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Localization of Two Cellular Forms of the Vesicular Stomatitis Viral Glycoprotein

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Two cell-associated forms of the glycoprotein (G) of vesicular stomatitis virus, termed G_1 and G_2, have been resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. G_1 has the higher electrophoretic mobility, but both forms migrate more slowly than G protein synthesized in a wheat germ cell-free system (G_0), which presumably is the unglycosylated form. G_1 is a kinetic precursor of the G_2 form, and the apparent cause of the electrophoretic difference between the two species is the presence of *N*-acetylneuraminic acid on the G_2 form. Conversion of G_1 to G_2 occurs 10 to 20 min prior to the appearance of the G_2 form of the protein on the cell surface. This suggests that the G protein may be completely glycosylated several minutes prior to its migration to the cell surface and that glycosylation is not the limiting step in its maturation. No glycoprotein comigrating with G_0 can be detected in the infected cells, even after 5-min labeling periods; this suggests that partial glycosylation of G occurs concomitantly with or immediately after its synthesis.

The lipoprotein envelope of vesicular stomatitis virus (VSV) contains external spikes made of a single glycoprotein (G) embedded in a lipid bilayer (15). This structure surrounds the helical nucleocapsid of the virus, with the matrix (M) protein forming a layer between them (17, 25). Because the virus matures by budding through the host cell plasma membrane, the glycoprotein appears to be inserted into the surface plasma membrane of the host cell. The G protein is synthesized on membrane-bound polyribosomes (4, 8, 16, 22) and is later found in the plasma membrane of the host cell, where it is picked up by the budding virus (10, 23, 33). We have separated two cytoplasmic species of the VSV G protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the two species appearing to differ only in their content of sialic acid. We have utilized these two species as markers for the determination of the cellular sites of glycosylation and the relationship between glycosylation and the course of appearance of the G protein on the surface of infected cells.

MATERIALS AND METHODS

Growth of cells and virus. Chinese hamster ovary (CHO) cells were grown in suspension culture at 12 $\times 10^4$ to 40 $\times 10^4$ cells/ml in Joklik modified minimal essential medium supplemented with 7% fetal calf serum and 1% nonessential amino acids. Mouse L cells were grown in similar medium lacking nonessential amino acids and maintained at 40 $\times 10^4$ to 80 $\times 10^4$ cells/ml.

The wild-type stock of VSV used in this work was derived from the large plaque variant of VSV Indiana isolated by Wagner et al. (24). All experiments were conducted with CHO cells. Virus stocks were prepared in L cells by infecting cells at 40 $\times 10^4$/ml with a multiplicity of 0.1 PFU/cell, using a stock prepared from plaque-purified virus (20). The infection was allowed to proceed for 20 h, the cells were removed by centrifugation, and the virus was purified as described previously (21).

Protocol for infecting and labeling cultures. CHO cells were concentrated to 4 $\times 10^6$ cells/ml in medium containing 1.8 mM $CaCl_2$, and virus was added to a multiplicity of 10 PFU/cell. Actinomycin D, the kind gift of Merck, Sharp, and Dohme, was added to 5 μg/ml. The culture was incubated for 30 to 45 min and then diluted with 2 volumes of complete medium. Cultures incubated at 31 or 39°C were buffered by the addition of 10 mM TES [*N*-Tris(hydroxylmethyl) - methyl - 2 - aminoethanesulfonic acid] and 25 mM HEPES (*N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4.

Cultures were generally labeled at 4 h postinfection with [^{35}S]methionine (New England Nuclear Corp.; 200 to 400 Ci/mmol) at 6 to 12 μCi/ml. Prior to labeling, the cells were collected by centrifugation, washed with Earle saline solution, and resuspended at 2 $\times 10^6$ cells/ml in methionine-free medium containing 1% nonessential amino acids, 7% dialyzed fetal calf serum (18), and 10 mM TES-25 mM HEPES, pH 7.4. Chase conditions were established by the addition of 1 mM unlabeled methionine. For labeling with ^{3}H-labeled sugars, the cells were col-

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lected and washed with phosphate-buffered saline (PBS; 7) and resuspended in glucose-free medium supplemented with 0.1 mg of glucose per ml and 10 mM TES-25 mM HEPES, pH 7.4.

