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Membrane Assembly: Synthesis and Intracellular Processing of the Vesicular Stomatitis Viral Glycoprotein

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The glycoprotein (G) of vesicular stomatitis virus (VSV) is synthesized on membrane-bound polyribosomes. Approximately 30 min after its synthesis, it reaches the surface plasma membrane where it is incorporated into budding virus. The first part of this paper focuses on the 2 intracellular, membrane-bound, glycosylated forms of the glycoprotein which are intermediates in its biogenesis. All glycosylation and processing is completed in the smooth microsome fraction before the protein reaches the surface.

Next, we turn to the mechanism by which G is synthesized on membrane-bound polyribosomes. All of the G mRNA is bound to membranes, and studies with puromycin suggest that this attachment of G mRNA is mediated by the nascent glycoprotein chain. After its synthesis G is a transmembrane protein with about 30 amino acids at the carboxyl terminus remaining on the cytoplasmic side of the endoplasmic reticulum. Since 95% of the glycoprotein, containing the carbohydrate residues, is resistant to attack by external proteases, it appears to be within the lumen of the endoplasmic reticulum or embedded within the lipid bilayer. Finally, we show that synthesis, glycosylation, and proper asymmetric insertion of G into the ER can be achieved in cell-free extracts. Both glycosylation of G and proper insertion into the ER membrane in this cell-free system require concomitant protein synthesis.

Key words: VSV, glycoprotein, membranes, cell-free synthesis

The envelope of vesicular stomatitis virus (VSV) contains a lipid bilayer in which external spikes, made up of a single glycoprotein (G), are embedded (1). This structure surrounds the RNA-containing nucleocapsid of the virus, with the virus matrix protein (M) forming a layer between them (2–4) (Fig. 1). The virus matures by budding from the host cell plasma membrane and contains lipids of the host cell surface membrane in closely conserved proportions (5). The glycoprotein is synthesized on membrane-bound polyribosomes (6–9) and, as we shall describe, later migrates to the surface membrane of the host cell where it is incorporated into the budding virus (10–12). As the genome of VSV encodes only 5 proteins, all of which are structural proteins of the virion particle (3), it

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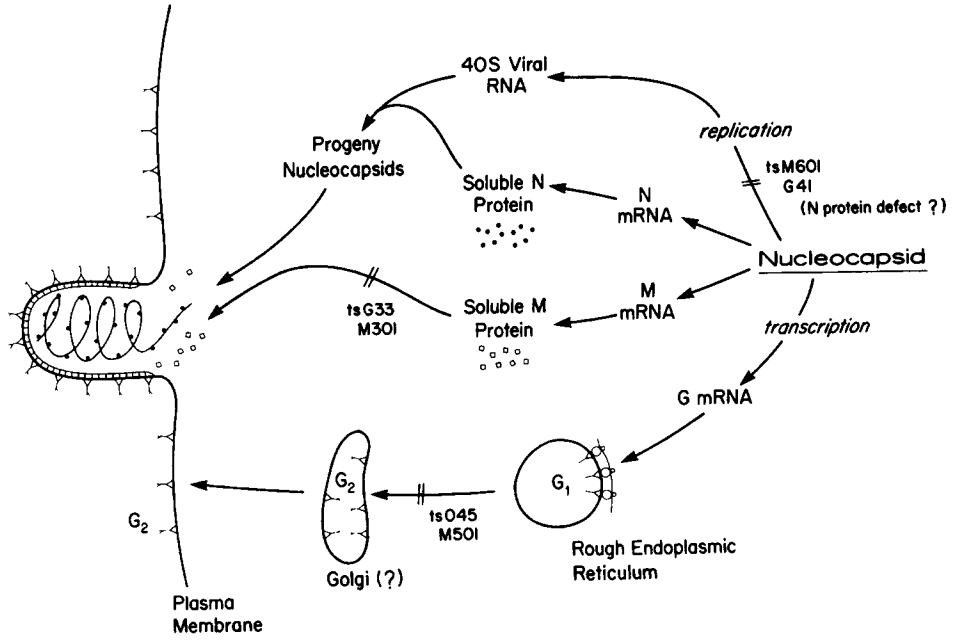

Fig. 1. Schematic diagram illustrating the pathways of maturation of the major structural proteins of VSV and the proposed site of block in virion assembly for certain temperature-sensitive mutants.

must make use of the host cell for the biosynthesis and assembly of viral components, including the viral membranes. Furthermore, the VSV G protein is, we shall show, an example of a major class of cellular membrane proteins which asymmetrically span the lipid bilayer. Thus, a study of the biogenesis of the G protein should provide insight into the mechanisms by which a large class of cell surface glycoproteins are manufactured and processed.

In this paper, we shall focus on the mechanism by which the G protein is inserted asymmetrically into the endoplasmic reticulum (ER) membrane. We shall show that G is synthesized as a transmembrane protein with about 30 amino residues at the carboxyl terminus remaining on the cytoplasmic face of the endoplasmic reticulum membrane. Most or all of the amino terminal 95% of the G protein, containing carbohydrate residues, appears to be within the lumen of the endoplasmic reticulum since it is resistant to attack by external protease. Synthesis, glycosylation, and proper asymmetrical insertion of G into the ER can be achieved in cell-free extracts. Both glycosylation of G and transmembrane insertion into the ER membrane in this cell-free system require concomitant protein synthesis. Attachment of G mRNA to membranes is apparently mediated by the nascent glycoprotein chain.

Multiple Forms of the VSV Glycoprotein: Intracellular Processing

Before discussing experiments on the mode of biosynthesis of G, it is first necessary to describe and characterize in some detail the several forms of G which can be isolated from infected cells or produced in a wheat germ cell-free system using exogenous VSV

mRNA (13). Indeed, the existence of multiple forms of the G protein with distinct subcellular localizations has revealed a complex sequence of steps in which the G protein participates between its synthesis in the rough endoplasmic reticulum and its incorporation into budding viral particles. This intracellular processing mechanism is believed to be of general significance and is, therefore, interesting in its own right.

Infected cultures were labeled with [^{35}S] methionine for 5 min at 4 h postinfection, chased for various periods of time, and the total cellular protein analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 2). Following the pulse label, the G protein was observed as a single species of apparent molecular weight 65,000 which we call G_1. During the period of chase, this species was progressively converted to a more slowly migrating species, called G_2, with an apparent molecular weight 67,000. The difference in mobility is related, in part, to differences in the extent of glycosylation (see below). No changes in mobility of the other VSV proteins (N, NS, M, L) were apparent.

