and NP pro-

teins but little or no viral membrane proteins. Furthermore, the P55 protein is likely to be a phosphoprotein (W. Gallager, M. Peeples, and M. A. Bratt, unpublished data; G. W. Smith, and L. E. Hightower, unpublished data).

Other paramyxoviruses have polypeptides with various molecular weights which have been called P protein. This protein is a nucleocapsid-associated (4) phosphoprotein (16) thought to be involved in transcription (4). It seems likely that the NDV P55 protein is analogous.

Proteolytic cleavage and the HN polypeptide. We have shown that in the presence of tunicamycin the NDV HN glycoprotein (molecular weight, 74,000) is synthesized as an unglycosylated polypeptide with a molecular weight of 67,000.

It is of interest that the HN protein synthesized in the presence of tunicamycin comigrates with the HN synthesized in a membrane-free, cell-free protein-synthesizing system directed by NDV 18 to 22S mRNA. The signal sequence (1) present on the amino-terminal end of the vesicular stomatitis virus glycoprotein was detected when it was found that the cell-free product was slightly larger than the unglycosylated protein made in infected cells (3, 12, 20). Comparison of the sizes of the two forms of the unglycosylated NDV HN protein has not revealed any detectable size differences. Either this protein has no cleaved signal sequence or the cleavage is too small to be detected under these conditions. It is also possible but unlikely that failure to glycosylate the HN protein inhibits the cleavage of its signal sequence.

Glycosylation and cleavage of the fusion protein. Tryptic peptide analysis of the P52 protein clearly demonstrated that this protein is derived from F₀. That P52 is in fact the cleaved unglycosylated form of F₀ is indicated by four different lines of evidence.

First, whereas the [35 S]methionine-containing tryptic peptide patterns derived from F₀ and F₁ are identical, the [35 S]methionine-containing chymotryptic peptide patterns derived from F₁ and F₀ show differences which are consistent with the known cleavage of F₀ to F₁ and F₂. The chymotryptic peptide pattern derived from P52 is identical to that of F₁ rather than F₀, suggesting that P52 is a cleaved version of F₀.

A second line of evidence came from analysis of polyacrylamide gels containing unreduced NDV proteins. It has been previously reported that unreduced NDV proteins contain an F₁-F₂ complex which migrates on polyacrylamide gels with F₀ (29). Reduction of this material eliminates the disulfide bond, and F₁ and F₂ migrate on polyacrylamide gels with molecular weights of 55,000 and 10,000, respectively (29). We have

found that unreduced proteins isolated from tunicamycin-treated cells do not contain material with a molecular weight of 52,000. However, reduction of material with a molecular weight of 55,000 to 56,000 yields P52. This result is consistent with the idea that the unglycosylated F₁-F₂ complex migrates with a molecular weight of 55,000 to 56,000.

A third line of evidence for cleavage of the unglycosylated fusion protein comes from analysis of cell-free translation of NDV proteins. It has been documented in several systems that proteolytic cleavages that take place in cells often do not occur in a cell-free protein-synthesizing system: the products of such a reaction are often the primary translation products (25). Translation of NDV mRNA does not yield P52; thus this protein is likely to be the result of a cleavage which occurs in infected cells.

Last, the amount of radioactively pulse-labeled P52 in an infected cell increases after a chase with nonradioactive methionine. The appearance of P52 kinetically mirrors the decrease in the appearance of the glycosylated F₀ in untreated cells. This result argues that P52 is not a primary translation product but, in fact, the result of a proteolytic cleavage.

There is, however, no evidence at this time that the cleavage of the unglycosylated F₀ to yield P52 is precisely the same cleavage as that which generates the glycosylated F₁.

Size of unglycosylated F₀ and glycosylated F₁. If the P52 polypeptide is the result of a cleavage of the unglycosylated F₀, then the uncleaved form of this protein (F_{0UG}) has not been directly detected. Analysis of viral polypeptides on a number of different gel systems has thus far failed to resolve this polypeptide. It seems most likely that this polypeptide comigrates with NP or P55, or both, but is present in such low amounts that peptides derived from it cannot be detected in tryptic peptide analysis of NP or P55.

Similarly, the glycosylated form of F₁ has not been directly detected on 10 or 12.5% polyacrylamide gels containing all the NDV polypeptides. We have demonstrated that P55 is not likely to be the glycosylated F₁. F₁ must, however, comigrate with NP or P55 since (i) [³H]mannose or [³H]glucosamine-labeled material comigrates with NP or P55, and (ii) reduction of F₀-sized material isolated from infected cells results in a polypeptide which comigrates with NP and which contains a methionyl tryptic peptide pattern identical to that of F₀.

Stability of unglycosylated glycoproteins. The role of carbohydrate on glycoproteins is often not clear, but it has been proposed that carbohydrate addition results in a conformation

which stabilizes the polypeptide, making it more resistant to protease (23, 30, 31). However, in a pulse-chase experiment with methionine, the unglycosylated HN protein and fusion protein remained quite stable. After a 60-min chase with cold methionine, only 20 to 30% of the glycosylated F₀ found after a 5-min label was present in infected cells. However, 90% of the unglycosylated HN as well as HN remained after a 60-min chase. In addition, the amount of P52 significantly increased during a 60-min chase, suggesting that the P52 is relatively stable. Thus, carbohydrate addition seems to have little or no protective role for these two glycoproteins inside CHO cells. We have no evidence concerning the stability of these proteins in an extracellular environment.

ACKNOWLEDGMENTS

We thank Donald J. Tipper for tunicamycin. We also thank M. A. Bratt, M. Peeples, P. Chatis, L. Hightower, and C. Madansky for critical reading of the manuscript and useful discussions and Patricia Theriault for preparation of the manuscript.

This work was supported by Public Health Service grant RO1 AI 13847-02 from the National Institutes of Health.

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