Lactoperoxidase surface iodination of cells. Cells to be labeled were washed three times with PBS containing Ca^{2+} and Mg^{2+}, and 10^7 cells were generally resuspended in 2 ml of PBS for the labeling reaction described by Sefton et al. (19). The following components were added: NaI, 5 nmol; glucose, 10 nmol; lactoperoxidase (Calbiochem), 20 μg; $Na^{125}I$ (New England Nuclear Corp., carrier-free), 25 to 100 μCi; glucose oxidase (Worthington Biochemicals Corp.), 0.1 U. The reaction was allowed to proceed for 10 min at 25°C, and the tube containing the cells was placed on ice and 1 mM unlabeled NaI was added to stop the labeling reaction. The cells were then washed three times with PBS prior to protease treatment or gel electrophoresis. Protease treatment (see below) was capable of removing greater than 80% of the acid-precipitable radioactivity from these iodinated cells, and separation of protease-treated samples on SDS-polyacrylamide gels indicated that the residual radioactivity must be in material not within the molecular-weight range on the gel or in some other material such as glycolipids, etc. (see Fig. 4). This indicated that the labeling was largely restricted to proteins on the surface of the cells.

Protease treatment of intact cells. After washing three times with PBS, cells were resuspended at approximately 10^7 cells/ml in PBS. Chymotrypsin (Worthington Biochemicals Corp.) was added to a final concentration of 1 mg/ml, and the cells were incubated at 37°C for 10 min. These conditions were shown to remove greater than 95% of the iodinated viral G protein on the surface of infected cells. After this incubation, phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co.) was added to a final concentration of 2 mM from a fresh stock solution of 0.1 M in ethanol, and the cells were further incubated for 1 to 5 min at 37°C. Chymotrypsin preincubated with PMSF under these conditions prior to exposure to iodinated infected cells was completely inactivated, as shown by its inability to remove any iodinated viral G protein. This allowed inactivation of the protease after removal of the surface polypeptides, but prior to breaking open the cells for fractionation or gel electrophoresis.

SDS-polyacrylamide gel electrophoresis of proteins. Labeled viral proteins were subjected to gel electrophoresis in 10% polyacrylamide slab gels by the method of Laemmli (12). To give maximal resolution of the viral proteins, the gels were run for 45 min after the bromophenol blue dye had reached the bottom of the gel. The slab gels were stained with Coomassie brilliant blue, destained, fixed in 10% acetic acid, and dried onto Whatman 540 paper. The dried gel was exposed to Kodak SB54 single-side emulsion, blue-sensitive X-ray film. For quantitation, the autoradiogram was scanned on a Joyce-Loebl microdensitometer, and the areas under peaks were determined by using a Keufel and Esser planimeter. For the detection of ^3H-labeled compounds, fluorography, using the method of Laskey and Mills (14), was performed.

RESULTS

Intracellular forms of the VSV glycoprotein. To study the forms of the G protein in the cytoplasm of infected cells, infected cultures were labeled with [^{35}S]methionine for 5 min at 4 h postinfection and chased for various periods of time, and the total cellular protein was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). After the pulse label, the G protein was observed as a single species, having an apparent molecular weight of 65,000, which we are calling G_1. During the chase period, this species was progressively converted to a more slowly migrating species, called G_2, with an apparent molecular weight of 67,000. No changes in mobility of the other VSV proteins were apparent, but the amount of L protein did