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 (6, 14, 15). To investigate this

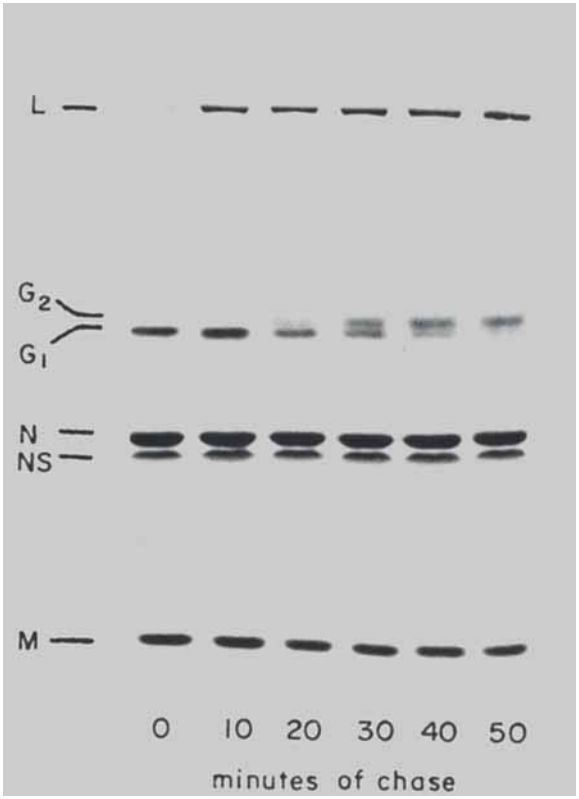

Fig. 2. Pulse-chase labeling of total cellular proteins of cells infected with VSV. Chinese hamster ovary (CHO) cells were infected at an MOI of 10 with VSV and incubated at 37°C. The culture was labeled for 5 min at 4 h postinfection with [^{35}S] methionine as described in Ref. 13, and then excess unlabeled methionine was added. Portions of the culture were removed at the times shown and transferred into cold Earle's 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.

question directly, the polypeptide made in a wheat germ cell-free system programmed with total VSV mRNA (Fig. 3, lane b) was 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 of cells, 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 (6, 8, 14, 15); its rapid migration relative to G_1 and G_2 would be consistent with the absence of carbohydrate in G_0. As is shown below, G_0 will not adhere to lectin columns which will adsorb G_1 and G_2. If the extents of glycosylation of G_0 and G_1 are different, 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 infected cells.

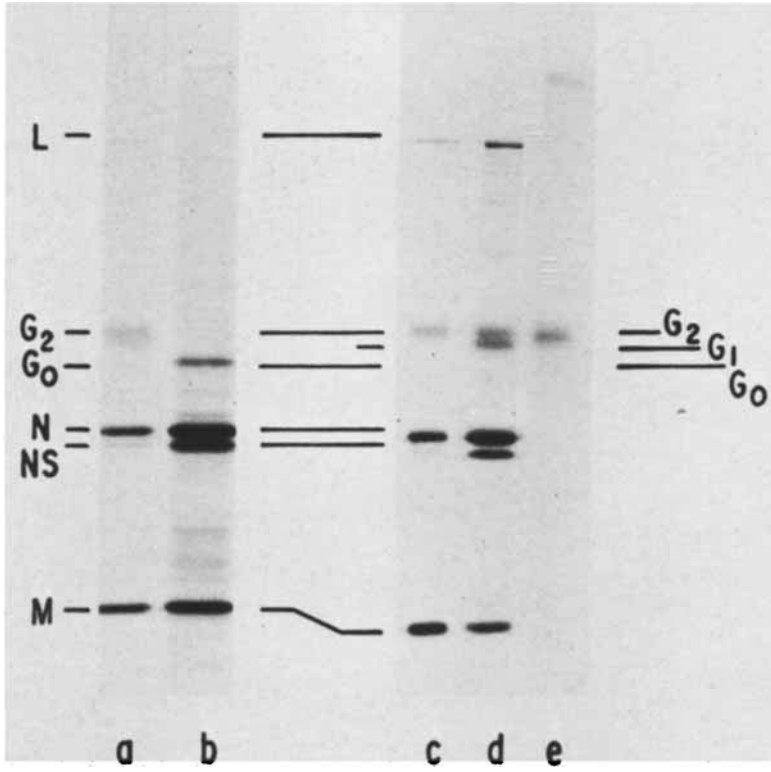

Fig. 3. Comparison of the electrophoretic mobilities of the various forms of the VSV glycoprotein on 10% SDS-polyacrylamide slab gels. Lanes a and b are from one gel, and c–e are from a second gel (run in parallel). Lane a. [^{35}S]methionine-labeled cytoplasmic VSV proteins from infected cells labeled for 30 min and chased for 30 min (10). 48-h exposure. Lane b. [^{35}S]Methionine-labeled proteins synthesized in a wheat germ cell-free extract in response to addition of total cytoplasmic VSV mRNA using the conditions of Morrison and Lodish (6). 16-h exposure. Lane c. [^{35}S]Methionine-labeled virion proteins. 48-h exposure. Lane d. [^{35}S]Methionine-labeled total cellular proteins from infected cells labeled from 4 to 4.5 hr post-infection. 48-h exposure. Lane e. ^{125}I-labeled surface proteins of infected CHO cells. Cells were labeled in a peroxide-lactoperoxidase-catalyzed reaction as detailed in Ref. 13. 48-h exposure. The few minor bands at the top of the gel are normal CHO cell surface proteins and are similar in intensity to iodinated proteins from uninfected cells.

The forms of the glycoprotein in virions and on the surface of infected cells (Fig. 3, lanes c–e) were also examined. Virions contained only the G_2 species (Fig. 3, lane c). In addition, lactoperoxidase-catalyzed iodination of the surface of infected cells revealed a virus-specific protein comigrating with G_2 (Fig. 3, lane e). 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.

In cells labeled with [^{3}H]glucosamine or [^{3}H]galactose, radioactivity is incorporated into only 2 species, comigrating with G_1 and G_2. Thus, both forms are at least partially glycosylated (13). Neuraminidase converts G_2 into a form comigrating with G_1; thus the difference in electrophoretic mobility between G_1 and G_2 is due largely, if not completely, to the presence of N-acetylneuraminic acid residues on the G_2 form (13).

Subcellular fractionation studies showed that all cellular forms of the glycoprotein are bound to membranes (10, 16). G protein labeled during a short pulse with radioactive amino acid was partially glycosylated (G_1) and was localized predominantly in fractions enriched in rough microsomes. Completion of glycosylation [conversion of G_1 to G_2] occurred only following movement to smooth membrane fractions. Addition of the terminal sialic acid residues and movement of G to smooth membranes appear to be coordinate events since some thermolabile mutant glycoproteins both remain localized in the rough microsomes and remain in the partially glycosylated G_1 form (17).

It is of considerable interest that glycosylation appears to be complete before the G protein reaches the surface membrane (13). To develop an assay for the time of appearance of the G protein on the cell surface, the ability of chymotrypsin to remove specifically proteins at the cell surface was exploited. Surface G protein, iodinated by lactoperoxidase catalysis (Fig. 3, lane 3), can be removed by chymotrypsin treatment (Fig. 4) without affecting the pattern of Coomassie blue-stained cell proteins or of N, NS, or L proteins in infected cells. 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. 4) to determine when G protein reaches the cell surface (i.e., when G protein becomes sensitive to treatment of intact cells with protease). 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 10–20 min lag period before the G_2 protein was converted to a protease-sensitive form. 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 precursor G_1 protein was resistant to treatment of intact cells with protease. Since sialic acid is the terminal sugar on each of the 2 carbohydrate chains bound to G, it appears that the G protein is completely glycosylated inside the infected cell approximately 10–20 min prior to its exposure on the surface of the cell. Glycosylation is evidently not the limiting factor in movement of G protein to the cell surface.

