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Maturation of the Envelope Glycoproteins of Newcastle Disease Virus on Cellular Membranes

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Based on subcellular fractionation data, the following maturation pathways were proposed for the Newcastle disease virus glycoproteins. During or shortly after synthesis in rough endoplasmic reticulum, hemagglutinin-neuraminidase (HN) and fusion (F_0) glycoproteins underwent dolichol pyrophosphate-mediated glycosylation, and HN assumed a partially trypsin-resistant conformation. HN began to associate into disulfide-linked dimers in rough endoplasmic reticulum, and at least one of its oligosaccharide side chains was processed to a complex form en route to the cell surface. During migration in intracellular membranes, F_0 was proteolytically cleaved to $F_{1,2}$. Neither HN nor $F_{1,2}$ required oligosaccharide side chains for migration to plasma membranes, and cleavage of F_0 also occurred without glycosylation. Virion- and plasma membrane-associated HN contained both complex and high-mannose oligosaccharide chains on the same molecule, and $F_{1,2}$ contained at least high-mannose forms. Several of the properties of HN were notable for a viral glycoprotein. The oligosaccharide side chains of HN were modified very slowly in chick cells, whereas those of the G glycoprotein of vesicular stomatitis virus were rapidly processed to a complex form. Therefore, their different rates of migration and carbohydrate processing were intrinsic properties of these glycoproteins. Consistent with its slow maturation, the HN glycopolypeptide accumulated to high levels in intracellular membranes as well as in plasma membranes. Intracellular HN contained immature oligosaccharide side chains, suggesting that it accumulated in the pre-Golgi/Golgi segment of the maturation pathway. The major site of accumulation of mature HN with neuraminidase activity was the plasma membrane.

Enveloped particles of the avian paramyxovirus Newcastle disease virus (NDV) are assembled at cellular plasma membranes (6, 30), and modified surface membranes containing viral glycoprotein may acquire hemadsorbing, neuraminidase, and cell-fusing activities (14). Previous fractionation studies of NDV-infected mammalian cells (41) and Sendai virus-infected avian and mammalian cells (34) established that the hemagglutinin-neuraminidase (HN) and fusion ($F_{1,2}$) glycoproteins follow maturation pathways to the cell surface different from those followed by the nonglycosylated viral membrane (M) protein. HN and the fusion glycoproteins are synthesized in close association with rough endoplasmic reticulum (ER) and delay in reaching plasma membranes, consistent with their migration through internal membranes; however, the M protein immediately associates with plasma membranes. The fusion glycoprotein is synthesized as a precursor F_0 which is proteolytically cleaved on internal membranes to form biologically active $F_{1,2}$.

Using these general maturation patterns as a starting point, we studied the role of carbohydrate side chains in the migration and proteolytic processing of the viral glycoproteins. The conformations of HN and $F_{1,2}$, their orientation in membranes, and the maturation state of their carbohydrate side chains were probed. Also, some information about the cellular location of the P protein (12, 40, 48) of NDV was obtained.

(Preliminary reports on parts of this work were presented at the 79th Annual Meeting of the American Society for Microbiology, Los Angeles, Calif., 4–8 May 1979, and at the ICN-UCLA Symposium on Biological Recognition and Assembly, Keystone, Colo., 4–9 March 1979 [50].)

MATERIALS AND METHODS

Growth of virus and cells. Strain AV (Australia-Victoria, 1932) of NDV was grown in 10-day-old embryonated chicken eggs (Spafas Inc.) and purified as described previously (48). The Indiana serotype of vesicular stomatitis virus (VSV) was grown in African green monkey kidney (Vero) cells and purified as described elsewhere (5). Secondary cultures of chicken embryo (CE) cells were prepared as described previously (11, 15). Confluent cultures (7.2×10^6 cells/100-mm plate) were used routinely.

Subcellular fractionation. Infected cultures were washed with a nuclear stabilizing buffer (NSB) containing 50 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Tris-hydrochloride (pH 7.4) and scraped into 3 ml of NSB containing 250 mM sucrose. The cell suspension was cooled for 10 min, equilibrated for 5 min at 250 lb/in^2 of nitrogen in a cell disruption bomb (Parr Instrument Co.), and disrupted by cavitation. The homogenate was subjected to low-speed centrifugation (1,000 × *g*) for 5 min to remove nuclei. To collect membranes, the supernatant was subjected to differential ultracentrifugation (100,000 × *g*) for 1 h in a Beckman type 65 rotor. The supernatant was removed, and the membrane pellet was resuspended in 1 ml of a low-salt buffer (LSB) containing 1 mM Tris-hydrochloride (pH 8.0) and 1 mM EDTA and then resedimented at 100,000 × *g* for 90 min. The washed membrane pellet was collected in 1 ml of 45% sucrose (wt/vol) in LSB, gently resuspended by using a Dounce homogenizer, and mixed with 2 ml of 60% sucrose in LSB to give a final concentration of 55% sucrose. A discontinuous sucrose gradient was constructed over the sample by adding sequentially 3 ml of 45% sucrose in LSB, 3 ml of 40%, 3 ml of 35%, 2 ml of 20%, and 2 ml of 25% sucrose. The gradient was centrifuged in a Beckman SW27.1 rotor for 16 h at 80,000 × *g* and collected in 0.5-ml portions. Samples were pooled into six fractions, M1 (1 to 4), M2 (5 to 10), M3 (11 to 16), M4 (17 to 23), M5 (24 to 33), and M6 (34 to 36). These pooled fractions were diluted with LSB and centrifuged at 100,000 × *g* for 30 min to sediment membranes. The supernatants were discarded, and the pellets were either dissolved in polyacrylamide gel sample buffer (48) or suspended in the appropriate assay buffer for further characterization. The bottom washes from discontinuous gradient tubes were treated in the same way.