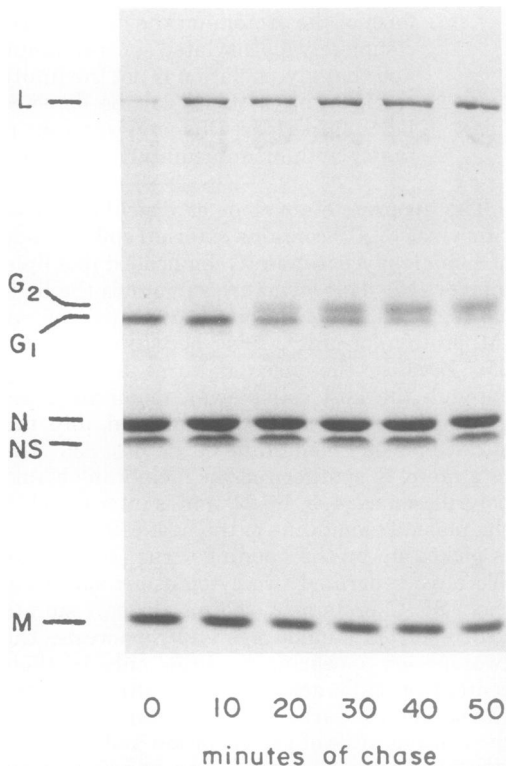

FIG. 1. *Pulse-chase labeling of total cellular proteins of cells infected with VSV. CHO cells were infected at a multiplicity of infection of 10 with VSV and incubated at 37°C. The culture was labeled for 5 min at 4 h postinfection with [^{35}S]methionine as described in the text, and then excess unlabeled methionine was added. Portions of the culture were removed at the times shown and transferred into cold Earle saline. The cells were washed and directly solubilized in gel sample buffer, and the cellular proteins were resolved by gel electrophoresis on a 10% SDS-polyacrylamide slab gel. The autoradiogram shown was a 3-day exposure of the dried gel.*

increase during the first 10 min of chase, presumably as a result of completion of nascent chains.

Both G_1 and G_2 appeared to have molecular weights greater than that of the polypeptide synthesized in vitro under the direction of G mRNA (3, 11, 16). To investigate this question directly, the polypeptides made in a wheat germ cell-free system programmed with total VSV mRNA (Fig. 2b) were compared by slab gel electrophoresis with the proteins of infected-cell membranes. The in vitro synthesized G protein, called G_0, migrated more rapidly than any G protein found in infected cells. Even after 2.5 min of labeling, the shortest feasible time, no G protein migrating with G_0 could be observed (data not shown). G_0 has all of the methionine-containing tryptic peptides of G_1 or G_2 (3, 4, 11, 16); therefore, its rapid migration relative to G_1 and G_2 presumably indicates a lower amount of carbohydrate, and possibly none. If the extent of glycosylation is the sole difference between G_0 and G_1, it would therefore appear that the G protein is glycosylated either during or very soon after its synthesis, so the G_0 form is never evident in the cytoplasm of infected cells.

We also examined the forms of the glycoprotein in virions and on the surface of infected cells (Fig. 2c to e). Virions contained only the G_2 species (Fig. 2c), thus accounting for the difference in mobility between virion and cytoplasmic G previously observed by Kang and Prevec (9). In addition, lactoperoxidase-catalyzed iodination of the surface of infected cells revealed a virus-specific protein comigrating with G_2 (Fig. 2e). Thus, the only form of the G protein present on the surface of the cells was G_2, a result consistent with the acquisition of only G_2 by virions as they bud.

Carbohydrate content of G_1 and G_2. To explore the nature of the difference between G_1