INTERACTION BETWEEN G mRNA AND THE ENDOPLASMIC RETICULUM

Newly made glycoprotein is associated with the rough microsome fraction, and all of the G mRNA is attached to the ER membrane (6–10, 16). This suggests a close coupling of G biosynthesis to insertion into the membrane. As we shall show in this section, puromycin, a drug which causes premature termination of growing peptide chains, also causes release of the G mRNA from the membrane fraction, presumably the result of dissociation

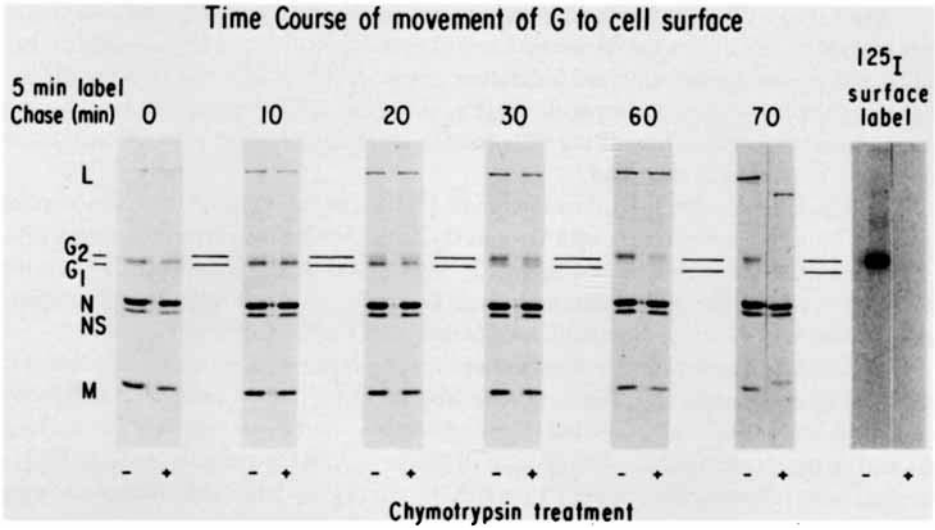

Fig. 4. The time course of movement of the G protein to the cell surface. A culture of infected cells was exposed to [^{35}S] methionine for 5 min at 4 h postinfection at 37°C. Following the labeling period, excess unlabeled methionine was added and the incubation continued at 37°C. At the times indicated, portions of the culture were removed and transferred into 4 volumes of ice-cold Earle's saline. Each sample of cells was washed with phosphate-buffered saline (PBS) 3 times. The cells were resuspended in PBS, and one half of each sample was incubated in PBS with 1 mg/ml chymotrypsin at 37°C for 10 min, while the remainder was incubated in PBS only for the same period. Following this incubation, the inhibitor phenylmethylsulfonylfluoride was added to 2 mM and incubation 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 Ref. 13 using infected cells at 4 h postinfection. Following 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.

of ribosomes from the ER membrane. This suggests that the attachment of ribosome-G mRNA complexes to membranes is mediated primarily by the nascent glycoprotein chain, a conclusion which is consistent with the close coupling of G protein polypeptide synthesis and insertion into the membrane, as revealed by in vitro biosynthesis studies (see below).

To determine the distribution of VSV G mRNA in the free and membrane-bound compartments of infected CHO cells, cells were labeled with [^{32}P] PO_4 from 1 to 4 h postinfection. Postnuclear supernatants from control cultures and from cells treated with puromycin were prepared in a buffer containing a low concentration of salt (0.01 M KCl). They were then analyzed in a sucrose density gradient of the same ionic composition; these contained a cushion of 55% (wt/wt) sucrose overlaid with a linear 15–40% sucrose (wt/wt) gradient. The centrifugation conditions were chosen to separate membranes, which banded isopycnically at the 40–55% sucrose interface, from free polysomes and ribosomes which sedimented in the top half of the gradient. Analysis of ^{32}P radioactivity in each gradient fraction which was extractable into a solution of chloroform:methanol (2:1) provided a measure of the distribution of phospholipids; as expected, the great majority of the labeled phospholipids were found at the 40–55% interface, coincident with the visible band of membranes (Fig. 5, panel II).

In control cells, about 35% of the VSV-specific mRNA cosedimented with the membrane fraction (Fig. 5, panel I). Polyacrylamide gel electrophoresis resolves VSV RNA into 4 classes (c.f., Fig. 6). Band 2 (mol. wt. = 7.4×10^5) is the mRNA for VSV G protein. Band

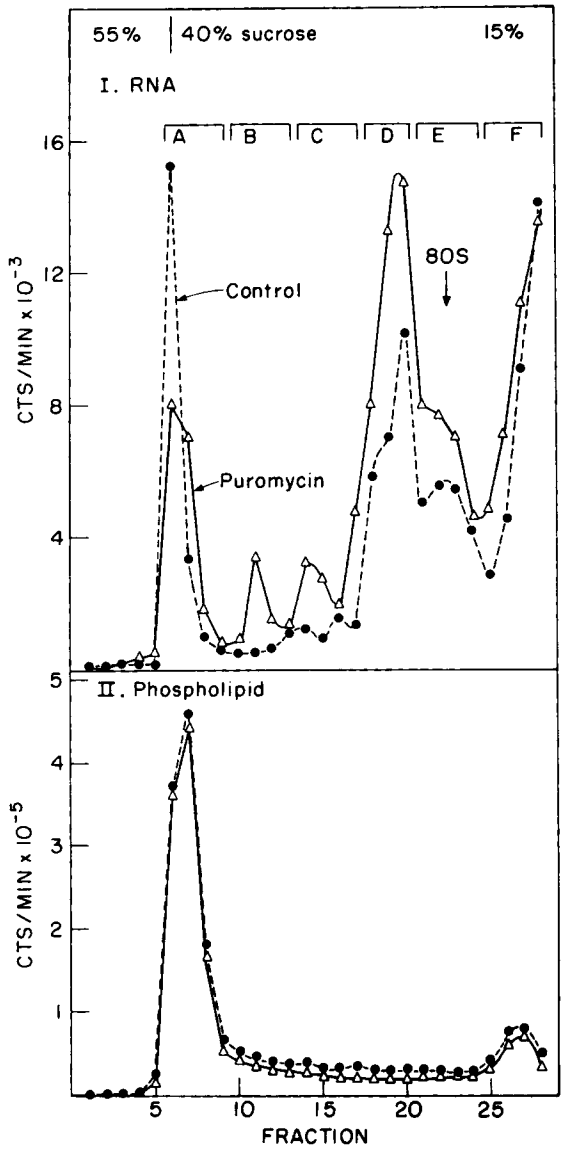

Fig. 5. Fractionation of ^{32}P-labeled RNA and phospholipid in VSV-infected cells. A postnuclear supernatant prepared from ^{32}P-labeled VSV-infected cells treated (triangles) or not (circles) with puromycin was fractionated on a sucrose gradient as detailed in Ref. 19 and in the text. Aliquots of each fraction were extracted with a mixture of chloroform and methanol. Shown in the top panel (I) is the distribution of radioactivity which was not extracted into the organic phase but which was precipitable with trichloroacetic acid. The bottom panel (II) shows the distribution of radioactivity which was extracted into the organic solvent. Sedimentation is from right to left. Brackets indicate fractions which were pooled for isolation of RNA.

