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Fatty Acid Modification of Newcastle Disease Virus Glycoproteins

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The fatty acid acylation of Newcastle disease virus hemagglutinin-neuraminidase and fusion glycoproteins was assayed. [^{3}H]palmitate label was associated with cytoplasmic fusion proteins (F_0 and F_1) and virion-associated F_1. In contrast, there was no detectable [^{3}H]palmitate label associated with the hemagglutinin-neuraminidase protein in Newcastle disease virus-infected Chinese hamster ovary cells or chicken embryo cells or in virions released from these cells. Thus, fatty acid modification may not be important for the maturation of some glycoproteins.

Many enveloped RNA viruses are assembled at and bud from the infected cell surface. The components of these viruses, the membrane proteins and the ribonucleoprotein core, migrate to the plasma membrane via different pathways (4, 13, 28). The pathway that results in insertion of viral glycoproteins into plasma membrane is complex and involves extensive posttranslational modification, including proteolytic cleavage, glycosylation, and sulfation (12, 17). Recently, it has been demonstrated that several viral glycoproteins are modified in a fourth way, fatty acid acylation. Schmidt and Schlesinger (25) have demonstrated that each glycoprotein of vesicular stomatitis virus (VSV) is modified by one to two molecules of fatty acid which become covalently attached to the protein during the late stages of maturation. Schmidt and Schlesinger (24) have also demonstrated that fatty acid is bound to the Sindbis virus glycoproteins, E_1 and E_2, and noted unpublished results which suggest that the influenza virus hemagglutinin glycoprotein is similarly modified.

The precise role(s) for fatty acid modification of proteins has not as yet been demonstrated. Schmidt and Schlesinger (24–26) have suggested that fatty acid attachment may modify the protein's conformation or may anchor a transmembrane protein in the membrane. Also, Zilberstein et al. (29) have reported that, at the nonpermissive temperature, the glycoprotein of a group V temperature-sensitive mutant of VSV migrates to the Golgi membranes, where its high-mannose oligosaccharides are trimmed. However, at the nonpermissive temperature, fatty acid addition does not occur, and the VSV glycoprotein does not reach the cell surface. On the basis of these data, Schlesinger et al. (23) have suggested that fatty acid acylation may be essential for glycoprotein transport to the plasma membrane.

We examined fatty acid modification of another enveloped RNA virus, Newcastle disease virus (NDV). This paramyxovirus has two viral glycoproteins, the hemagglutinin-neuraminidase protein (HN; molecular weight, 74,000) and the fusion protein (F_0; molecular weight, 66,000) (4, 11, 20, 21). F_0 is cleaved to F_1 and F_2 (molecular weights, 56,000 and 10,000, respectively) (21, 22). The cleaved fusion protein is the biologically active form of the glycoprotein (21, 22).

We present evidence that the NDV glycoprotein HN is unlike the other glycoproteins previously examined (23–26). There appears to be no fatty acid modification of this viral glycoprotein. The fusion protein does contain fatty acid.

To ensure that fatty acid acylation was occurring in our experimental system, VSV-infected Chinese hamster ovary (CHO) cells were labeled with [^{3}H]palmitic acid, and the proteins in these extracts were analyzed by polyacrylamide gel electrophoresis. As previously reported, there is only one detectable [^{3}H]palmitic acid-labeled polypeptide found in VSV-infected cells (25, 26; Fig. 1A, lane 2). This polypeptide comigrates with the glycoprotein from [^{35}S]methionine-labeled VSV-infected cells (Fig. 1A, lane 1).

