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Vesicular Stomatitis Virus Glycoprotein Is Anchored to Intracellular Membranes near Its Carboxyl End and Is Proteolytically Cleaved at Its Amino Terminus

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The intracellular vesicular stomatitis virus glycoprotein (G) is inserted into membranes such that a small portion of one end of the molecule is exposed on the cytoplasmic surface of the endoplasmic reticulum and is susceptible to proteolytic digestion (T. G. Morrison, C. O. McQuain, and D. Simpson, J. Virol. **28**:368-374). We have determined that this region of the G protein contains two methionyl tryptic peptides. The methionyl tryptic peptides of the G protein have been ordered by the use of the antibiotic pactamycin, and the two methionyl tryptic peptides removed by proteolytic digestion of intracellular G protein have been shown to be derived from the carboxyl terminal end of the protein. In addition, we have found that the unglycosylated G protein synthesized in a reticulocyte cell-free reaction migrates on polyacrylamide gels slightly slower than the unglycosylated G protein synthesized in tunicamycin-treated infected cells. We have also compared these G proteins derived from different sources by partial proteolysis (D. W. Cleveland, S. G. Fischer, M. W. Kirschner, and V. K. Laemmli, J. Biol. Chem. **252**:1102-1106) and by chymotryptic peptide analysis. We have found minor differences between the two proteins consistent with the removal of 10 to 15 amino acids from the amino terminus of the intracellular G protein.

Vesicular stomatitis virus (VSV) is an enveloped RNA virus composed of an inner ribonucleoprotein core surrounded by a membrane (5, 11, 38). On the external surface of this membrane are spikes which are composed of a glycoprotein (G) (6, 9, 22, 28), one of the five viral-encoded proteins (5, 6, 11, 38). The glycoprotein is synthesized on membrane-bound polyribosomes (4, 10, 23). The nascent polypeptide is transported across intracellular membranes, from the cytoplasmic side of the membrane into the lumen of the endoplasmic reticulum (13, 18, 25, 33, 37). However, the intracellular glycoprotein is transmembranal, and a small portion of one end of the protein extends from the cytoplasmic side of the membrane (13, 25, 33, 37).

It has been proposed that secretory proteins initially interact with the endoplasmic reticulum because of a hydrophobic sequence of amino acids located at the amino terminus of the nascent protein chain (1, 2). This sequence of amino acid residues (signal sequence) is thought to trigger the attachment of the nascent protein chain to the endoplasmic reticulum and the transfer of the nascent chain into the lumen of the endoplasmic reticulum. The signal sequence is then cleaved off the protein by a membrane-bound signal peptidase (2). The signal sequence hypothesis was originally proposed for secretory proteins which are transported from the endoplasmic reticulum to the outside of the cell. A similar mechanism is thought to be responsible for membrane glycoproteins, like the VSV G protein, which are transported to the external surface of the cell (13, 33).

Many membrane proteins appear to be attached to the plasma membrane by their carboxyl terminal end whereas the amino terminal end is exposed on the external surface of the cell (20). In light of the signal sequence hypothesis, such an orientation suggests that the amino terminal ends of membrane proteins pass through membrane channels in the rough endoplasmic reticulum, whereas the carboxyl terminal ends are never released from the membrane. Such considerations lead to the prediction that the carboxyl terminal end of the VSV G protein may be exposed on the cytoplasmic face of the endoplasmic reticulum. The signal sequence hypothesis also suggests that a proteolytic cleavage may occur at the amino terminus of the VSV G protein.

We present evidence here that the VSV G protein is indeed inserted into intracellular

membranes such that a region of the molecule derived from the carboxyl terminal end remains on the cytoplasmic side of the membrane. Furthermore, a small (10 to 15 amino acids) segment is cleaved from the amino terminus of the G protein, a result consistent with the removal of a signal sequence.

MATERIALS AND METHODS

Cells and virus. Cells used were Chinese hamster ovary cells. VSV, Indiana serotype, was grown and purified as described previously (35).