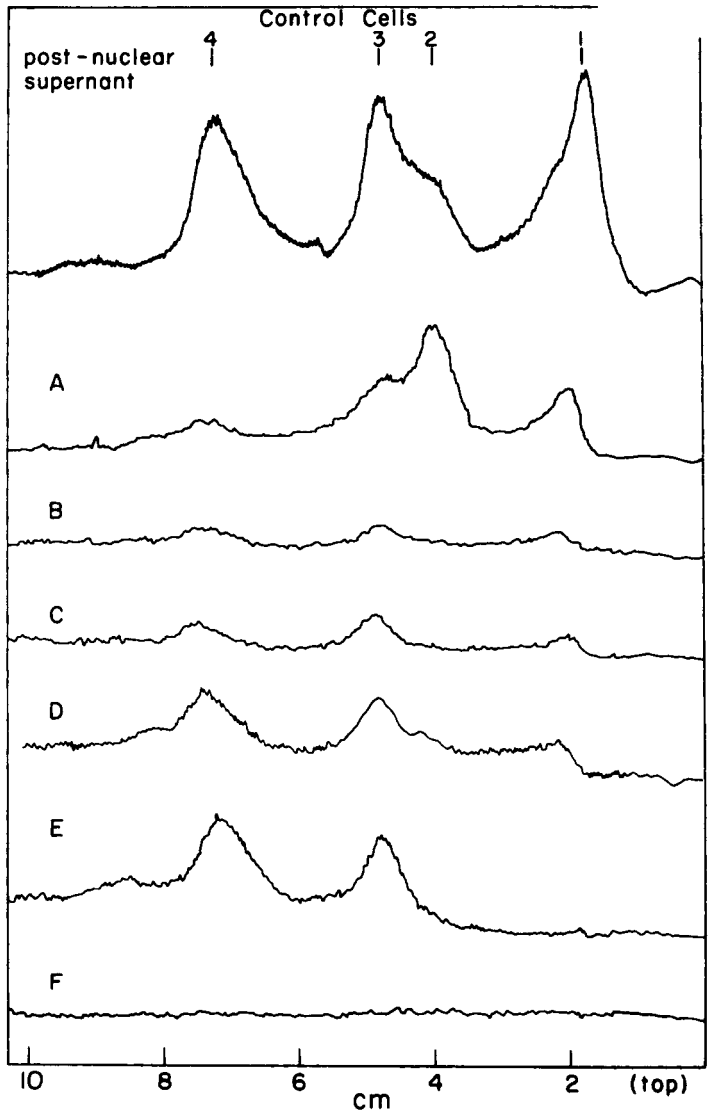

Fig. 6. Formamide-polyacrylamide gel analysis of ^{32}P-labeled RNA isolated from subcellular fractions from control VSV-infected cells. RNA was extracted from the postnuclear supernatant or from pooled gradient fractions of the experiment depicted in Fig. 5. Shown are scans of the radioautograms of the polyacrylamide gel.

1 is a mixture of 42S virion RNA and 26S mRNA for L protein. Band 3 (mol. wt. = 5.5 × 10^5) encodes the N protein, and band 4 (mol. wt. = 2.8 × 10^5) is a mixture of mRNAs for the M and NS proteins (15, 18). As is shown in Fig. 6, over 90% of the G mRNA (band 2) is associated with the membrane fraction. Over 80% of the ^{32}P radioactivity in bands 3 and 4, by contrast, sediments with free polyribosomes, in agreement with our earlier results (6). The interaction of G mRNA with membranes is maintained when the latter are floated to equilibrium in a sucrose density gradient (19).

Treatment of cells with puromycin 15 min before harvesting results in selective release of G mRNA from membranes (Figs. 5, 7). Only 20% of G mRNA remains associated with membranes; 80% sediments as free polysomes (fractions B–D) or ribosomes (panel E). In both puromycin-treated and control cells, about 20% of the VSV mRNA bands 3 and 4 – encoding the soluble proteins N, NS, and M – cosediments with the membrane

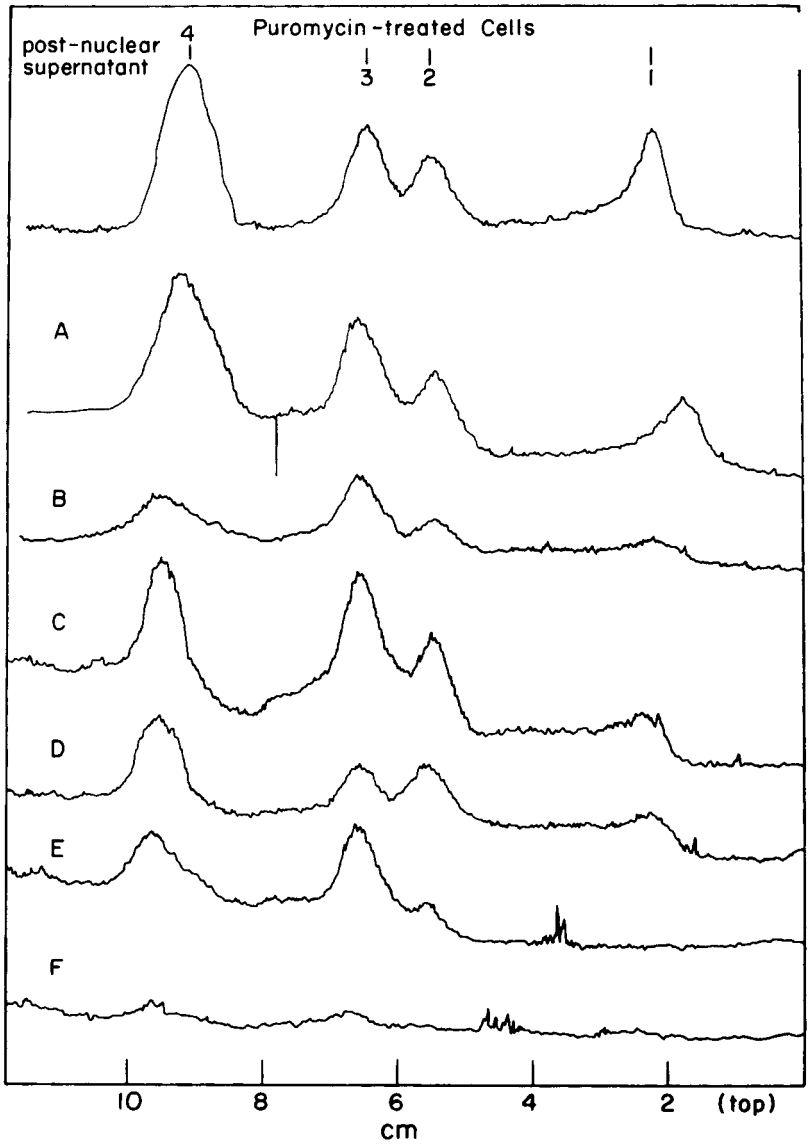

Fig. 7. Polyacrylamide gel analysis of ^{32}P-labeled RNA isolated from subcellular fractions from puromycin-treated VSV-infected cells. RNA was extracted from the postnuclear supernatant or from pooled gradient fractions of the experiment depicted in Fig. 5. One twentieth of each preparation of RNA was analyzed, except for fraction A, for which 0.1 of the preparation was used. Hence, to compare A with the other fractions, areas under the peaks in A should be divided by 2.

fraction. Presumably this is due to nonspecific adsorption since mRNAs for bands 3 and 4 are selectively lost during equilibrium centrifugation of the membranes. The 20% of the G mRNA remaining in the membrane fraction of puromycin-treated cells might also be due to nonspecific adsorption.

Treatment with puromycin in a buffer of high ionic strength (100–500 mM KCl) is required to dissociate from pancreatic, myeloma, and liver ER membranes most of the ribosomes and also mRNA active in directing synthesis of secretory proteins (20–22). A similar result was obtained with the Sindbis 26S mRNA, which directs the synthesis of 2 virus glycoproteins and 1 soluble protein: removal of ribosomes and of 26S mRNA from ER membranes required treatment of cells with puromycin and also addition of high-salt buffer to the isolated membranes (23). Presumably, ionic linkages between the ribosomes and/or nascent chains and the ER membrane, supposedly those disrupted by high-salt solutions, are of less importance for binding of VSV G mRNA than for Sindbis 26S RNA or mRNAs encoding secretory proteins.