Characterization of fractions. Isolated membrane fractions were characterized by the following methods. Total protein was measured by a colorimetric protein assay (Bio-Rad) with bovine serum albumin as the standard (9). Cell surface membranes were labeled before disruption, using ^{3}H-labeled wheat germ agglutinin (WGA; 3.7 Ci/mmol; New England Nuclear Corp.) according to the procedure of Hunt and Summers (29), and using $Na^{125}I$ in a reaction catalyzed by lactoperoxidase (Miles Laboratories) and glucose oxidase (Sigma Chemical Co.) according to the method of Hubbard and Cohn (28). Rough ER was marked by allowing cells to incorporate [5,6-^{3}H]uridine (40 Ci/mmol; New England Nuclear Corp.) into rRNA for 24 h before infection and fractionation (31). NADPH-cytochrome *c* reductase, assayed by the method of Ragnotti et al. (44), was used to locate ER. Galactosyltransferase activity, a marker for Golgi membranes, was assayed according to Fleischer et al. (22). Plaque assays of membrane fractions were performed by routine methods on CE cells at 40°C. Hemagglutination assays were done by the pattern method, using chicken erythrocytes, and neuraminidase assays were done by the thiobarbituric acid method, using fetuin as a substrate (2).

Preparation of radioactive proteins. For pulse-chase experiments, infected cultures were exposed to 25 μCi of [^{35}S]methionine (500 to 1,300 Ci/mmol; New England Nuclear Corp.) per ml of Eagle minimal essential medium containing 1% (0.15 mg/liter) of the normal methionine concentration and 2% dialyzed calf serum. After a 2-min incubation at 40°C, the radioactive medium was removed and replaced with minimal medium containing 10 times (150 mg/liter) the normal methionine concentration and 2% dialyzed calf serum. After chase periods ranging from 3 to 180 min, the cultures were washed with cold NSB and prepared for N_2 cavitation as described above. To monitor total accumulation of radioactive proteins in infected cultures, a 30-min labeling period was used with no chase.

Polyacrylamide gel electrophoresis. Radioactive proteins extracted from membrane fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 11.5% polyacrylamide slab gels, using the procedure of Laemmli (33). Electrophoresis was performed at a constant current of 20 mA for 16 h. After electrophoresis, the gels were fixed and prepared for fluorography by the method of Laskey and Mills (35). The dried gels were exposed to Kodak XR-5 X-Omat R film for 2 to 10 days at −70°C.

Endo H treatment. Endo-β-*N*-acetylglucosaminidase (endo H) digestions were performed on extracts of infected cells according to the methods of Robbins et al. (45). To treat membranes recovered from discontinuous gradient fractions, the membrane pellets were dissolved in 100 μl of H buffer (45) and processed as above. For treatment of purified virions, 2 μl of an [^{35}S]methionine-labeled virion preparation (2 mg of protein/ml) was diluted to 50 μl in phosphate-buffered saline before enzymatic digestion. [^{35}S]methionine-labeled virions were prepared as described previously (48).

Tunicamycin treatment. At 4 h postinfection, the antibiotic tunicamycin was added to a final concentration of 1 μg/ml of culture medium. At 6 h postinfection, cells were labeled for 30 min with [^{35}S]methionine and fractionated. Membrane fractions were analyzed by SDS-PAGE as described earlier.

Trypsin treatment. Membrane fractions from cells labeled for 30 min with [^{35}S]methionine were suspended in 100 μl of phosphate-buffered saline. Portions were either made 1% in Triton X-100 (scintillation grade; Eastman Kodak Co.) or mock treated. After addition of Trypsin-tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) (Worthington Biochemicals Corp.) to a final concentration of 50 μg/ml, the samples were incubated at 37°C for 1 h. The samples were mixed with equal volumes of twice-concentrated gel sample buffer containing aprotinin (10^3 U/ml; Sigma Chemical Co.) for analysis by SDS-PAGE. For treatment of virions, 2 μl of a purified preparation of [^{35}S]methionine-labeled virions (2 mg of protein/ml) was diluted to 50 μl with phosphate-buffered saline and processed as above.

RESULTS

Characterization of subcellular fractions. Various cellular membrane systems were located after fractionation, using enzymatic and radiochemical markers. Table 1 shows the distributions of these markers among the three crude fractions generated by differential centrifugation of infected cell homogenates. Most of the membrane marker activities were recovered in a pellet designated membrane fraction. The specific activities of the enzymatic markers in this

TABLE 1. Characterization of crude subcellular fractions[a]

Fraction[b]	Protein	Incorporated [^{3}H]thymidine	Bound [^{3}H]WGA[c]	Membrane-bound [^{3}H]-uridine-labeled RNA	NADPH-cytochrome *c* reductase activity	Galactosyl-transferase activity
Cytosol	70.0	0.3	5.1	12.9	19.8	4.8
Nuclear	15.3	94.7	17.9	19.7	35.9	24.9
Membrane	14.7	5.0	77.0	67.4	44.3	70.3

[a] Data are expressed as percentage of total amount in homogenate.
[b] The cytosol fraction was the 100,000 × *g* supernatant; the nuclear fraction was the pellet of the 1,000 × *g* centrifugation step; the membrane fraction was the 100,000 × *g* pellet.
[c] [^{3}H]WGA binds to *N*-acetylglucosamine moieties on glycoproteins and glycolipids (1, 24). Control experiments were done to ascertain that [^{3}H]WGA remained quantitatively bound to surface membranes when exposed to an unlabeled homogenate.

fraction were three- to sixfold higher than those in the homogenate.