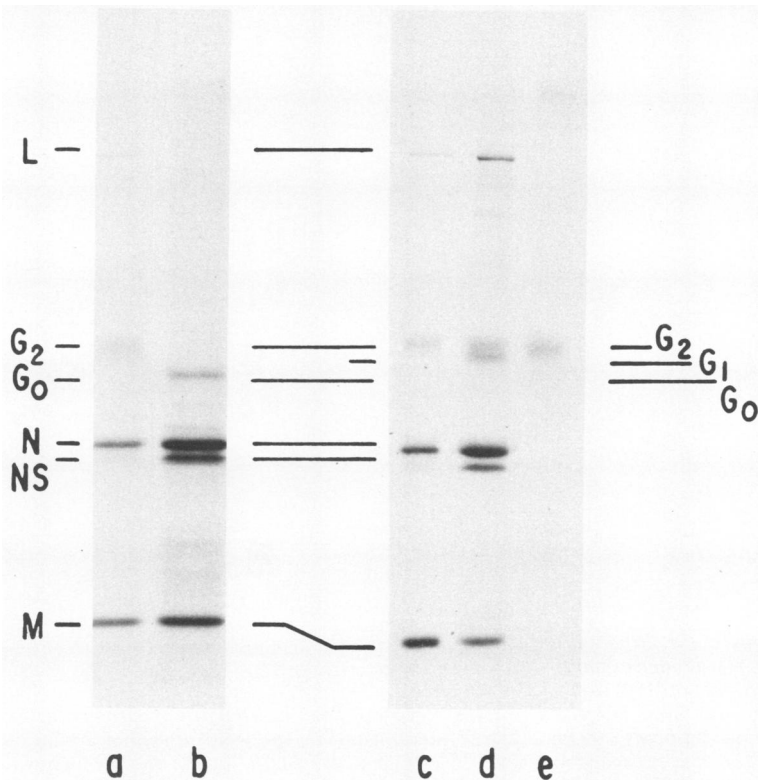

FIG. 2. *Comparison of the electrophoretic mobilities of the various forms of the VSV glycoprotein on 10% SDS-polyacrylamide slab gels. (a) and (b) are from one gel, and (c to e) are from a second gel (run in parallel). (a) [^{35}S]methionine-labeled cytoplasmic VSV proteins from intermediate density membranes (ρ = 1.175) of infected cells labeled for 30 min and chased for 30 min (10)–48-h exposure; (b) [^{35}S]methionine-labeled proteins synthesized in a wheat germ cell-free extract in response to the addition of total cytoplasmic VSV mRNA, using the conditions of Morrison and Lodish (16)–16-h exposure; (c) [^{35}S]methionine-labeled virion proteins–48-h exposure; (d) [^{35}S]methionine-labeled total cellular proteins from infected cells labeled from 4 to 4.5 h postinfection–48-h exposure; (e) ^{125}I-labeled surface proteins of infected CHO cells– 48-h exposure.*

and G_2, we labeled infected cells with ^{3}H-labeled sugars. In cells labeled with [^{3}H]glucosamine for 30 min, two labeled bands were apparent, comigrating with G_1 and G_2 (Fig. 3). Both forms of G, therefore, appeared to be at least partially glycosylated. In addition, when cells were labeled with [^{3}H]galactose, a sugar found only in the subterminal position in the major glycopeptides of the G protein of VSV grown in BHK cells (J. Etchison, personal communication), a band comigrating with G_2 and a second band comigrating with G_1 or slightly behind it were found. These results suggested that the carbohydrates on G_1 and G_2 are not very different.

To determine whether terminal sialic acid residues might be responsible for the electrophoretic differences between G_1 and G_2, the effect of neuraminidase treatment on their mobility was investigated. Cells were pulse-labeled with [^{35}S]methionine for 15 min and chased for 60 min with cold methionine to allow most of the labeled G protein to accumulate on the cell surface (see below). The intact cells were then incubated with 50 units of neuraminidase per ml for 30 min at 37°C. Prior to the enzyme treatment, a large percentage of the G protein was in the G_2 form (Fig. 4b). After the enzyme treatment of the cell surface, all of the protein comigrated with the G_1 species (Fig. 4c). Similar treatment of [^{3}H]galactose-labeled cells showed a shift in mobility of the galactose-labeled G_2 species to the G_1 position, with no loss of radioactivity (data not shown). These results were supporting evidence that the enzyme preparation was not contaminated with other glycosidases, and no sugars other than sialic acid were removed. Incubation of VSV proteins synthesized in vitro with neuraminidase showed no degradation of the viral proteins, especially G_0, demonstrating that there was no specific proteolytic activity in the preparation which was capable of removing a small