To determine whether the NDV glycoproteins are modified by the addition of fatty acid during maturation, [^{3}H]palmitic acid-labeled cytoplasmic extracts from NDV-infected CHO cells and NDV-infected chicken embryo (CE) cell (9) extracts were analyzed by polyacrylamide gel electrophoresis (Fig. 1A, lanes 4 and 6, respectively). Uninfected cells were radioactively labeled in parallel (Fig. 1A, lanes 3 and 7). Both infected cell extracts contained polypeptides which comigrated with the F_0 and F_1 proteins, whereas no radioactively labeled polypeptides of F_0 or F_1 size were found in uninfected cell extracts. In addition, under nonreducing conditions, infect-

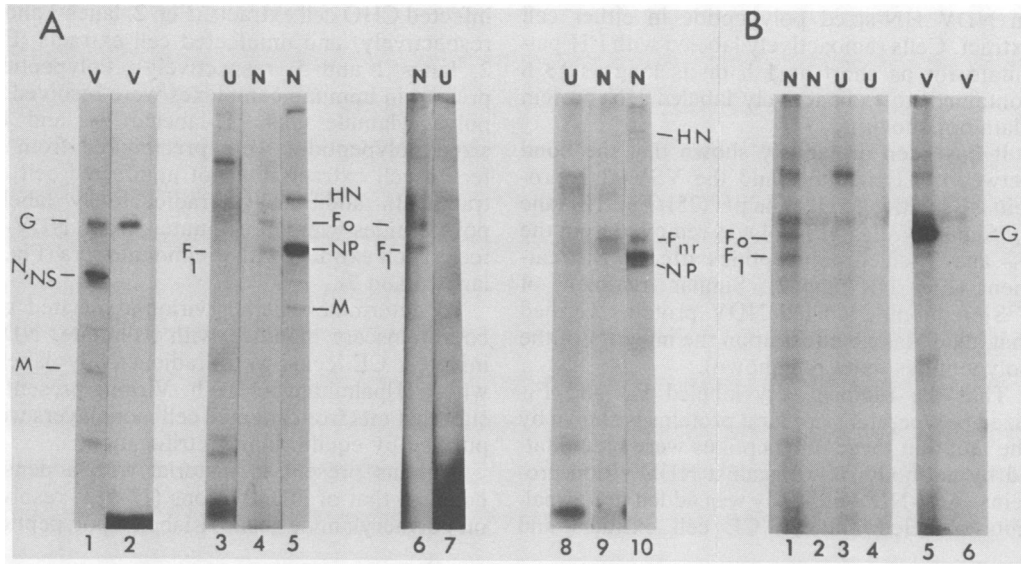

FIG. 1. Polyacrylamide gel electrophoresis of NDV and VSV cell-associated polypeptides. Confluent monolayers (2×10^6) of CHO or CE cells were infected with virus at a multiplicity of 5 PFU per cell. The VSV prototype strain was obtained from Donald Summers. NDV (Australia-Victoria) was obtained from M. Bratt. For labeling with [^{35}S]methionine, the medium was removed after 4 h of incubation at 37°C, and the monolayers were washed three times with methionine-free minimal essential medium supplemented with nonessential amino acids and 7.5% dialyzed fetal calf serum. [^{35}S]methionine (20 μCi/ml, 500 Ci/mmol; Amersham Corp.) was added to the monolayers. For labeling with [^{3}H]palmitic acid, 9,10-[^{3}H](*N*)-palmitic acid (23.5 Ci/mmol; New England Nuclear Corp.) in 80% ethanol was desiccated in a glass tube. Dialyzed fetal calf serum was added to the tube, sonicated for 30 s, and then placed at 37°C for 30 s. This procedure was repeated three times. The sonicated fetal calf serum containing the [^{3}H]palmitate was added to supplemented minimal essential medium at a final concentration of 7.5% fetal calf serum and 100 μCi of [^{3}H]palmitic acid per ml. At 4 h after the beginning of infection at 37°C, the monolayers were washed three times with serum-free minimal essential medium supplemented with nonessential amino acids. To each culture, 1 ml of the above [^{3}H]palmitate labeling media was added, and labeling was continued for 4 h. After incubation, medium from both the [^{35}S]methionine- and [^{3}H]palmitate-labeled cell cultures was removed. The monolayers were washed once with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride [pH 7.4]) and then each monolayer was lysed in 0.2 ml of NET–1% Triton X-100. After addition of NET-Triton, the plates were scraped with a rubber policeman, and the resulting cell extract was blended on a Vortex mixer. Samples (10 μl) were then analyzed on 10% polyacrylamide gels as described by Laemmli (14). (A) Autoradiograms of polyacrylamide gels containing VSV-infected cell extracts, NDV-infected cell extracts, and uninfected