Preparation of cytoplasmic extracts. Chinese hamster ovary cells (4×10^6) growing at 37°C were infected with purified B particles of VSV at a multiplicity of 5 PFU/cell as described previously (25). Tunicamycin (gift to Donald J. Tipper from G. Tamura), when used, was added at 1 h postinfection at a concentration of 0.5 μg/ml. At 4.5 h postinfection, the cells were harvested by centrifugation, washed once, and then resuspended in 0.5 ml of modified Eagle medium (MEM) containing no methionine but supplemented with nonessential amino acids and dialyzed fetal calf serum (7.5%). [^{35}S]methionine (1 mCi, 750 Ci/mmol; Amersham) was added to the cells. After 15 min at 37°C, the cells were harvested by centrifugation and washed in cold 5% sucrose. A total of 2×10^7 uninfected cells resuspended in 5% sucrose were added as carrier. The cells were disrupted with a tight-fitting Dounce homogenizer, and the nuclei were removed from the cytoplasmic extract by centrifugation. The nuclei were washed once in 5% sucrose, and the resulting supernatant was combined with the cytoplasmic extract.

Cell-free protein synthesis. VSV proteins were synthesized in cell-free extracts derived from rabbit reticulocytes prepared as described previously (12). Exogenous, RNA-dependent cell-free protein synthesis was carried out in these extracts by modification of the procedures of Pelham and Jackson (30). Pactamycin added to these reactions was a gift of the Upjohn Company.

Polyacrylamide gel electrophoresis. Polypeptides were resolved on 10 or 13% polyacrylamide slab gels (14 by 22 by 0.15 cm), prepared and run as described by Laemmli (15). The gels were then fixed and stained with Coomassie brilliant blue as described by Clinkscales et al. (8), dried, and subjected to autoradiography (X-ray film X-OMAT; Eastman Kodak Co.). The resulting autoradiograms were scanned with an Ortec microdensitometer.

Peptide mapping by limited proteolysis. Proteins isolated from 10% polyacrylamide gels were digested with chymotrypsin in the presence of sodium dodecyl sulfate, and the resulting cleavage products were resolved by electrophoresis on 15% polyacrylamide gels as described by Cleveland et al. (7).

Tryptic and chymotryptic peptide analysis. Trypsin digestion of individual polypeptides was done as described previously (23). Tryptic peptides were resolved by paper electrophoresis at pH 3.5 (17, 24).

RESULTS

Orientation of the glycoprotein in intracellular membranes. Trypsin digestion of intracellular membrane-associated G protein reduces the size of the molecule by approximately 3,000 daltons (13, 25, 37). To determine which methionine-containing tryptic peptides are lost from the molecule under these conditions, digested and undigested G proteins were isolated on polyacrylamide gels and further digested with trypsin. The resulting tryptic peptides were resolved by paper electrophoresis at pH 3.5. Figure 1 shows densitometer scans of the resulting autoradiogram. Two methionine-containing tryptic peptide peaks (indicated by the arrows) were lost when membrane-associated G protein was digested with trypsin.

To determine which end of the G protein is removed by trypsin digestion, it was necessary to determine which methionine-containing tryptic peptides are derived from the amino terminal end and which from the carboxyl terminal end of the molecule. The order of the methionine-containing tryptic peptides was determined by the use of the antibiotic pactamycin which, at