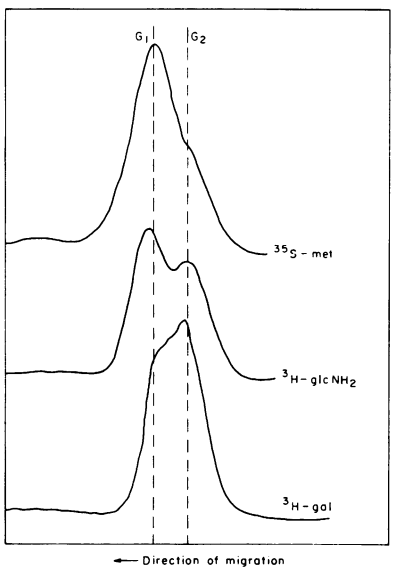

FIG. 3. *Analysis of the carbohydrate content of the G_1 and G_2 species. Microdensitometer tracings of the G region of fluorograms of 10% slab gels on which the total cellular proteins were subjected to SDS-polyacrylamide gel electrophoresis from cultures incubated with the indicated radioactive labels. ^{35}S-met, [^{35}S]methionine; ^{3}H-glcNH$_2$, D-[6-^{3}H]glucosamine (25 μCi/ml; New England Nuclear Corp.); ^{3}H-gal, D-[1-^{3}H]galactose (25 μCi/ml; New England Nuclear Corp.). All cultures were labeled 4 to 4.5 h postinfection in the appropriate medium (see text). Although not shown in the tracings, no radioactivity was incorporated into the other viral proteins in the case of the sugar labels.*

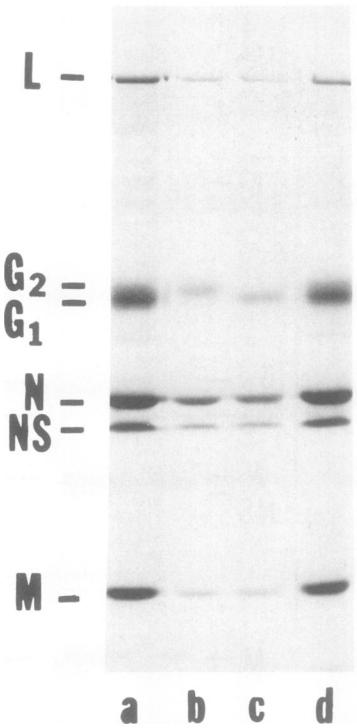

FIG. 4. *Analyais of carbohydrate content of the G_1 and G_2 species. Autoradiogram of a 10% slab gel on which the following samples were subjected to electrophoresis: (a and d) total cellular proteins labeled 4 to 4.5 h postinfection with [^{35}S]methionine; (b) total cellular proteins from the cells labeled with [^{35}S]methionine for 15 min, chased for 60 min, and incubated in PBS for 30 min at 37°C; (c) same as sample (b), except that incubation in PBS contained 50 U of neuraminidase (Vibrio cholerae; Calbiochem) per ml.*

portion of the G protein (data not shown). We conclude that the difference in electrophoretic mobility between the G_1 and G_2 forms of the glycoprotein is due largely, if not completely, to the presence of *N*-acetylneuraminic acid residues on the G_2 form.

Movement of the G protein to the cell surface. To develop an assay for the time of appearance of the G protein on the cell surface, we first used the ability of lactoperoxidase-catalyzed iodination to label G protein on the infected cell surface (Fig. 2e). Such ^{125}I-labeled surface G protein can be removed by protease treatment. In preliminary experiments, it was shown that treatment of intact cells with 1 mg of chymotrypsin per ml for 10 min at 37°C, followed by inhibition of the enzyme, was capable of removing more than 95% of the ^{125}I-labeled G protein from the cell surface (Fig. 5). Such treatment did not affect the pattern of Coomassie blue-stained cell proteins or of labeled N, NS, or L proteins in infected cells. These conditions were then used on [^{35}S]methionine-labeled cells to determine when G protein reaches the cell surface (i.e., when G protein becomes sensitive to treatment of intact cells with protease).