These experiments suggest that there is a direct interaction between the nascent G chain and the ER membrane and also indicate an absence of a direct interaction between G mRNA and membranes.

THE TOPOLOGY OF NEWLY MADE G – SYNTHESIS AS A TRANSMEMBRANE PROTEIN

Our efforts were next directed toward elucidating the manner in which the newly completed G protein is bound to the ER membrane. To this end, infected cells were labeled for 10 min with [^{35}S] methionine, and a crude microsomal membrane fraction was prepared. By comparison with marker proteins, it was shown that this preparation (Fig. 8, lane 7) contained only the G_1 form of the G protein and also various amounts of other VSV structural proteins, L, N, NS, and M. To determine which polypeptides were accessible to added protease, a portion of the preparation was digested with trypsin. As can be seen (column 9), essentially all of the labeled L, N, M, and NS proteins are sensitive to proteolysis. [The amount of N which is resistant to trypsin remains so when the membranes are destroyed by sodium deoxycholate (column 8); it is probably in nucleocapsids.]

By contrast, G is largely resistant to proteolysis, and this protection is afforded by the permeability barrier of membrane vesicles. Trypsin does convert G to a form which migrates slightly faster on polyacrylamide gels; this change in mobility is consistent with a loss of 30–50 amino acids from the polypeptide chain. All of the methionine-containing tryptic peptides found in this fragment are also found in authentic G protein; this establishes that this fragment indeed is derived from G (Fig. 9). The resistance of newly made G to protease is dependent on the integrity of a membrane barrier; treatment of the preparation with the detergent sodium deoxycholate (Fig. 8, lane 8) before proteolysis results in essentially complete digestion of G.

Note that the proteolytic fragment of G lacks 3 methionine-containing tryptic peptides characteristic of authentic G (arrows in Fig. 9). By the use of a procedure devised by Dintzis (24), it has been possible to establish the relative order of the methionine-containing peptides of the G protein (25). The 3 peptides which are lost from G upon proteolysis are located closest to the carboxyl terminus of the protein than are any other

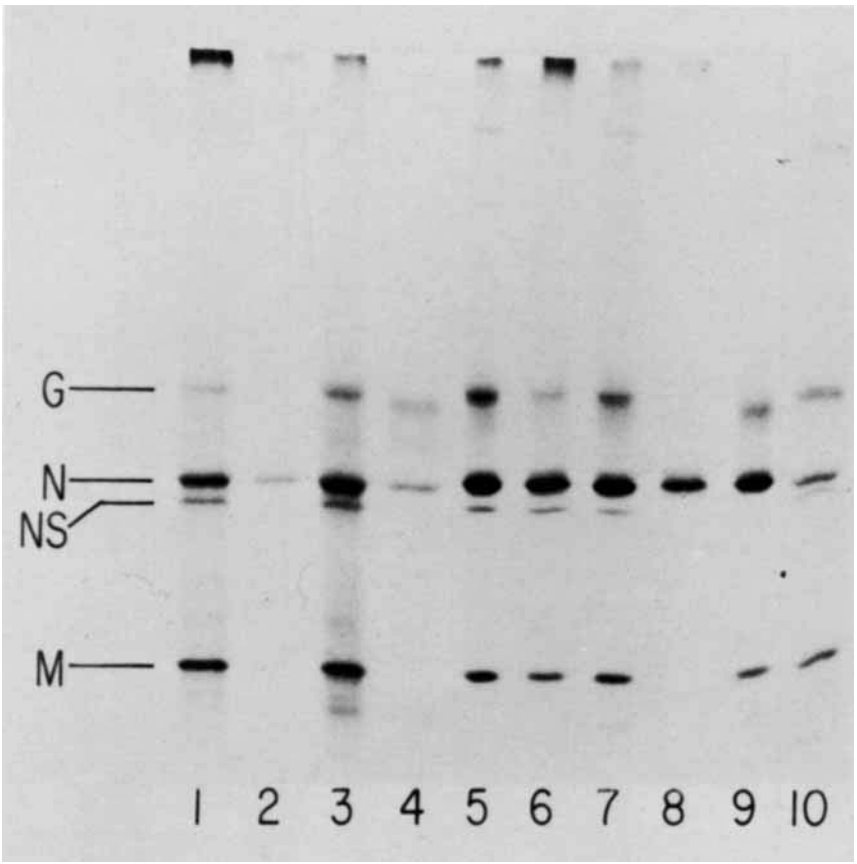

Fig. 8. Transmembrane biogenesis of VSV glycoprotein. Lanes 1–4: CHO cells infected with VSV (3 PFU/cell) in the presence of actinomycin D were harvested at 4 h postinfection. The cells were swelled in a hypotonic buffer (10) and broken by 40 strokes of a tight dounce homogenizer. Nuclei were removed by low speed centrifugation. The postnuclear supernatant was centrifuged at 20,000 × g for 30 min and the pellet resuspended in buffer S (50 mM KCl, 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$). This fraction was added to an S100 preparation from uninfected cells and a protein synthesis mixture containing [^{35}S] methionine, as previously described (23). After a 30 min incubation at 37°C, aliquots were treated for 30 min at 37°C with protease and/or detergent as indicated below. All reactions were subsequently treated with soybean trypsin inhibitor at 100 μg/ml for an additional 30 min at 37°C before being run on 10% SDS-polyacrylamide gels. Additions: 1) 1% DOC (sodium deoxycholate); 2) 1% DOC + trypsin (100 μg/ml); 3) trypsin (100 μg/ml) + soybean trypsin inhibitor (100 μg/ml) added simultaneously; 4) trypsin (100 μg/ml).

Lanes 5–10: At 4 h postinfection, cells were pelleted, resuspended in MEM supplemented with 1% dialyzed calf serum but lacking methionine, and labeled for 10 min with [^{35}S] methionine (100,000 mCi/mmole) at 24 μCi/ml. These cells were harvested, broken, and a 20,000 × g pellet prepared as above. The pellet was resuspended in buffer S and incubated for 30 min at 37°C in the presence of protease and/or detergent as indicated below. All reactions were incubated an additional 30 min in the presence of soybean trypsin inhibitor (100 μg/ml) and run on 10% SDS-polyacrylamide gels. Additions: 5) H_2O, 6) 1% DOC + soybean trypsin inhibitor (100 μg/ml); 7) trypsin (100 μg/ml) + soybean trypsin inhibitor (100 μg/ml); 8) trypsin (100 μg/ml) + 1% DOC; 9) trypsin (100 μg/ml); 10) marker from VSV-infected cells.