cell extracts. Lane 1, [^{35}S]methionine-labeled VSV-infected CHO cell extracts; lane 2, [^{3}H]palmitate-labeled VSV CHO cell extracts; lane 3, [^{3}H]palmitate-labeled uninfected cell extracts (reduced); lane 4, [^{3}H]palmitate-labeled NDV-infected CHO cell extracts (reduced); lane 5, [^{35}S]methionine-labeled NDV-infected CHO cell extracts (reduced); lane 6, [^{3}H]palmitate-labeled NDV-infected CE cell extracts (reduced); lane 7, [^{3}H]palmitate-labeled uninfected CE cell extracts (reduced); lane 8, [^{3}H]palmitate-labeled uninfected CHO cell extracts (nonreduced); lane 9, [^{3}H]palmitate-labeled NDV-infected CHO cell extracts (nonreduced); lane 10, [^{35}S]methionine-labeled NDV-infected CHO cell extracts (nonreduced). The polyacrylamide gels containing ^{3}H-labeled protein were impregnated with 1 M sodium salicylate as described by Chamberlain (2). Lane 2 was exposed to pre-flashed film for 24 h. Lanes 3, 4, 6, 7, 8, and 9 were exposed to pre-flashed film for 5 days. Lanes containing [^{35}S]methionine were exposed to film for 8 h. (B) Autoradiograms of polyacrylamide gels containing [^{3}H]palmitate-labeled NDV- and VSV-infected cell extracts treated with alkali. [^{3}H]palmitate-labeled NDV-infected, uninfected, and VSV-infected CHO cell extracts were treated with alkali as described previously by Schmidt and Schlesinger (25). Lane 1, untreated NDV-infected CHO cell extracts; lane 2, alkali-treated NDV-infected CHO cell extracts; lane 3, untreated uninfected cell extracts; lane 4, alkali-treated uninfected cell extracts; lane 5, untreated VSV-infected CHO cell extracts; lane 6, alkali-treated VSV-infected CHO cell extracts. Abbreviations: V, VSV; N, NDV; U, uninfected.

ed cell extracts contained a polypeptide which comigrated with the F_0 and the F_1-F_2 complex (F_{nr}) (Fig. 1A, lane 9), whereas uninfected cell extracts contained no polypeptides of this size (Fig. 1A, lane 8). No ^{3}H-labeled polypeptides the size of F_2 were detected. In addition, the amount of label in F_0 and F_1 was equivalent to the amount in F_{nr}, suggesting that F_2 is not modified by fatty acid.

Significantly, there was no ^{3}H label found in

an NDV HN-sized polypeptide in either cell extract. Cells radioactively labeled with [^{3}H]palmitate for as short as 1 h or as long as 18 h contained no radioactively labeled HN protein (data not shown).

It has been previously shown that the bond between [^{3}H]palmitate and the VSV glycoprotein is sensitive to alkaline pH (25) (Fig. 1B, lane 6). Similarly, the ^{3}H label was removed from the F_0- and F_1-sized polypeptides after alkali treatment (Fig. 1B, lane 2). Similar treatment of [^{35}S]methionine-labeled NDV proteins verified that alkali has no effect upon the integrity of the polypeptides (data not shown).