Cells were labeled with [^{35}S]methionine for 5 min and then chased for various periods of time. Samples were taken and half of each was treated with chymotrypsin. The treated and control samples were then analyzed in parallel by gel electrophoresis (Fig. 5). The amounts of G_1 and G_2 were quantitated, and the percentage of label in G_2 as well as the percentage of G removed by protease was plotted (Fig. 6). As is most evident between 10 and 30 min of the chase, there was protein migrating as G_2 that was not sensitive to protease treatment. The conversion of G_1 to G_2 had a half-time of about 30 min, but there was a 10- to 20-min lag period before the G_2 protein was converted to a protease-sensitive form. The protease-resistant G_2 did not appear to result from conversion of G_1 to G_2 and movement onto the surface during the 5-min inactivation of chymotrypsin with PMSF because, in another experiment utilizing a 1-min incubation with PMSF, similar results

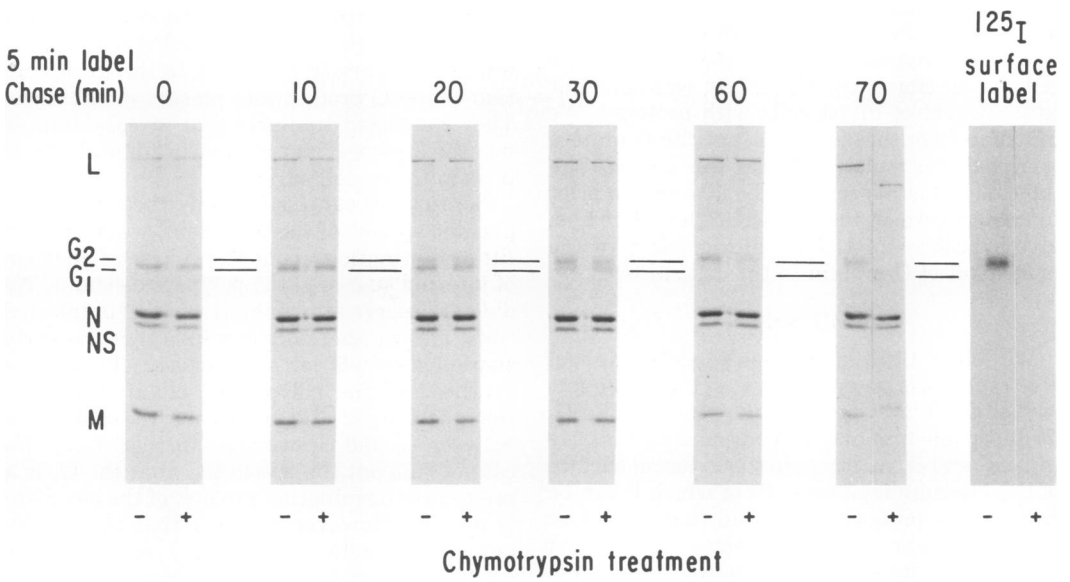

FIG. 5. *Time course of movement of the G protein to the cell surface. A culture of infected cells was exposed to [^{35}S]methionine for 5 min at 4 h postinfection at 37°C. After the labeling period, excess unlabeled methionine was added and the incubation was continued at 37°C. At the times indicated, portions of the culture were removed and transferred into 4 volumes of ice-cold Earle saline. Each sample of cells was washed with PBS three times. The cells were resuspended in PBS, and one-half of each sample was incubated in PBS with 1 mg of chymotrypsin per ml at 37°C for 10 min while the remainder was incubated in PBS only for the same period. After this incubation, PMSF was added to 2 mM and incubation was continued for 5 min at 37°C. The cells were washed with PBS and dissolved directly in gel sample buffer. The sample of iodinated cells was labeled as described in the text, using infected cells at 4 h postinfection. After the labeling reaction, the cells were extensively washed and suspended at the same concentration as the [^{35}S]methionine-labeled cells in PBS for the protease treatment. The remainder of the protease reaction was conducted as above. The samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% slab gels. The autoradiogram shown is from a 4-day exposure of the dried gel.*