The membrane fraction was separated further according to buoyant density in discontinuous sucrose gradients (Table 2). [^{3}H]WGA bound to plasma membranes and radioiodinated cell surface proteins were concentrated in low-density fraction M5. To ensure that only cell surface proteins were radioiodinated under our conditions, ^{125}I-labeled intact infected cells were solubilized, and radioactive proteins were analyzed by SDS-PAGE. As expected, the only labeled viral proteins were HN and $F_{1,2}$ (data not shown). Based on membrane densities and the distribution of markers for internal membranes, we considered that membranes in MB and M1 were derived mainly from rough ER. Fraction M2 contained a mixture of rough ER and intermediate-density membranes including Golgi membranes, as indicated by the accumulation of galactosyltransferase activity in M2. Fraction M3 also contained significant Golgi activity, whereas M4 was not highly enriched for any of our markers.

The infectivity in fraction M2 (Table 3) had the appropriate density (1.20 g/ml) for mature virions released from plasma membranes either after assembly or during homogenization. Little infectivity was associated with fraction M5, enriched in surface membranes from which virions bud. However, hemagglutinating and neuraminidase activities, presumably contributed by biologically active HN in plasma membranes, were concentrated in this fraction.

Kinetic analysis of viral proteins in membrane fractions. During the steady state of NDV infection (26, 27), a radioisotopic pulse-chase experiment was done to monitor the kinetics of association of viral proteins with cellular membranes. After each chase period, infected cells were harvested, homogenized, and fractionated. Radioactive proteins were analyzed with SDS-PAGE (Fig. 1). After a brief chase period, fraction M2 (Fig. 1a) and other dense fractions were relatively rich in HN (95% in M1 to M3) and F_0 glycoproteins, whereas fraction M5 (Fig. 1b) was enriched for M and nucleocapsid proteins (L, NP, and P). During a 60-min chase, fraction M2 (Fig. 1c) and other dense fractions lost HN (48% remained in M1 to M3), whereas M5 accumulated 38% of the HN in the gradient (Fig. 1d). After the long chase, F_0 was not detectable in any gradient fraction. However, one of its cleavage products, F_1, was detected on gels of M5 as a relatively broad band (Fig. 1d) that migrated slightly slower than the character-

TABLE 2. Characterization of discontinuous gradient fractions[a]

Fraction[b]	Density[c] (g/ml)	Protein	Bound [^{3}H]WGA (^{125}I-surface proteins)	Membrane-bound [^{3}H]-uridine-labeled RNA	NADPH-cytochrome *c* reductase activity	Galactosyl-transferase activity
MB	—	9.1	5.1 (1.0)	17.2	0.7	4.6
M1	1.214	7.3	9.4 (4.4)	32.3	21.8	10.1
M2	1.195	16.9	15.7 (15.4)	23.0	44.0	45.8
M3	1.169	11.5	9.1 (15.6)	9.7	19.1	27.7
M4	1.145	10.4	11.5 (5.4)	8.3	8.4	11.3
M5	1.108	42.4	49.0 (50.8)	7.9	5.6	0.4
M6	1.022	2.3	0.2 (7.4)	1.5	0.4	0.1

[a] Data are expressed as percentage of total amount in discontinuous gradient.
[b] Gradient fractions were pooled as described in Materials and Methods.
[c] Density of pooled fractions was determined by refractometry.

TABLE 3. Viral activities in discontinuous gradient fractions[a]

Fraction	Neuraminidase activity	Hemagglutinating activity	PFU	[35S]methionine-labeled virions[b]	
				−M	+M
MB	2.8	0.0	0.1	1.0	1.4
M1	0.9	1.3	0.9	29.8	19.4
M2	16.6	8.2	87.3	46.0	48.0
M3	3.1	4.1	3.5	13.7	14.8
M4	8.5	4.1	1.7	5.5	8.6
M5	68.0	81.8	6.5	3.1	7.1
M6	0.1	0.5	0.0	1.0	0.8

[a] Data are expressed as percentage of total amount in discontinuous gradient.

[b] M was the 100,000 × *g* pellet derived from unlabeled infected cells. The M fraction was mixed with purified radioactive virus and incorporated into the 55% sucrose layer (M1 and M2) of the gradient.

istically narrow band of P protein (Fig. 1b). Both F_1 and P were identified previously by peptide mapping (48).

When the radioactive viral proteins in membrane fractions were analyzed with SDS-PAGE under nonreducing conditions, HN was present as monomers and disulfide-linked dimers throughout the gradient (data not shown). Plasma membrane-rich fraction M5 contained predominantly dimer, the only form of HN detected on nonreducing gels of strain AV virions (48).

The M protein was the most highly localized in membranes. At the end of a 3-min chase, fraction M5 contained 95% of M in the gradient (Fig. 1b). During the chase period M protein appeared transiently in fraction M2 (probably in virions; Table 3), but by 60 min it was no longer detectable in the gradient (Fig. 1c, d).