That the radioactively labeled F_0- and F_1-sized polypeptides are viral proteins is shown by the fact that these polypeptides were precipitated by antibody raised against NDV virion proteins. Anti-NDV antibody was added to [^{3}H]palmitate-labeled infected CE cell extracts and infected CHO cell extracts (Fig. 2, lanes 3 and 4, respectively) and uninfected cell extracts (Fig. 2, lanes 2 and 5, respectively). Polypeptides present in immune complexes were resolved on polyacrylamide gels. ^{3}H-labeled F_0- and F_1-sized polypeptides were precipitated from infected cell extracts but not uninfected cell extracts. In addition, no radioactively labeled polypeptides were precipitated from NDV-infected cell extracts with preimmune sera (Fig. 2, lanes 6 and 7).

To determine whether virion-associated glycoproteins are modified with palmitate, NDV-infected CE cells were radioactively labeled with [^{3}H]palmitate for 18 h. Virions present in supernatants from infected cell monolayers were purified by equilibrium centrifugation.

Proteins present in material with a density equal to that of NDV virions (1) were resolved on polyacrylamide gels. ^{3}H-labeled polypeptides

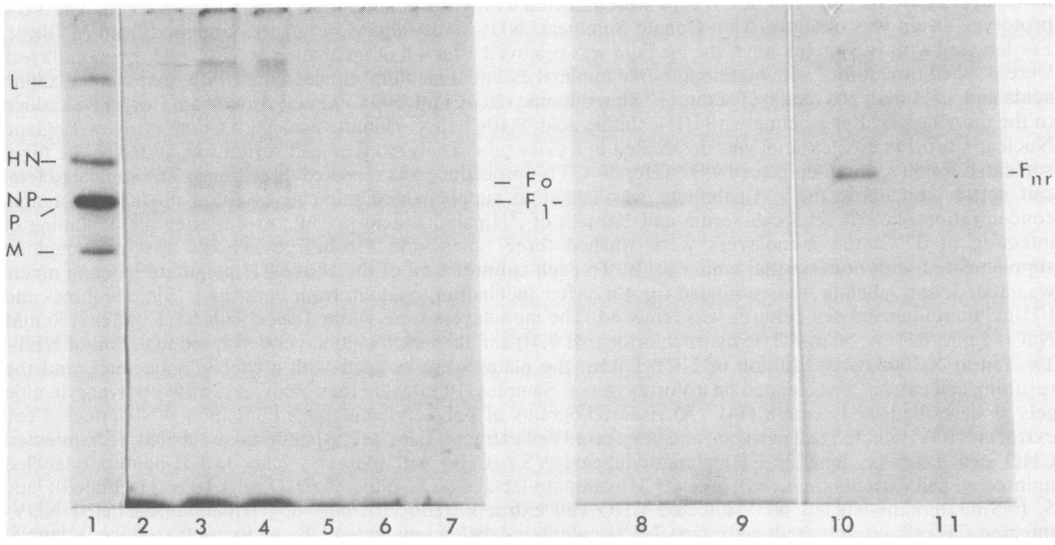

FIG. 2. [^{3}H]palmitate-labeled NDV proteins present in immune complexes and in virions. Infected and uninfected CE or CHO cells were labeled with [^{3}H]palmitate as described in the legend to Fig. 1. The resulting cell extracts were incubated with rabbit anti-NDV antiserum or preimmune sera, and immune complexes were precipitated with Immunobeads (Bio-Rad Laboratories) to which goat anti-rabbit antisera were coupled (3). Before use, Immunobeads were washed once in NET containing 1% Nonidet P-40 and 5 mg of bovine serum albumin per ml. Immune complexes were precipitated and washed in the presence of 0.04% sodium dodecyl sulfate. Immune complexes were released from the Immunobeads by boiling in 6 M urea and 6% sodium dodecyl sulfate. Precipitated proteins were resolved on 10% polyacrylamide gels, impregnated with sodium salicylate, and exposed to pre-flashed film for 7 days. Lane 1, [^{35}S]methionine-labeled, NDV-infected cell extracts (2-h label; thus, amounts of F_0 are quite low); lane 2, uninfected CE cell extracts immunoprecipitated with anti-NDV sera; lane 3, NDV-infected CE cell extracts immunoprecipitated with anti-NDV sera; lane 4, NDV-infected CHO cell extracts immunoprecipitated with anti-NDV sera; lane 5, uninfected CHO cell extracts immunoprecipitated with anti-NDV sera; lane 6, infected CE cells immunoprecipitated with preimmune sera; lane 7, infected CHO cell extracts immunoprecipitated with preimmune sera; lane 8, virion-associated [^{3}H]palmitate-labeled protein (reduced); lane 9, [^{3}H]palmitate-labeled material released from uninfected cells with a density of 1.9 g/cm^3 (reduced); lane 10, virion-associated [^{3}H]palmitate material (nonreduced); lane 11, [^{3}H]palmitate-labeled material released from uninfected cells with a density of 1.9 g/cm^3 (nonreduced). The apparent increase in radioactivity in the F_{nr} polypeptide over F_1 is a loading artifact and does not reflect an increase in modification of the nonreduced polypeptide.