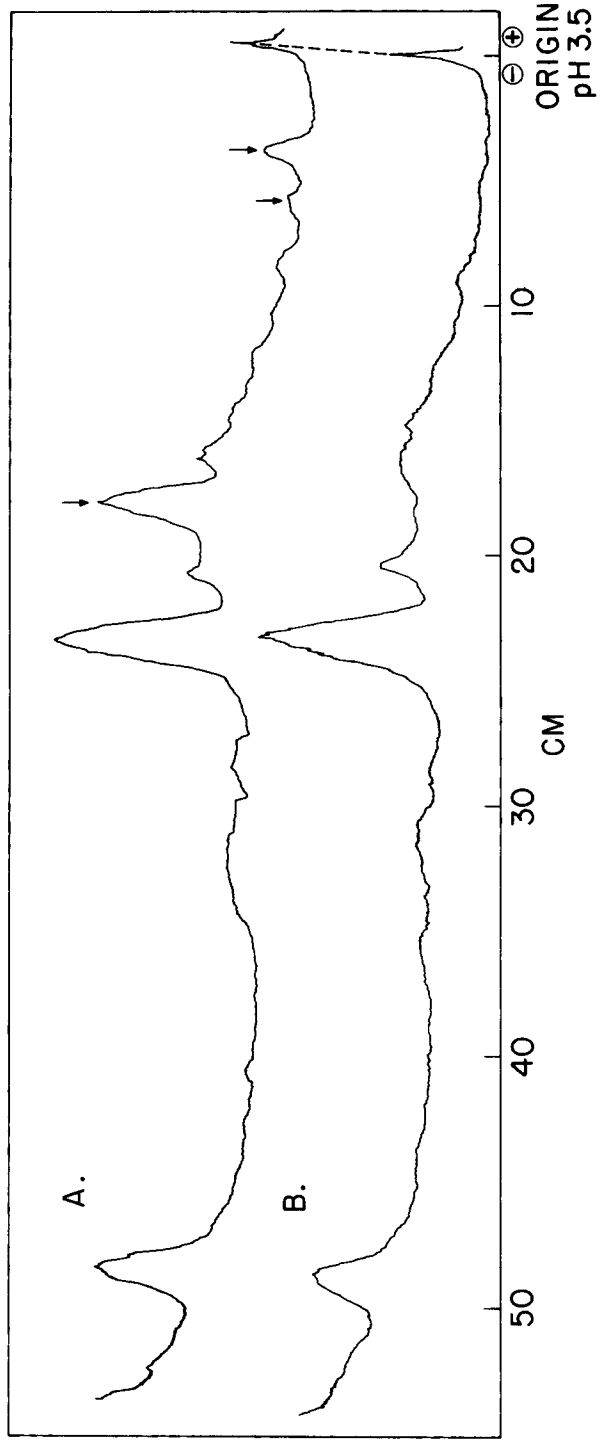

Fig. 9. Tryptic digests of [^{35}S]methionine-labeled G_1 and its protease-resistant fragment. Tryptic peptides of G protein (A) and it protease-resistant derivative (B) found associated with microsomal membranes derived from labeled infected CHO cells were prepared and paper ionophoresis at pH 3.5 performed as previously described (15). Microdensitometer tracings were made from autoradiograms of the electrophoretograms.

methionine-containing peptides. Indeed, these peptides must reside in the carboxy-terminal 5% of G_1 since about 95% of the polypeptide chain is protected from proteolysis.

Similar results were obtained if a microsome fraction was prepared from (unlabeled) cells and allowed to complete synthesis of nascent chains initiated in vivo in a cell-free system. Labeled G_1 protein is made in vitro (Fig. 8, lane 1); digestion of the reaction products with trypsin again results in conversion of G to a smaller fragment (Fig. 8, lane 4). Again, treatment with deoxycholate results in complete digestion of G (lane 2).

It is important to point out that all of the G protein completed in the cell-free system is in the G_1 form. Since it is unlikely that any movement between membrane fractions takes place in this extract, and since G is made on rough microsomal vesicles, this glycosylation of G takes place in the rough microsomes.

We conclude that G is a transmembrane protein immediately after its synthesis in the rough ER. The 30–50 amino acid residues, and the 2 methionine-containing peptides removed from the extreme carboxy-terminal region, are located on the outside of the vesicle, that side which faced the cytoplasm in the cell. This follows from the fact that the factors required for completion of G chain in vitro could only have interacted with ribosomes located on the outside of the membrane vesicles, the same side to which protease was added. From the requirement for disruption of the lipid bilayer by treatment with detergent for the extensive proteolysis of G_1, it is inferred that G must have protease-sensitive sites on the lumenal side of the ER. Thus, it appears to be transmembrane. While it may also be tentatively concluded that most or all of the protected amino-terminal 95% of the polypeptide is either facing the lumen of the ER or is imbedded in the lipid bilayer, these studies do not rigorously eliminate the possibility that more than 5% of the protein is exposed to the cytoplasmic face.

As was noted previously, G is partially glycosylated (G_1 form) concomitantly with or immediately after its synthesis. To show this (25), we took advantage of the finding that G_1 is specifically adsorbed to a column of Sepharose to which is covalently linked the lectin Concanavalin A (Con A). Presumably, this is due to the specific binding of α-mannosyl residues of G_1 to the Con A since the binding is prevented by α-methylmannoside but not by galactose, a sugar which does not bind to Con A. [Fig. 11 illustrates a similar experiment on G_1 made in the reconstituted cell-free system.]

IN VITRO GLYCOSYLATION AND ASYMMETRIC INSERTION OF THE GLYCOPROTEIN INTO THE ENDOPLASMIC RETICULUM MEMBRANE

To understand how specific membrane proteins are inserted only into the appropriate cytoplasmic membrane, to elucidate the molecular basis of asymmetry of membrane proteins such as G (26–28), and to understand how the extracytoplasmic portions of G cross the membrane, it is necessary to develop a cell-free system capable of proper synthesis and insertion of this protein. On the hypothesis that secreted proteins and the external portions of transmembrane glycoproteins such as G cross the endoplasmic reticulum membrane by similar mechanisms (28, 29), we have employed a modification of the cell-free system devised by Blobel and Dobberstein for secretion (30). In this system, secreted proteins such as immunoglobin are specifically transferred into the lumen of vesicles from pancreatic rough endoplasmic reticulum. This segregation process requires that the light chains be synthesized in the presence of the membrane vesicles. Free ribosomes, initially unbound to membranes, will suffice for proper segregation (30). In this section, we report experiments (31) done in collaboration with G. Blobel and V. Lingappa in which the VSV

G protein is translated by extracts of wheat germ in the presence of canine pancreatic rough ER vesicles. The G protein is incorporated into the membrane during or immediately after its synthesis, spans the ER membrane asymmetrically and properly, and is glycosylated.

It will be recalled that translation of VSV mRNA by wheat germ extracts results in production of a form of G, termed G_0, which is apparently unglycosylated (Fig. 3). Figure 10A, lane 3, shows that when VSV mRNA is translated by wheat germ ribosomes in the presence of "stripped" rough pancreatic microsomes (the ribosomes having been removed with 20 mM EDTA), a polypeptide appears which is absent when membranes are not included during protein synthesis (lane 1). This protein comigrates with G_1 found in the cytoplasm of infected cells and has thus far proven to be indistinguishable from G_1 in all respects. Increasing concentration of membranes results in increasing yields of G_1 relative to G_0 (Fig. 10A, lanes 3, 5, 7). Production of G_1 is not accomplished by ribosomes endogenous to the pancreatic membrane since these were removed by treatment with 20 mM EDTA before and since the total level of G made does not increase with increasing membrane concentration. Note that when membranes are added after synthesis, only G_0 is found (Fig. 10B, lanes 3, 6).

As is the case for the cytoplasmic G_1, the G_1 formed in this cell-free system is glycosylated. Figure 11, panels 1 and 2, shows that of the products made in the cell-free system, only G_1 but not G_0 or other VSV proteins will adhere specifically to Con A Sepharose. Binding of G_1 is specifically prevented by the ligand α-methylmannoside (lane 3) but not by the nonspecific sugar galactose (lane 4). Thus, G_1 formed in vitro contains at least the mannose-rich core region of the mature G_2 form of the glycoprotein. By inference, G_0 contains few, if any, mannose residues.