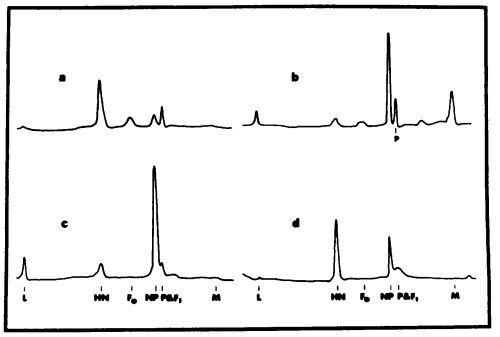

FIG. 1. Radioactive proteins from membrane fractions M2 and M5. At 6 h postinfection, cultures were exposed for 2 min to label medium containing [35S]methionine followed by either a 3- or a 6-min chase with excess unlabeled methionine. Membrane fractions were isolated and analyzed with SDS-PAGE as described in Materials and Methods. Approximately 40,000 cpm of 35S per well was loaded on slab gels in this and subsequent experiments. Fluorograms of the dried gels were scanned with a Joyce-Loebl densitometer. Representative scans of the radioactive proteins associated with M2 (a, 3-min chase; c, 60-min chase) and M5 (b, 3-min chase; d, 60-min chase) are shown.

The association of core proteins with fraction M2 (and M1) may have been gratuitous since preliminary experiments indicated that nucleocapsids isolated from virions could bind to high-density (but not low-density) membranes under our fractionation conditions (J. C. Schwalbe and L. E. Hightower, unpublished data). Free nucleocapsids with a density of 1.27 g/ml (18) sedimented to the bottom of the type of discontinuous gradient used here (see densities, Table 2).

NP was the only viral protein detected in the cytosol fraction. This result agreed with previous studies of NDV (41) and Sendai virus (34) in which 20 to 25% of total NP extracted from cells was in a high-speed supernatant but no other viral proteins were found.

Migration of HN and F on cellular membranes in the absence of glycosylation. The antibiotic tunicamycin inhibits the formation of *N*-acetylglucosamine–lipid intermediates and consequently stops dolichol pyrophosphate-mediated transfer of large oligomannosyl cores to asparagine residues on polypeptide chains (52). Treatment of CE cells for 2 to 3 h with tunicamycin at concentrations of 1 μg/ml almost completely inhibits the incorporation of radioactive mannose and glucosamine into NDV proteins without significantly affecting rates of protein synthesis (40, 50, 53).

To determine if oligosaccharide side chains were required for migration of HN and F, infected cells were either pretreated or mock treated with tunicamycin, exposed to [35S]methionine for 30 min to allow time for protein movement, and then fractionated. Radioactive proteins were separated by SDS-PAGE under reducing conditions. No proteins with the expected mobilities of glycosylated HN or F_0 were detected in tunicamycin-treated fractions (Fig. 2). However, a new 67-kilodalton form (HN_T) accumulated in all fractions, and a 52-kilodalton protein (F_T) was detected in M4 and M5. No proteins having similar mobilities were detected in untreated control fractions. These new species had

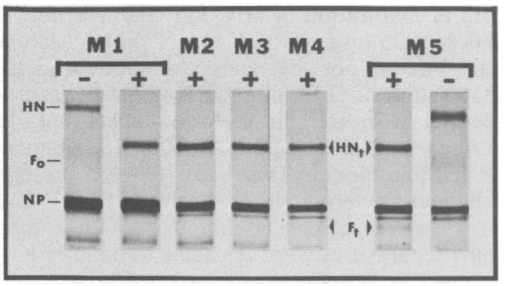

FIG. 2. Analysis of membrane fractions from tunicamycin-treated cells. Cultures were either mock-treated (−) or treated with tunicamycin (+) at 4 h postinfection and then labeled for 30 min at 6 h with [^{35}S]methionine. Membrane fractions were obtained and analyzed by SDS-PAGE. A fluorogram of the 45- to 80-kilodalton region of the dried gel is shown.

the same apparent sizes as the nonglycosylated forms of HN and F_1 extracted from tunicamycin-treated cells and identified by Morrison and Simpson (40). We concluded that unglycosylated forms of both HN and F_1 migrated from high- to low-density membranes and that cleavage of unglycosylated F_0 occurred.

Modest quantitative differences between the amount of radioactive proteins in low-density membranes from tunicamycin-treated cells and from untreated cells were found. The M5 fraction from tunicamycin-treated infected cultures contained 35% less [^{35}S]methionine-labeled protein than M5 from untreated infected cells. This difference may have been due to altered rates of migration or to reduced metabolic stability of HN_T and F_T relative to their glycosylated counterparts.

Maturation of the oligosaccharide units of HN and F. High-mannose forms of asparagine-linked oligosaccharides are cleaved by endo H between the polypeptide-proximal and second *N*-acetylglucosamine residue (3, 45, 54). However, complex oligosaccharides containing branch sugars (sialic acid, fucose, galactose, and *N*-acetylglucosamine) and only three mannose residues are resistant. Glycoproteins become endo H resistant during migration on cellular membranes, and the transferases that add branch sugars are located in Golgi membranes (10, 21, 46).

As the first step in studying the maturation of the oligosaccharide chains of HN and F, we determined the endo H sensitivities of the most mature forms of the NDV glycoproteins, those found in released virions (Fig. 3). Purified [^{35}S]methionine-labeled virions were solubilized in gel sample buffer, either treated with endo H or mock treated, and analyzed by SDS-PAGE. On reducing gels, mock-treated HN (channel a) had an apparent size of 74 kilodaltons as expected, but endo H-treated HN (channel b) shifted to 70 kilodaltons (HN_{PR}). As described later, the completely endo H-sensitive form HN_s that accumulated in infected cells was 67 kilodaltons (position marked in channel b). Therefore, we concluded that HN in virions was partially resistant to endo H, indicating that each molecule contained both high-mannose and complex oligosaccharides. A 52-kilodalton polypeptide (marked F_s? in channel b) with the expected size for unglycosylated F_1 (see F_T, Fig. 2) was detected in gels of endo H-treated samples under reducing conditions. However, the low abundance of this protein and its proximity to another protein precluded further identification.