which comigrated with F_1 (Fig. 2, lane 8) or the F_1-F_2 complex (Fig. 2, lane 10) were present in this material. Uninfected cells released no labeled fusion protein-sized polypeptides. Again, no label was found in an HN-sized polypeptide. This result further verified that the ^{3}H-labeled F_1- and F_{nr}-sized polypeptides were indeed viral proteins. Furthermore, typical of the fusion protein, cell extracts contained both labeled F_0 and the cleaved form of the fusion protein F_1, whereas virions contained only the cleaved forms of the polypeptide (21).

In an attempt to roughly estimate the degree of fatty acid acylation of the NDV glycoproteins, we calculated the molar ratios of intracellular VSV and NDV glycoproteins in parallel infections of CHO cells (Fig. 3, lanes 1 through 4). Given the assumption that the number of methionine residues in the VSV glycoprotein is comparable to the number of methionines in the

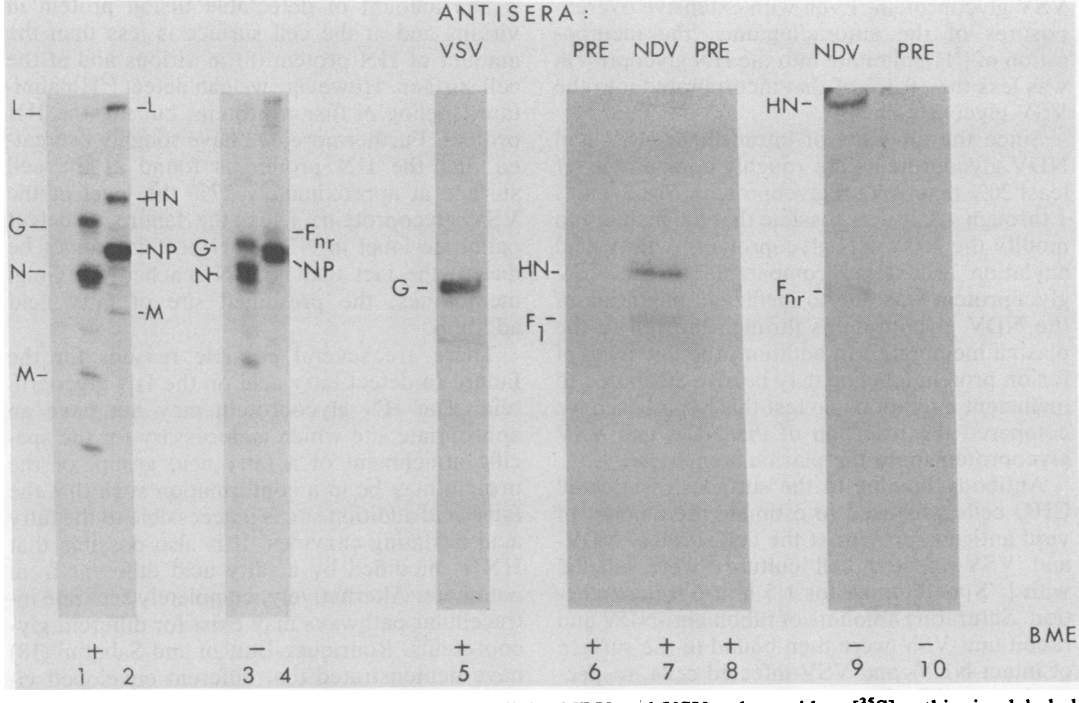

FIG. 3. Comparison of cell surface and intracellular NDV and VSV polypeptides. [^{35}S]methionine-labeled VSV-infected and NDV-infected CHO cell extracts were prepared as described in the legend to Fig. 1. Samples corresponding to equivalent numbers of infected cells were electrophoresed on polyacrylamide gels under reducing (lanes 1 and 2) or nonreducing (lanes 3 and 4) conditions. The resulting polyacrylamide gel was exposed to pre-flashed film for 5 h. The autoradiographs were scanned with an Ortec microdensitometer to quantitate the amount of label in the VSV glycoprotein, NDV HN, and NDV fusion (F_{nr}) proteins. Similar results were obtained from films exposed for 2 and 9 h. To calculate molar ratios, the values obtained for VSV glycoprotein were divided by 10 (the number of methionine residues determined by direct sequencing [19] and assuming that the glycoprotein of the prototype strain has the same number of methionine residues as the San Juan strain). Values obtained for the NDV HN and F_{nr} were divided by 10 (6, 16) and 9 (6, 16), respectively. To compare the amounts of cell surface glycoproteins, NDV- and VSV-infected CHO cell monolayers (2×10^6) were labeled with [^{35}S]methionine (25 μCi/ml) for 1.5 h at 6 h postinfection. Excess heat-inactivated rabbit anti-NDV and rabbit anti-VSV were bound to the surface of intact NDV- and VSV-infected cells, respectively, at 4°C. After 60 min, excess antibody was washed away in ice-cold