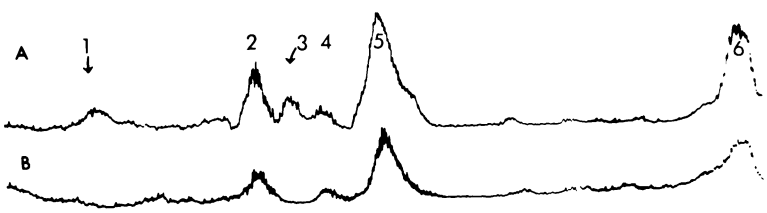

FIG. 1. *[^{35}S]methionyl tryptic peptides of trypsin-digested, intracellular membrane-associated G protein. Cell extracts of tunicamycin-treated VSV-infected cells radioactively labeled for 15 min with [^{35}S]methionine were prepared as described in the text. The extracts were digested with trypsin (20 μg/ml) for 30 min at 25°C, and the proteins were acetone precipitated and isolated on 10% polyacrylamide gels (15). Proteins in extracts undigested with trypsin were subjected, in parallel, to electrophoresis. The regions of the gel containing the G proteins were excised and further digested with trypsin (23). The resulting tryptic peptides were resolved by paper electrophoresis at pH 3.5 (17, 23, 24). The paper was exposed to X-ray film (X-OMAT; Kodak) for 5 weeks. The autoradiogram was scanned with an Ortec microdensitometer. (A) Intact glycoprotein; (B) glycoprotein digested with trypsin. Each peptide was assigned a number.*

tne appropriate concentration, inhibits polypeptide initiation but not polypeptide chain elongation (19). Six cell-free protein-synthesizing reactions directed by VSV mRNA were incubated for 2 min at 22°C, pactamycin at a concentration of 8×10^{-7} M was added to all six reactions (the concentration determined to inhibit initiation but not elongation in our system), and then [^{35}S]methionine was added to each reaction at various times. All reactions were incubated for a total of 24 min. Under these conditions, G protein initiated in the first 2 min of the reaction was completed within 20 min (not shown). Since [^{35}S]methionine was added after the pactamycin, the radioactivity should have been incorporated only into elongating molecules. The later the [^{35}S]methionine was added, the greater the chance of being incorporated near the carboxyl terminal end (31, 36). The products of the cell-free reactions were resolved on polyacrylamide gels, the G protein was digested with trypsin, and the resulting tryptic peptides were resolved by paper electrophoresis at pH 3.5. Figure 2 shows densitometer scans of the autoradiogram. Panel A shows the pattern of radioactively labeled tryptic peptides obtained when methionine was added at the same time as pactamycin. Panels B-F show the patterns obtained when [^{35}S]methionine was added 3, 8, 10, 12, and 16 min, respectively, after pactamycin addition. With later addition of methionine, fewer tryptic peptides were radioactively labeled. In addition, radioactivity present in each peptide disappeared at a different rate.

The regions of the electrophoresis paper containing each peptide were excised, and the radioactivity present was determined by liquid scintillation counting. Figure 3 shows the reduction in radioactivity in each peptide peak. Assuming that each peak contains one tryptic peptide, these data suggest that peptide 6 is closest to the amino terminus and peptide 1 is closest to the carboxyl terminus.

Comparison of Fig. 1 and Fig. 2 reveals that the intracellular G protein digested with trypsin is missing peptides 3 and 1. Thus, a carboxyl terminal region of the G protein must be exposed on the cytoplasmic side of intracellular membranes.

Comparison of unglycosylated G proteins derived from a cell-free protein-synthesizing system and infected cells. If the amino terminus of the membrane-bound glycoprotein is processed by a "signal peptidase" (1, 2), then one would expect a size difference between the cell-free G protein and the intracellular G protein. However, previous comparisons of the sizes of cell-free G protein and intracellular G protein have thus far been difficult to interpret due to the carbohydrate moieties present on the intracellular G protein. Cell-free extracts directed by VSV 13 to 15S RNA and infected cells treated with tunicamycin both synthesize an unglycosylated form of the VSV G protein (14, 16). To compare their sizes, these two glycoproteins were subjected, in parallel, to electrophoresis on 13% polyacrylamide gels. Figure 4 shows densitometer scans of the resulting autoradiogram. G protein derived from tunicamycin-treated infected cells (Fig. 4B) migrated slightly faster than the G protein synthesized in a cell-free system (Fig. 4A). There was an apparent molecular weight difference of 1,500. To determine whether the difference in apparent