To evaluate $F_{1,2}$ from virions, endo H-treated samples were analyzed by SDS-PAGE under nonreducing conditions. The $F_{1,2}$ protein from mock-treated samples (channel c) migrated at 65 kilodaltons as expected; however, no protein of that size was detected on gels of endo H-treated samples (channel d). A deglycosylated form of $F_{1,2}$ was not detected; however, if $F_{1,2}$ were completely sensitive to endo H, the deglycosylated form would comigrate with NP/P (17, 40).

Next, we studied the maturation of HN_s to HN_{PR} by analyzing extracts of infected cells (Fig. 4A). Cultures were exposed to [^{35}S]methionine for 5 min and then either solubilized immediately in gel sample buffer or chased with excess unlabeled methionine for 60 min and then

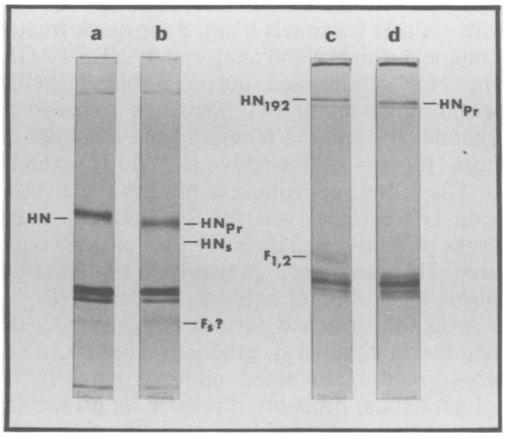

FIG. 3. Analysis of endo H-treated proteins from virions. [^{35}S]methionine-labeled, purified virions were disrupted and either mock treated (channels a and c) or treated with endo H (channels b and d) as described in Materials and Methods. The samples were then analyzed by SDS-PAGE under reducing (channels a and b) and nonreducing (channels c and d) conditions. A fluorogram of the resulting dried gel is shown. A question mark indicates a protein of uncertain identity. The position of the most sensitive form of HN, taken from an adjacent gel channel, was marked for reference. This form is not found among endo H-treated virion proteins.

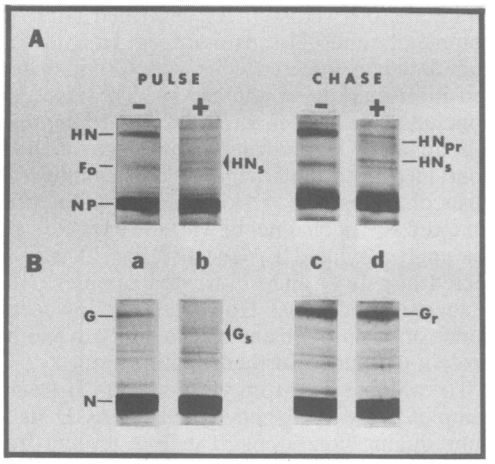

FIG. 4. Comparison of endo H-treated proteins from NDV (A)- and VSV (B)-infected cultures. At 6 h postinfection, cultures infected with either VSV or NDV were pulse-labeled for 5 min with [^{35}S]methionine followed by either no chase or a 1-h chase with excess unlabeled methionine. The cultures were solubilized, and samples of each were either mock treated (channels a and c) or endo H treated (channels b and d). The samples were then analyzed by SDS-PAGE. A fluorogram of the 45- to 80-kilodalton region of channels containing NDV proteins and the 40- to 70-kilodalton region of channels containing VSV proteins is shown.

solubilized. The extracts were either treated with endo H (channels b and d) or mock treated (channels a and c) and analyzed by SDS-PAGE. The HN synthesized during a brief labeling period (channel a) was sensitive to endo H (channel b). After a 60-min chase (channel c), most HN was still sensitive to endo H (channel d). The protein remaining in the HN region after endo H treatment was the 71-kilodalton avian stress protein (16, 25). Some loss of deglycosylated HN occurred, probably by proteolysis during incubation of extracts with endo H.

After longer chase periods HN_{PR}, barely detectable in channel d, gradually appeared. The HN_{PR} region contained multiple partially resolved bands, probably representing processing intermediates varying in sensitivity of endo H. Roughly 2.5 h of chase was required for conversion of one-half of HN_s to HN_{PR}. The deglycosylated forms of HN were identified by peptide mapping (not shown). The F_0 glycoprotein also appeared to be endo H sensitive after a 60-min chase. The protein remaining in the F_0 region after endo H treatment (channel d) comigrated with a major cellular protein.

The G glycoprotein of VSV becomes endo H resistant in CE cells with a half-time of 30 to 40 min (45). By comparison, the processing of HN_s was very slow. To determine whether the slow rate of maturation of HN was characteristic of this protein or a consequence of the physiological state of our CE cells, we monitored the maturation of G to an endo H-resistant form in parallel with the pulse-chase experiment described for NDV in Fig. 4A. After a 5-min pulse of [^{35}S]methionine (Fig. 4B), G glycoproteins (channel a) were endo H sensitive (G_s, channel b). However, after a 60-min chase, G glycoproteins (channel c) were resistant to endo H (G_R, channel d). We concluded that our CE cells were capable of rapidly processing the VSV G glycoprotein and that the relatively slow maturation of HN to partial endo H resistance was characteristic of this glycoprotein. NDV infection did not slow glycoprotein maturation in general, since G was processed rapidly in cultures mixedly infected with NDV and VSV (not shown).