phosphate-buffered saline (6). The cells were then lysed with NET containing 1% Triton X-100, and immune complexes were precipitated with Immunobeads to which goat anti-rabbit antisera were coupled (Bio-Rad Laboratories) as described previously (2). Preliminary experiments determined the amount of Immunobeads necessary to precipitate all immune complexes in the cell extract. The immunoprecipitated polypeptides from 10^6 cells were suspended in gel sample buffer and subjected to electrophoresis on 10% polyacrylamide gels as described previously (2). The figure shows an autoradiogram of a fixed, dried gel (5) containing cell surface radiolabeled glycoprotein (lane 5) and radiolabeled NDV proteins analyzed under reducing and nonreducing conditions (+ and − BME, respectively) (lanes 7 and 9, respectively). No radioactive VSV (lane 6) or NDV (lanes 8 and 10) polypeptides were precipitated with preimmune antisera. The molar ratios of the three glycoproteins were determined as described above. Similar results were obtained in three separate experiments.

NDV HN and in the NDV F_0, the ratio of VSV glycoprotein molecules to fusion protein molecules is 4.8:1, and the ratio of VSV glycoprotein molecules to HN protein molecules is 2.4:1. This assumption is valid, since estimates of the number of methionine residues in each protein from published peptide maps are roughly comparable (6, 16, 19). The labeling of the fusion protein (F_0 and F_1) with palmitate is only 6% that of VSV glycoprotein. Therefore, on a molar basis, [^{3}H]palmitate labeling of total cytoplasmic fusion protein is 24.8% the level of labeling in VSV glycoprotein. Even with extensive overexposures of the autoradiograms, the incorporation of [^{3}H]palmitate into the HN glycoprotein was less than 0.1% of that incorporated into the VSV glycoprotein.

Since the amounts of intracellular VSV and NDV glycoproteins are roughly comparable (at least 20% that of VSV glycoprotein [Fig. 3, lanes 1 through 4]), it was possible that the inability to modify the NDV HN glycoprotein by fatty acid acylation at a level comparable to the VSV glycoprotein was due to inefficient migration of the NDV glycoproteins through the cell to the plasma membrane. In addition, the low level of fusion protein labeling may be also attributed to inefficient migration. To test this hypothesis, we compared the insertion of the NDV and VSV glycoproteins into the plasma membrane.