As is the case with the cytoplasmic form of G_1, G_1 formed in vitro asymmetrically spans the membrane bilayer. Figure 10A, lane 2, shows that the G_0 form of G is completely sensitive to exogenous protease, irrespective of whether membranes are present or not (Fig. 10B, lanes 5, 6). By contrast, the G_1 formed in vitro is resistant to the action of trypsin, apart from the removal of a discrete portion of G_1, resulting in a fragment which is shorter by 30–50 amino acids (Fig. 10A, lanes 4, 6, 8). This partial fragment of G comigrates with the analogous fragment of G_1 produced by protease digestion of ER vesicles isolated from infected cells (Fig. 8). Furthermore, tryptic maps of these 2 [^{35}S]-methionine-labeled G_1 fragments are similar (data not shown). The protection afforded most of the G_1 polypeptides formed in vitro results from the permeability barrier of the pancreatic membranes since if membranes are treated with 1% Triton X-100 (Fig. 10B, lane 8) or 1% deoxycholate (not shown) before proteolytic digestion, no G_1 is protected. Because Triton X100 is a nondenaturing detergent which disrupts membranes by displacing native lipid molecules from hydrophobic binding sites of native membrane proteins (32), it is extremely unlikely that the increased susceptibility of G_1 to protease induced by it is due to partial denaturation of the protein.

Figure 11 (lanes 5–8) shows that this proteolytic fragment of G contains at least some mannosyl residues. It binds specifically to columns of Con A, and its binding is inhibited by the ligand α-methylmannoside, but not by the nonspecific sugar galactose. Thus, some, if not all, of the mannose residues on G_1 are protected by the ER membrane from proteolytic digestion and are almost certainly located within the lumen of ER-derived vesicles.

We conclude that when the VSV glycoprotein is synthesized in vitro in the presence of pancreatic microsomes, a form of the glycoprotein is produced which is indistinguishable

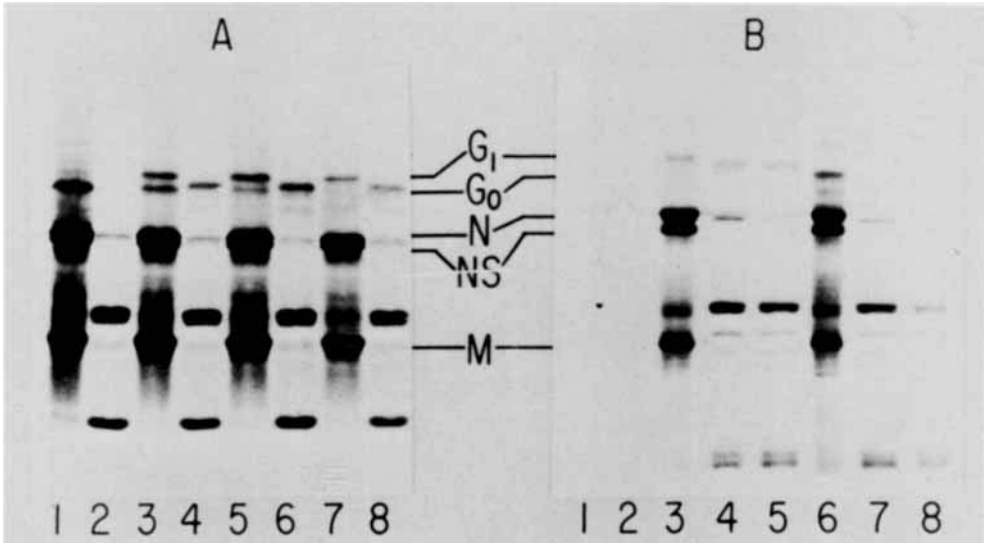

Fig. 10. Synthesis of the vesicular stomatitis viral glycoprotein in the presence of pancreatic endoplasmic reticulum. A) The effect of increasing concentrations of membranes and of trypsin treatment. Proteins were synthesized in 100 μl incubations for 1 h in the presence of pancreatic membranes. The latter were prepared (31) by treatment of pancreatic microsomes with EDTA (0.01 M), followed by centrifugation through a sucrose cushion, a procedure modified from Ref. 30. Reactions in lanes 1 and 2 contained no membranes; those in 3 and 4, 0.5 A_{280} units; 5 and 6, 1.0 A_{280} units; 7 and 8, 2.5 A_{280} units. Standard wheat germ reaction conditions (6, 36) containing VSV mRNA (37) were employed. After 1 h of incubation, pancreatic ribonuclease A (10 μg/ml) was added to inhibit further protein synthesis, and each incubation was divided in half. To one half of each incubation (lanes 2, 4, 6, and 8), trypsin (0.5 mg/ml final concentration) was added in 5-μl volume. After 20 min of subsequent incubation at 23°C, proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Shown is a radioautogram of the dried gel. B) Features of the reconstitution reaction: Lane 1, no VSV mRNA or membranes; Lane 2, 0.025 A_{280} units of membranes per 50-μl reaction; Lanes 3–5, A 150-μl reaction containing 0.375 A_{280} units of membranes and VSV mRNA was incubated for 60 min at 23°C. Then pancreatic ribonuclease (10 μg/ml) was added. The incubation was divided into 3 portions which were incubated for 30 min more without trypsin (lane 3), 15 min with 0.5 mg/ml trypsin (lane 4), or 30 min with 0.5 mg/ml trypsin (lane 5).

Lanes 6–7: Proteins were synthesized for 30 min in an incubation (100 μl) to which no membranes were added. At 30 min, pancreatic ribonuclease (10 μg/ml) was added. Five minutes later, membranes (0.05 A_{280} units) were added, and incubation was continued for another 30 min. Then one half of the reaction was incubated with 0.5 mg/ml trypsin for 15 min (lane 7) while the other half was incubated for the same period without trypsin (lane 6).

Lane 8: Proteins were synthesized in the presence of 0.025 A_{280} units of membrane in a total volume of 50 μl for 60 min. The pancreatic ribonuclease, Triton X-100 (1% wt/vol) and trypsin were added, in that order, and incubation was continued for 15 min more.

from the G_1 form found in association with intracytoplasmic membranes from infected cells (Figs. 2–4, 8). Furthermore the product of the cell-free system has the same topographical relationship to the pancreatic microsomal vesicles as does the G_1 form of intracytoplasmic membranes (Figs. 8, 10). Specifically, both cell-free and in vivo G_1 forms span the membrane asymmetrically with their carboxy termini outside the vesicles, are glycosylated, and appear to have most, and possibly all, of their N-terminal portion inside the