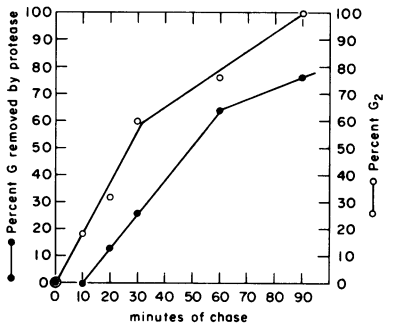

FIG. 6. *Quantitation of the time course of the conversion of G_1 to G_2 and appearance of total G protein on the cell surface. These data are from the experiment shown in Fig. 5. Symbols: ●, percentage of total G protein removed by protease treatment, normalized relative to the amount of NS protein in each sample to account for differences in total material in each sample (the amount of G protein in each sample was determined by microdensitometry of the autoradiogram shown in Fig. 5); ○, percentage of total G in G_2 form in the control sample for each time point.*

were obtained (data not shown). By 90 min of chase, all of the G protein was in the G_2 form, and nearly 80% of it was removed by chymotrypsin and appeared to be on the surface of the cell. At all times, the G_1 protein was resistant to treatment of intact cells with protease. We conclude from these results that the G protein is probably completely glycosylated inside the infected cell at approximately 10 to 20 min prior to its exposure on the surface of the cell. Thus, glycosylation is not the limiting factor in the movement of G protein to the cell surface.

DISCUSSION

We have utilized SDS-polyacrylamide gel electrophoresis to separate two cytoplasmic species of the VSV G protein, G_1 and G_2. The G_1 form is made first and converted intracellularly into G_2, and G_2 migrates to the external surface of the plasma membrane, from which it can be removed by proteases. We find that G protein does not appear on the cell surface until at least 15 min after its synthesis, a much longer time than previously proposed by David (6). Because neuroaminidase could convert G_2 to G_1, the apparent cause of the difference in mobility is the difference in sialic acid content. The large difference in mobility between G_1 and G_2 could be due to either the additional mass added by the sialic acid or the additional negative charge of the acidic residues. This could lower the binding of SDS to the glycoprotein and lessen its electrophoretic mobility.

Either form of the glycoprotein could be heterogeneous in its carbohydrate content. The protein could be sialylated to varying extents, but the various forms could migrate together as G_2. Similarly, G_1 might contain forms with heterogeneous carbohydrate chains, but with the heterogeneity not affecting the mobility. It is only clear that the G_1 band contains glycoprotein that has label from both galactose and glucosamine.

The resolution of these two species has allowed us to define the series of events involving the sialylation of the G protein and its migration onto the surface of infected cells. There appears to be a 10- to 20-min lag period between the addition of sialic acid and the appearance of the protein on the cell surface.

Because sialic acid is the terminal sugar of the major glycopeptides of VSV (5), it would appear that these chains are nearly complete several minutes prior to the emergence of the protein on the surface of the cell. This would imply that the addition of sugars is not the limiting step in the maturation of the G protein. However, there could be additional glycosylation or sialylation that we do not detect which limits maturation. Atkinson et al. (2) observed that the incorporation of fucose, considered to be the last sugar added to glycoproteins (1), occurs 20 min prior to the incorporation of the G protein into plasma membranes. They concluded similarly that glycosylation is not the limiting step in maturation of the G protein to the cell surface.

Because we can resolve only these two cytoplasmic species of the G protein, we have very little information about the course of addition of internal sugars to the polypeptide chain. We did not observe any of the G_0 species in infected cells, and we therefore conclude that the early steps of glycosylation occur soon after or during synthesis of the polypeptide chain. However, the question of the nature of the difference between G_0 and G_1 warrants further study. We cannot rule out the possibility that the G_0 is a premature termination product of the complete G protein. However, the fact that the 63,500-molecular-weight protein (G_0) is the major product of the G mRNA in several cell-free systems (3, 11, 16, 22) argues that this is the complete protein moiety of the glycoprotein. It remains to be conclusively demonstrated that the sole difference between G_0 and G_1 is the presence of carbohydrate chains on G_1.

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