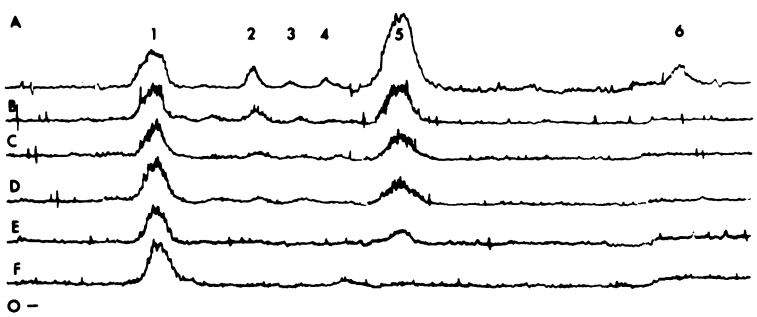

FIG. 2. *[^{35}S]methionyl tryptic peptides of VSV G protein made in cell-free reactions containing pactamycin. Six reticulocyte extracts directed by optimal concentrations of VSV 13 to 15S RNA were incubated for 2 min at 22°C. [^{35}S]methionine (10 μCi) was added to reaction A, and pactamycin was added to reactions A-F. [^{35}S]methionine (10 μCi) was added to reactions B-F after 5, 10, 12, 15, and 18 min of incubation, respectively. The reactions were incubated for a total of 25 min. The products of the reaction were subjected to electrophoresis on a 10% polyacrylamide gel, the regions of the gel containing the G protein were excised and digested with trypsin (23), and the resulting tryptic peptides were resolved by paper electrophoresis at pH 3.5 (17, 23, 24). The paper was exposed to X-ray film for 5 weeks. The figure shows the densitometer scans of the resulting autoradiogram.*

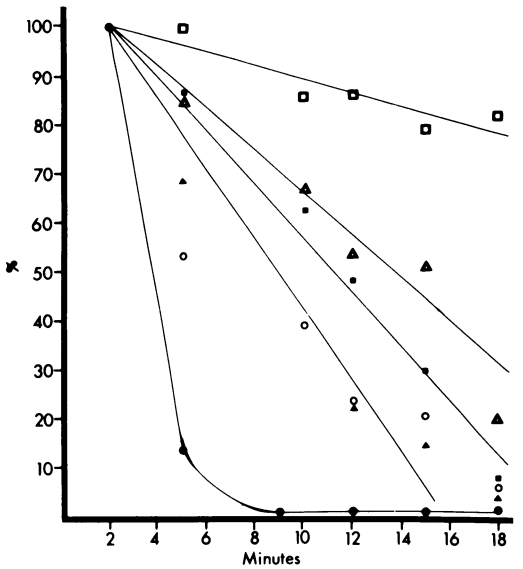

FIG. 3. *Order of [^{35}S]methionyl tryptic peptides within the G protein. The figure shows a quantitative determination of the decrease in the radioactivity present in each tryptic peptide in the patterns shown in Fig. 2. The location of each tryptic peptide on the electrophoresis paper was determined by autoradiography, the region of the paper containing each peptide was excised, and the radioactivity present was determined by liquid scintillation counting. The amount of radioactivity present in each peptide in sample A was taken as 100%. The figure shows the decrease in radioactivity in each peptide (as a percentage of the amount found in sample A) with later addition of [^{35}S]methionine. Symbols: ●, peptide 6; ○, peptide 2; ▲, peptide 4; ■, peptide 5; △, peptide 3; □, peptide 1.*