The data presented in Fig. 4A indicated that the bulk of newly synthesized HN in cells was in an endo H-sensitive form after a 60-min chase. However, HN is incorporated into released virions within 30 min after synthesis (49), and virion-associated HN was partially endo H resistant (Fig. 3). These observations predict that at least some newly synthesized HN reaches the plasma membrane relatively fast. In search of rapidly maturing HN, we labeled infected cultures for 30 min with [^{35}S]methionine at 6 h postinfection. The radioactive cells were fractionated; then isolated membrane fractions were solubilized in gel sample buffer and either treated with endo H or mock treated. The samples were analyzed by SDS-PAGE, and representative fluorograms are shown in Fig. 5. HN_s was the dominant form in M1 to M3, whereas roughly equal amounts of HN_s and HN_{PR} accumulated in M4, and HN_{PR} dominated fraction M5. These data indicated that cells contain a small amount of processed HN (15% of the HN in the gradient in Fig. 5 was HN_{PR}) in low-density membranes within 30 min after synthesis.

Quantification of HN migration. Based on the experiment summarized in Fig. 1, we calculated the absolute amounts of radioactive HN recovered in rough ER fractions M1 and M2 and in plasma membrane fraction M5 after various chase periods (Fig. 6). About 85% of the radioactive HN present in fractions M1 and M2 at the shortest chase time was lost during longer chases at a constant rate (half-time, ≈30 min). The remainder in M1 and M2 may have been in membrane systems other than rough ER. The amount of HN that accumulated in fraction M5 reached a maximum between 1 and 3 h of chase. The largest amount of HN detected in this fraction was only about 20% of the HN initially present in fractions M1 and M2.

It was possible to compare the absolute levels

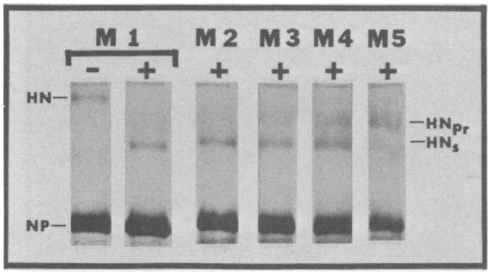

FIG. 5. Analysis of endo H-treated membrane fractions. NDV-infected cultures were labeled with [^{35}S]methionine for 30 min at 6 h postinfection. Membrane fractions were obtained and either mock (−)- or endo H (+)-treated. Radioactive proteins were analyzed with SDS-PAGE, and a fluorogram of the 50- to 80-kilodalton gel region is shown.

of HN in rough ER (M1 and M2) and plasma membrane (M5) fractions because approximately 70% of the marker activities in the original homogenate for each of these membrane systems was recovered in the crude membrane pellet (Table 1), and 50% of each of the recovered activities was localized in gradient fractions M1-M2 and M5, respectively (Table 2). Fractions containing intermediate-density internal membranes, presumably the sites of accumulation for 65% of HN, were not quantified directly because the overall recovery of these membranes was substantially lower than recoveries of rough ER and plasma membranes.

Trypsin-resistant forms of the viral proteins. The surface glycoproteins as well as internal proteins of purified [^{35}S]methionine-labeled virions were highly resistant to trypsin (Fig. 7, channels a and b). However, when the viral envelope was disrupted with Triton X-100, all of the major proteins with the possible exception of M were sensitive to trypsin (channel c). Protease-resistant fragments of 67 and 43 kilodaltons were identified as pieces of HN and NP, respectively, by peptide mapping (not shown). In the presence of SDS, trypsin digested HN_{67} and NP_{43}, indicating that both were protected by their molecular conformations.

To determine where in cellular membranes HN and NP became partially resistant to trypsin, infected cultures were labeled with [^{35}S]methionine for 30 min, and membrane fractions were prepared. Portions of each membrane fraction were mock treated, treated with trypsin alone, or treated with trypsin in combination with Triton X-100. The samples were then solubilized in gel sample buffer containing the serine protease inhibitor aprotinin and analyzed by SDS-PAGE. Representative fluorograms are shown in Fig. 7 (channels d through i). In rough ER fraction M1 (channel d), HN and NP were partially accessible to trypsin in the absence (channel e) or presence (channel f) of detergent. They were digested to trypsin-resistant fragments that comigrated with HN_{67} and NP_{43} from virions. A membrane-protected fragment NP_{45}, identified by peptide mapping, was found in M1 fraction only. Otherwise, the behavior of viral proteins in M1 was representative of those found in M2 to M4 as well.

Fraction M5 was the only membrane fraction in which the resistance of HN and NP to trypsin was similar to that of virions and intact cells. NDV glycoproteins on the surface of intact CE cells are highly resistant to trypsin. Treatment of whole cells with trypsin (1 mg/ml) for 1 h at 38°C did not cause detectable degradation of either HN or $F_{1,2}$ on plasma membranes (not shown). When fraction M5 (channel g) was exposed to trypsin (channel h), roughly one-half of all HN and NP molecules were insensitive to proteolysis. In the presence of Triton X-100, trypsin digested all detectable HN and NP molecules to their resistant cores, suggesting that they were protected originally by membranes (channel i).