Antibody binding to the surfaces of infected CHO cells was used to estimate the amount of viral antigens present at the cell surface. NDV- and VSV-infected cell cultures were labeled with [^{35}S]methionine for 1.5 h at 6 h postinfection. Saturating amounts of rabbit anti-NDV and rabbit anti-VSV were then bound to the surface of intact NDV- and VSV-infected cells, respectively, at 4°C. Excess unbound antibody was washed away, leaving antibody molecules which were bound to exposed viral proteins. Since internal proteins are not accessible to antibody binding, only cell surface proteins are precipitated in antigen-antibody complexes after cell disruption (15; T. G. Morrison, manuscript in preparation). The [^{35}S]methionine-labeled viral antigen-antibody complexes isolated after binding antibody to cell surfaces were resolved on polyacrylamide gels (Fig. 3, lanes 5, 7, and 9). Proteins precipitated in immune complexes after incubating preimmune sera with intact infected cells are shown in Fig. 3, lanes 6, 8, and 10. After such a procedure, the VSV glycoprotein and small amounts of nucleocapsid are precipitated (T. G. Morrison and C. O. McQuain, manuscript in preparation). Only the NDV HN and fusion protein are precipitated (15). The molar amounts of NDV HN and fusion protein accessible by this procedure are 27 and 6.5%, respectively, the amount of VSV glycoprotein detected at the cell surface. Again, these values assume that the number of methionine residues in VSV glycoprotein is comparable to the number of methionine residues in NDV HN and in fusion protein. These results suggest that migration of fusion protein to the cell surface is indeed inefficient. The low level of fatty acid acylation of the fusion protein may reflect an inefficient migration of the fusion protein to the site of fatty acid acylation. The molecules which do reach the surface may in fact be modified at least to the same extent as the VSV glycoprotein.

The amount of detectable fusion protein in virions and at the cell surface is less than the amount of HN protein (8) in virions and at the cell surface. However, we can detect [^{3}H]palmitate labeling of fusion protein, but not the HN protein. Furthermore, we have roughly estimated that the HN protein is found at the cell surface at approximately 27% the level of the VSV glycoprotein. Thus, the failure to detect palmitate label in NDV HN protein cannot be due to the fact that no HN reached the Golgi membranes, the presumed site of fatty acid addition.

There are several possible reasons for the failure to detect fatty acid on the HN glycoprotein. The HN glycoprotein may not have an appropriate site which is necessary for the specific attachment of a fatty acid group, or the protein may be in a conformation such that the fatty acid addition site is inaccessible to the fatty acid acylating enzymes. It is also possible that HN is modified by a fatty acid different from palmitate. Alternatively, completely separate intracellular pathways may exist for different glycoproteins. Rodriquez-Boulan and Sabatini (18) have demonstrated that different enveloped viruses bud from different plasma membrane domains, suggesting that there may be alternate maturational pathways for viral proteins.

Strous and Lodish (27) suggest alternate pathways for transferrin and the VSV glycoprotein from results that showed different kinetics of intracellular modification of the two proteins. Therefore, it is possible that the NDV HN glycoprotein may migrate through the cell by a different pathway and therefore is not modified by fatty acid addition like the NDV fusion protein, VSV glycoprotein, Sindbis virus E_1 and E_2, or the influenza virus HA protein.

In conclusion, our data clearly demonstrate that fatty acid palmitate modification is not essential for maturation of the NDV HN protein. Even in the absence of fatty acid, the HN glycoprotein migrates to the cell surface and is incorporated into virus particles. Therefore, fatty acid addition may not be a general processing phenomenon for all viral transmembranal glycoproteins. It is, however, possible that fatty acid

acylation may be an essential component of the pathway of some membrane glycoproteins to the cell surface.

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