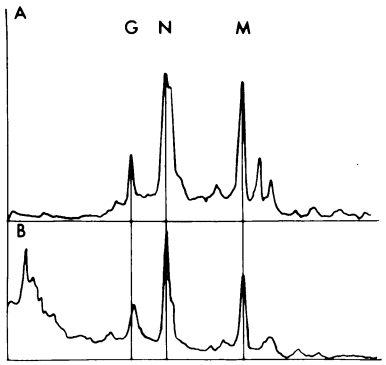

FIG. 4. *Comparison of the sizes of the G protein synthesized in a cell-free reaction and the G protein synthesized in tunicamycin-treated infected cells. The figure shows densitometer scans of an autoradiogram of a stained, dried polyacrylamide gel containing the VSV proteins synthesized in a cell-free reaction directed by VSV 13 to 15S RNA (A) and the proteins made in tunicamycin-treated, VSV-infected cells (B). [^{35}S]methionine labeled polypeptides, prepared as described in the text, were resolved on 13% slab gels (15) and subjected to electrophoresis for approximately 3 h at a constant current of 30 mA. The stained, dried gel was exposed to X-ray film for 24 h.*

molecular weight between the two unglycosylated G proteins was due to a proteolytic cleavage and not an artifact in the gel preparation, the technique of partial proteolysis devised by Cleveland et al. (7) was used to compare these two polypeptides. [^{35}S]methionine-labeled G protein was treated with increasing concentrations of chymotrypsin in the presence of SDS, and the resulting polypeptide fragments were resolved on 15% polyacrylamide gels (Fig. 5). Channels 1, 4, 7, and 10 show the pattern obtained from G protein made in a cell-free system, whereas channels 2, 5, 8, and 11 show the pattern obtained from the G protein synthesized in tunicamycin-treated cells. The patterns are quite similar, with the exception of the peptides marked X and X-1. Peptide X is missing in the cellular G protein. However, cellular G contains a peptide (X-1) which migrated slightly faster than peptide X and the G protein made in the cell-free system is missing peptide X-1.

Comparison of channels 1 and 2 reveals another difference between the cell-free product and the intracellular protein—the intracellular protein is missing a band which migrated slightly faster than the band marked W. However, with increasing chymotrypsin concentrations this band appeared in the pattern obtained from the intracellular protein (see channel 5). Thus this difference in the two patterns probably reflects only different degrees of partial digestion of the samples shown in channel 1 and 2.

Partial proteolysis of intracellular G protein missing its carboxyl terminus is also shown in Fig. 5 (channels 3, 6, 9, and 12). Two bands (Y and Z) show increased mobility, and a peptide fragment (W) is absent. However, peptide fragment X-1 was unaffected. Thus, the differences between the intracellular G protein and the cell-free G protein were unaffected by removing the carboxyl terminus from the protein.

Chymotryptic peptide analysis was used to verify the differences between the G protein synthesized in a cell-free reaction and the unglycosylated G protein made in infected cells. The two G proteins were isolated on 10% polyacrylamide gels and exhaustively digested with chymotrypsin, and the resulting chymotryptic peptides were then resolved by paper electrophoresis at pH 3.5. The [^{35}S]methionine-labeled chymotryptic peptide patterns obtained from these G proteins were identical except that one or two peptides present in the G protein made in the

free system were absent in the pattern obtained from G protein made in VSV-infected cells (Fig. 6).

DISCUSSION

The signal sequence hypothesis (1, 2) leads to the prediction that the VSV G protein is anchored to intracellular membranes at its carboxyl terminal end and that the amino terminal end of the protein is proteolytically removed. To explore these predictions, we have utilized tryptic and chymotryptic peptide mapping procedures and partial proteolysis patterns (7, 17, 24). In these experiments, we have compared the G protein made in a cell-free protein-synthesizing system devoid of membranes and the G protein synthesized in infected cells treated with the antibiotic tunicamycin. We have previously found that detailed comparison of the fully glycosylated G protein and the unglycosylated G protein by peptide mapping procedures and by patterns of partial proteolysis were difficult to interpret. There were many minor differences which were probably due to the carbohydrate