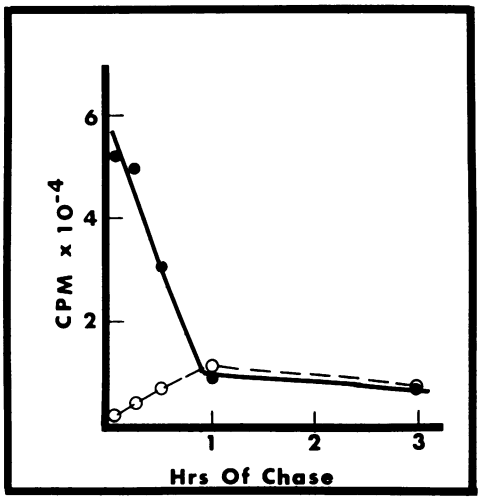

FIG. 6. Kinetics of migration of HN from dense to low-density cellular membranes. Areas under the HN peaks and under the entire scan of each channel of gels from the experiment in Fig. 1 were measured by planimetry. Background optical densities of the films were subtracted to establish baselines. The absolute amount of HN in a membrane fraction at each chase point equaled the area under the HN peak divided by the total area under all peaks in the scan, multiplied by the cpm loaded in the gel well divided by the fraction of the entire sample loaded in the well. An adjustment was made for the small amount (approximately 10% of the total cpm loaded) of low-molecular-weight radioactive material which ran off the gels. The absolute amounts of radioactive HN in membrane fractions M1 plus M2 (●) and M5 (○) after various chase times are shown.

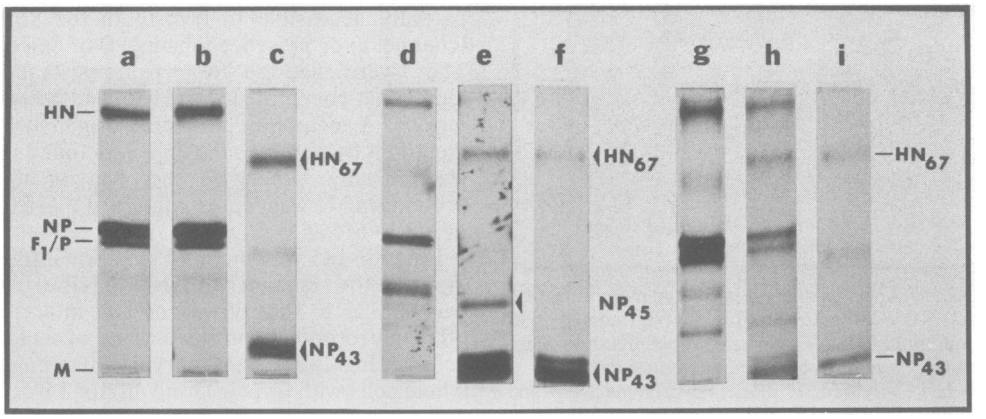

FIG. 7. Analysis of trypsin- and detergent-treated virions (channels a through c) and membrane fractions (d through i). [^{35}S]methionine-labeled, purified virions were mock treated (channel a), treated with trypsin-TPCK (channel b), or treated with trypsin in the presence of 1% Triton X-100 (channel c) as described in Materials and Methods. Infected cultures were labeled with [^{35}S]methionine for 30 min, and membrane fractions were obtained. The membrane fractions were either mock treated (channels d and g), treated with trypsin (e and h), or treated with trypsin and detergent (f and i) as described above for virions. Samples of radioactive proteins from treated virions and membrane fractions were analyzed by SDS-PAGE, and fluorograms of the 40- to 80-kilodalton region of the resulting gels are shown. Virions and membrane fractions were analyzed on separate slab gels. The results from membrane fractions M1 (d through f) and M5 (g through i) are shown.

DISCUSSION

Intracellular maturation and assembly of proteins into virions. A fundamental test of our fractionation protocol was that the kinetics of association of viral proteins with plasma membranes agree with their kinetics of appearance in released virions (49). The proteins (L, P, NP, and M) associated with plasma membrane fraction M5 met this criterion. The rapid and selective association of M with this fraction is consistent with proposed roles for M as a mediator in the recognition of modified cell surfaces by nucleocapsids during budding (4, 37; reviewed in 19). The P protein of NDV had not been characterized at the time of previous fractionations. P is associated with nucleocapsids from virions (48) and from infected cells (40). Although we did not isolate nucleocapsids in the present study, it seems likely that both the P and L proteins in our membrane fractions were components of membrane-associated nucleocapsids.

Synthesis and maturation of fusion glycoproteins. Since tunicamycin blocked mannose addition to both F_0 and HN, we inferred that dolichol pyrophosphate-mediated glycosylation of the NDV glycoproteins began in the lumen of the rough ER, probably with the transfer of the common oligosaccharide intermediate $Glc_3Man_9GlcNAc_2$ (45) to asparagine residues. Pulse-labeled fusion glycoproteins migrated from high- to low-density membranes during radioisotopic chases, and they along with HN were accessible to cell surface-selective radioiodination. Carbohydrate side chains were not required for the migration and cleavage of F_0 in intracellular membranes, since polypeptides with the expected size of unglycosylated F_1 accumulated in low-density membranes from tunicamycin-treated cells. Whether cleavage of unglycosylated and glycosylated F_0 occurs at the same position on the polypeptide chain remains to be determined.

We cannot rule out the possibility that $F_{1,2}$ contained complex chains in addition to high-mannose units because the deglycosylated form was not resolved from other viral proteins under our conditions of SDS-PAGE. A previous analysis of Sendai virions produced in embryonated chicken eggs showed that the Sendai fusion glycoprotein contained only complex chains (32). Another intriguing and possibly related difference between these viruses is that proteolytic cleavage of the Sendai F_0 is primarily an extracellular event, whereas NDV F_0 undergoes intracellular cleavage (47).