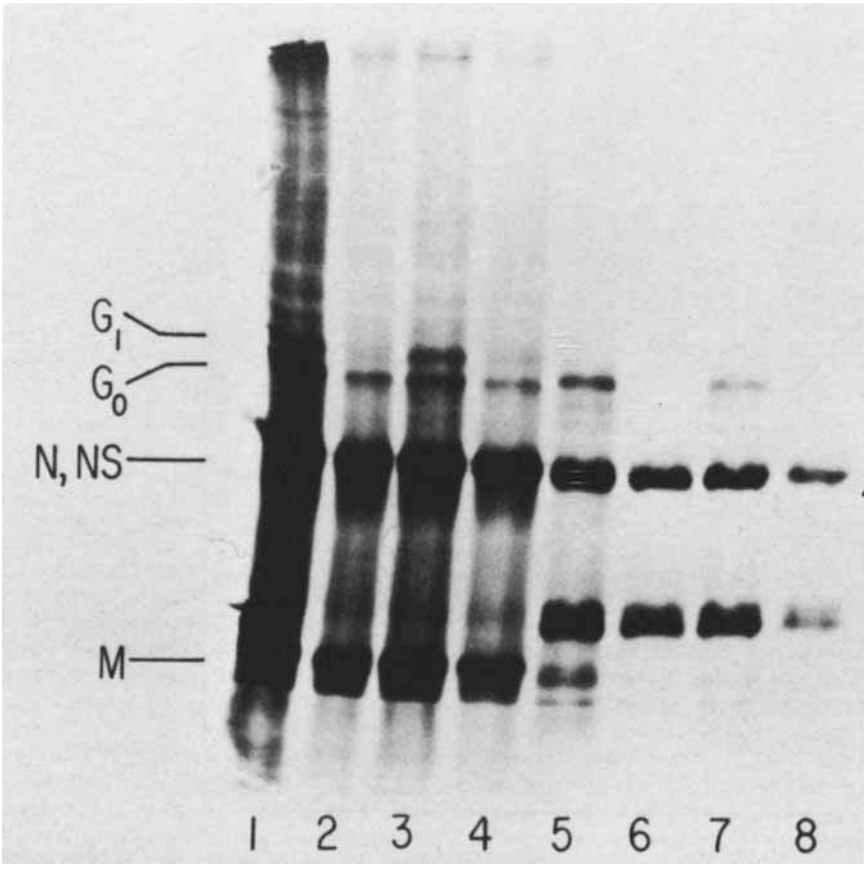

Fig. 11. Binding of vesicular stomatitis viral glycoprotein forms to columns of Concanavalin A Sepharose. Columns of Concanavalin A-derived Sepharose 4B (Pharmacia), 2 cm in length, were formed in Pasteur pipettes. All manipulations were at room temperature. Each column was washed with 5 ml of a buffer containing Hepes (25 mM, pH 7.5), $MnCl_2$ (1 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), bovine serum albumin (0.1 mg/ml), and Triton X-100 (1% w/v). Then columns were washed with 5 ml of Hepes (25 mM, pH 7.5) containing Triton X-100 (1%) and, where appropriate, either galactose (0.2 M) or α-methyl-mannoside (0.2 M). Samples (25 μl) of radioactive polypeptides from protein synthesis incubations were loaded onto the columns and were washed with 2 ml of a buffer containing Hepes (25 mM, pH 7.5) and Triton X-100 (1%). Where appropriate, sugar (galactose or α-methylmannoside) was added to the sample to a final concentration of 0.2 M and was also included (0.2 M) in the buffer used to wash unbound proteins from the column. Proteins were precipitated from the 2-ml fraction containing unbound proteins by adding 8 ml acetone. After 15 min at −20°C, the precipitates were collected by low speed centrifugation, were lyophilized, and were subjected to gel electrophoresis. Gel represents proteins not bound to column.

Lanes 1–4: VSV proteins were synthesized in a total volume of 100 μl which contained 0.05 A_{280} units of membranes. After 60 min, pancreatic ribonuclease (10 μg/ml) was added, and incubation was continued for 5 min more. Then Triton X-100 (1%) was added. Lane 1, total reaction, before affinity chromatography on Concanavalin A Sepharose. Lane 2, chromatography in the absence of sugar. Lane 3, chromatography in the presence of α-methylmannoside. Lane 4, chromatography in the presence of galactose.

Lanes 5–8: VSV proteins were synthesized in a total volume of 100 μl which contained 0.25 A_{280} units of membranes. After 60 min, ribonuclease was added, as above, followed by trypsin (0.25 mg/ml) treatment for 15 min. Then soybean trypsin inhibitor (0.5 mg/ml) was added, and incubation was continued for 10 min more. Immediately before chromatography, Triton X-100 (1%) was added. Lane 5, proteins before chromatography. lane 6, chromatography in the absence of sugar. Lane 7, chromatography in the presence of α-methylmannoside. lane 8, chromatography in the presence of galactose.

vesicle permeability barrier. The glycosylation of an identified membrane protein in a cell-free system represents a novel observation which may potentially be of value in elucidating the temporal sequence, biochemical mechanisms, and the basis for specificity in protein glycosylations.

The observation that synthesis of the glycoprotein in the presence of membranes is required for protection of portions of the glycoprotein from external proteolysis shows that the intravesicular portions of the glycoprotein can only cross the membrane during or immediately following protein synthesis. Together with the fact that the C-terminus of the protein is exposed on the surface of vesicles following synthesis, these observations support a model (28, 29) in which the intravesicular portions of the glycoprotein pass N-terminus first across the membrane during polypeptide chain synthesis. The passage of the chain through the membrane must certainly depend on membrane proteins since when membranes are first treated with N-ethylmaleimide or are heated at 60°C for 10 min, only the G_0 form of the glycoprotein is found, and this is not protected from proteolysis (J.E. Rothman and H.F. Lodish, unpublished experiments).

All of the sugar in the G protein in virions is readily released with external proteases (5), yet sites for binding to Con A are retained in the large fragment of G_1 protected by membranes from trypsin in vitro. Taken together with the observation that synthesis of G in the presence of membranes is absolutely required to yield a glycosylated product, this suggests that glycosylation is restricted to the intravesicular surface (26) corresponding to the lumenal side of the endoplasmic reticulum in cells. In relating the apparently opposite overall orientations of the glycoprotein when synthesized in vitro to that found in virions, it is concluded that the plasma membrane glycoprotein is derived from glycoprotein in intracellular vesicles by a membrane fusion process (33, 34) (Fig. 1).

It cannot be excluded that other forms of the glycoprotein exist as transient intermediates in the process of incorporation into the membrane, or in glycosylation. Thus, a broad spectrum of proteins secreted by pancreas have been shown to possess short-lived hydrophobic N-terminal sequences which are cleaved off during or immediately after protein synthesis (35). These sequences have been proposed (30) to function as "signals" which direct the ribosomes which carry them to specific receptors in the rough endoplasmic reticulum and set up a ribosome-membrane junction which permits the nascent chain to cross the membrane during synthesis. It is entirely possible that a similar N-terminal cleavage may take place during the insertion of the VSV glycoprotein, but is masked by the anomalously high apparent molecular weight of the G_1 form due to its carbohydrate constituents. If so, intermediates which, for example, are cleaved at the N-terminus but not glycosylated (or vice versa) would have to exist, but probably would not be detected in the present experiments since these would be short-lived forms, comprising only a small fraction of the total in the steady state.

The cell-free system for membrane assembly described here appears to be capable of faithfully reproducing all known aspects of the topography and structure of an important class of membrane proteins, ectoproteins (28). These characteristics include an absolute asymmetry in transmembrane orientation, substantial mass located on the extracytoplasmic side of the lipid bilayer, and generally, the presence of sugar substituents on the extractyoplasmic portions of the polypeptide (28). The mechanisms used in this system in the synthesis and assembly of the VSV glycoprotein are almost certainly not unique to this one ectoprotein. This is indicated both by the vast evolutionary disparity of the sources of materials (plants and animals) which function together in the cell-free extracts, and by the fact that none of these components is obtained from viral-infected cells, so that these steps could not depend on the prior expression of viral genes. Therefore,

a detailed biochemical and kinetic examination of the steps involved in the synthesis, processing, and incorporation into membranes of the VSV glycoprotein in the cell-free system described here can be expected to yield valuable insight into fundamental aspects of membrane assembly.

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