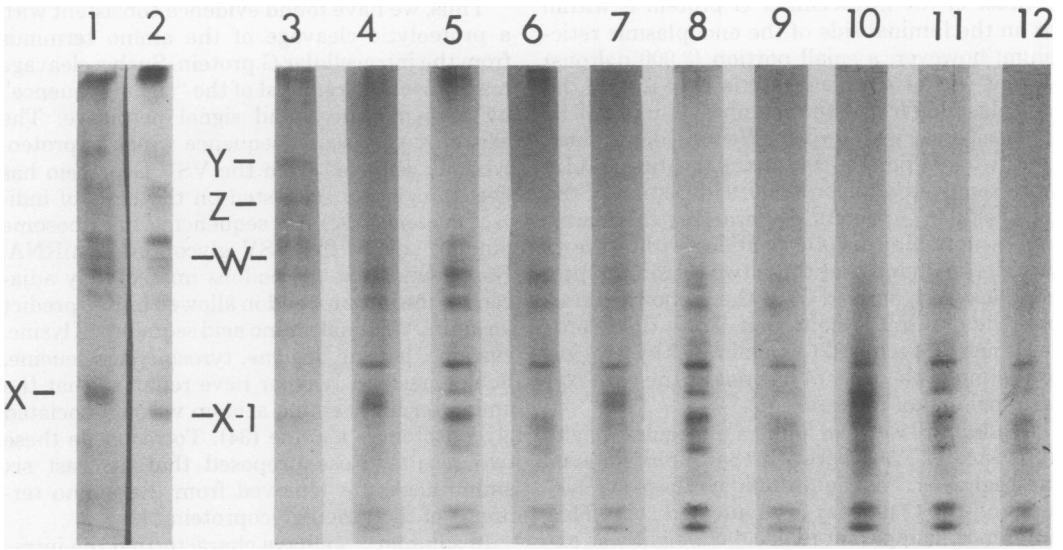

FIG. 5. *Comparison of polypeptide fragments of G protein derived from different sources. The figure shows an autoradiogram of the polypeptides generated from partial proteolysis of the G protein synthesized in a cell-free reaction (slots 1, 4, 7, and 10), G protein synthesized in tunicamycin-treated, VSV-infected cells (slots 2, 5, 8, and 11), and G protein from tunicamycin-treated, infected extracts digested with trypsin (slots 3, 6, 9, and 12). Partial proteolysis was carried out by the method of Cleveland et al. (7). [^{35}S]methionine-labeled proteins were excised from dried 10% polyacrylamide gels, the gel slices were soaked for 30 min in 5 ml of buffer (0.125 M Tris-hydrochloride, pH 6.8, 0.1% sodium dodecyl sulfate and 1 mM ethylenediaminetetraacetic acid), the gel slices containing the various G proteins were digested with 25 μl of chymotrypsin in increasing concentrations: slots 1–3, 5 μg/ml; slots 4–6, 25 μg/ml; slots 7–9, 50 μg/ml; and slots 10 to 12, 100 μg/ml), and the resulting polypeptide fragments were resolved on a 15% polyacrylamide gel (7). Electrophoresis was carried out at 30 mA constant current for approximately 4 h, except that the current was turned off for 30 min when the dye front reached the bottom of the stacking gel. The gel was impregnated with 2,5-diphenyloxazole (3) and exposed to X-ray film for 10 days.*

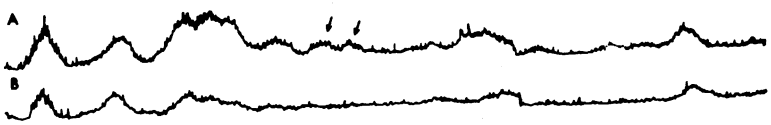

FIG. 6. *Comparison of [^{35}S]methionyl chymotryptic peptides of G protein synthesized in a cell-free reaction and in infected cells. The figure shows densitometer scans of autoradiograms of Whatman 3MM paper containing chymotryptic peptides from G protein synthesized in a cell-free reaction (A) and G protein made in tunicamycin-treated, VSV-infected cells (B). Regions of a 10% polyacrylamide [^{35}S]methionine-labeled G proteins were excised and digested with chymotrypsin as previously described (23), and the resulting chymotryptic peptides were subjected to paper electrophoresis at pH 3.5 (17, 23, 24). After electrophoresis, the dried paper was exposed to X-ray film for 4 weeks.*