Maturation of the HN glycoprotein. HN accumulated in rough ER as partially trypsin-resistant (HN_{67}), disulfide-linked dimers. Therefore, it is possible that HN acquires these properties during or shortly after synthesis in the lumen of the rough ER. En route to the cell surface, one or more of the oligosaccharide chains on HN was processed to a partially endo H-resistant form, suggesting that HN passed through Golgi membranes. The migration of the VSV G protein through Golgi membranes and to the plasma membrane was demonstrated directly in a recent

ultrastructural study (7). Previously this maturation route for G had also been inferred from its acquisition of endo H resistance, presumably through the activity of Golgi-based sugar transferases. Like the NDV fusion glycoprotein and also the HA glycoprotein of influenza virus (42), HN did not require oligosaccharide side chains for migration to the plasma membrane. Based on current theories about glycoprotein conformation and intracellular migration (13, 23), we suggest that carbohydrate does not strongly affect the conformations of the polypeptide domains involved in migration of the NDV glycoproteins. In contrast, glycosylation is required for the formation of biologically active HN (39).

The pattern of endo H resistance of HN indicated the presence of both complex and high-mannose oligosaccharide side chains in the same molecule. Previously the HN of Sendai virus produced in embryonated chicken eggs was shown to have both types of chains (32). In another study of paramyxoviral oligosaccharides, an unusual type of low-mannose oligosaccharide carrying aldehydes in a terminal linkage was found on simian virus 5 glycoproteins produced by MDBK cells (43). This type of oligosaccharide was found characteristically on both cellular and viral glycoproteins synthesized by these cells.

The kinetics of migration of HN had several notable features. Both radioisotopic pulse-chase and long-accumulation studies indicated that internal as well as plasma membranes were major sites of accumulation of HN. Although HN left rough ER with a half-time of about 30 min, it acquired partial endo H resistance relatively slowly ($t_{1/2}$ = 2.5 h), and the partially endo H-resistant form accumulated in plasma membrane fraction M5. Therefore, it is likely that most intracellular HN accumulated either in pre-Golgi intermediate-density membranes such as transitional ER or in Golgi membranes where it may have been processed slowly. Recently, Strous and Lodish (51) reported that the secretory protein transferrin is externalized more slowly than the G glycoprotein in VSV-infected rat hepatoma cells. The differences in transit appeared to be in the rough ER to Golgi segment of the maturation pathways. Our evidence suggests that different rates of migration of two intrinsic membrane glycoproteins HN and G also involves this part of intracellular migration.

A second unexpected finding was that maximal levels of HN were attained in plasma membrane fraction M5 at a time when most newly synthesized HN was still in intracellular membranes. Other experiments similar to the one shown in Fig. 6 but covering different chase intervals supported our interpretation that the accumulation of HN in fraction M5 displayed saturation kinetics and reached a maximal level by 1 h of chase. Additional studies will be needed to determine whether the saturation level of HN in plasma membranes is a static level contributed by a small pool of rapidly migrating HN or whether it is a dynamic steady-state level. We favor the latter alternative. We suspect that the relatively slow maturation of HN is balanced at this level by the loss of HN from the cell surface. We know from previous studies (27) and unpublished observations that neither the incorporation of HN into virions nor shedding of HN into the extracellular medium could account for more than a small fraction of the loss of HN from the cell. Thus, if a substantial amount of HN is lost from the surface of infected cells, endocytosis is the most likely route. It is also possible that HN is sequestered on a region of the cell surface that was not included in fraction M5.

Whereas HN molecules accumulated both intracellularly and in plasma membranes, our fractionation studies showed that biologically active HN accumulated mainly in plasma membrane-rich fraction M5. Merz et al. (38) recently proposed that viral neuraminidase may be expressed before arrival of HN at the plasma membrane. If this hypothesis is correct, then it follows from our studies that active HN moves rapidly to the cell surface and does not accumulate intracellularly.

Nitrogen cavitation causes extensive vesicularization of cellular membranes (55). Our fractionation procedure was carried out in the cold and with EDTA-containing buffers to minimize redistribution of membrane components by aggregation and vesicle fusion. Vesicles derived from rough ER generally retain a native orientation (20), whereas those from plasma membranes are thought to be a mixture of native and inside-out vesicles (56). Our results are consistent with the interpretation that at least some of the plasma membrane-derived vesicles in fraction M5 retained a native orientation capable of causing hemagglutination. We could not rule out the possibility that intracellular HN had hemagglutinating activity, since the receptor-binding portions of HN could have been inside vesicles derived from internal membranes. However for neuraminidase activity, detergent-permeabilization of cellular membranes did not reveal additional activity.

Fraction M5 was also the only fraction that contained significant amounts of completely trypsin-resistant HN. We consider this to be the most mature cell-associated form of HN, probably comparable to the HN spikes on virions in biological activity and morphology. Our results are consistent with several previous studies which suggest that the final maturation of HN

into morphologically distinguishable spikes occurs on the cell surface (41; reviewed in 14). We were not able to obtain information on possible transmembrane orientations of HN or $F_{1,2}$, since trypsin treatment of most membrane fractions yielded similar results in the presence or absence of detergent. The most probable interpretation of this result is that our membrane vesicles were permeable to trypsin. In light of the findings by Lyles and co-workers that both HN and F in Sendai virions (36) and infected cell surfaces (8) contain 1- to 2-kilodalton segments which span the lipid bilayer, it seems unlikely that the 7-kilodalton fragment removed from the NDV HN in isolated membranes by trypsin represented a transmembrane segment. However, a more interesting interpretation of our results is that immature HN has a larger segment exposed on the cytoplasmic side of internal cellular membranes than the mature forms located in plasma membranes and virions.

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