moieties of the fully glycosylated G protein (unpublished data). Thus, we have inhibited the addition of carbohydrate to the G protein made in infected cells by treating infected cells with tunicamycin (16). We have previously shown that the VSV G protein made in the presence of tunicamycin is fully membrane associated and is transported across the membrane of the endoplasmic reticulum in the same way as the fully glycosylated G protein (26). Thus, at this time there is no indication that failure to glycosylate the G protein interferes with these initial events in the maturation of the G protein.

Most of the intracellular G protein is within or on the luminal side of the endoplasmic reticulum; however, a small portion (3,000 daltons) of one end of the molecule is exposed on the cytoplasmic face of the membrane and can be digested away with trypsin. We have determined that two methionine-containing tryptic peptides were removed after proteolytic digestion of the intracellular G protein. By ordering the methionine-containing tryptic peptides within the G protein, we found that these two missing tryptic peptides were derived from the carboxyl terminal region of the glycoprotein. Thus, the G protein must be anchored to membranes by a region of the molecule close to but not at the carboxyl terminal end of the molecule.

In addition, we have found a molecular weight difference of 1,500 between the G protein synthesized in a cell-free protein-synthesizing system and the G protein from infected cells. This difference in apparent molecular weight was explored in two different ways. First, we compared the pattern of partial proteolysis obtained from the two proteins. By this procedure we have found that one of the polypeptide fragments derived from the infected cells' protein appears to migrate faster on polyacrylamide gels than its counterpart derived from the cell-free G protein. Secondly, we have compared the pattern of [^{35}S]methionine-containing chymotryptic peptides by ionophoresis. We have found one to two peptides missing from the protein isolated from infected cells. These results are consistent with a proteolytic cleavage of the protein derived from infected cells.

To determine which end of the molecule has been cleaved, we utilized our finding that the carboxyl terminal end of the G protein is removed when the intracellular, membrane-bound G protein is digested with trypsin. The pattern of polypeptides derived from partial proteolysis of this shortened G protein was compared to the pattern obtained from the two full-sized G proteins. Removing the carboxyl terminal end of the molecule generated differences in the pattern of partial proteolysis products, but the differences seen between the cell-free G protein and the intact G protein were unaffected by the removal of the carboxyl terminal end of the molecule. This result leads us to suggest that the difference between the cell-free G and the intracellular, intact G protein resides at the amino terminus and not the carboxyl terminus. We cannot, however, eliminate the possibility that there is a minor internally located modification in either the intracellular G protein or the cell-free G protein which causes the differences in these two proteins.

Thus, we have found evidence consistent with a proteolytic cleavage of the amino terminus from the intracellular G protein. Such a cleavage may reflect the removal of the "signal sequence" by a membrane-bound signal peptidase. The existence of a signal sequence which is proteolytically removed from the VSV G protein has been previously suggested on the basis of indirect evidence (32). By sequencing the ribosome binding site of the VSV glycoprotein mRNA, Rose found that the codons immediately adjacent to the initiator codon allowed him to predict an amino terminal amino acid sequence of lysine, cysteine, leucine, leucine, tyrosine, and leucine. Schloemer and Wagner have reported that the amino terminal amino acid on virion-associated glycoprotein is alanine (34). To reconcile these two results, Rose proposed that at least six amino acids are removed from the amino terminus of the virion glycoprotein (32).

In summary, we have characterized the intracellular, membrane-associated VSV G protein made in infected cells treated with tunicamycin. We have found that the glycoprotein is bound to membranes near its carboxyl terminal end. Furthermore, the amino terminal end appears to be subjected to a minor proteolytic cleavage.

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