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Membrane assembly *in vitro*: Synthesis, glycosylation, and asymmetric insertion of a transmembrane protein

(vesicular stomatitis viral glycoprotein/cell-free protein synthesis/pancreatic microsomal membranes/membrane asymmetry)

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ABSTRACT **Membrane assembly was observed to proceed in cell-free extracts. Specifically, the membrane glycoprotein of vesicular stomatitis virus was synthesized in crude extracts of wheat germ in the presence of membrane vesicles derived from pancreatic endoplasmic reticulum. The resulting glycoprotein spans the lipid bilayer asymmetrically, is glycosylated, and is indistinguishable in these respects from the form of the glycoprotein found in the rough endoplasmic reticulum of virus-infected cells. Both glycosylation and asymmetric transmembrane insertion of the glycoprotein into membranes *in vitro* require protein synthesis in the presence of membranes. The carboxyl-terminal 5% of the polypeptide chain is located on the external surface of vesicles, corresponding to the cytoplasmic surface of the endoplasmic reticulum in cells. Most, or all, of the amino-terminal portion of the glycoprotein, as well as the protein-bound carbohydrate, appears to be located within the lumen of the membrane vesicles. These findings demonstrate that insertion of this membrane protein occurs during or immediately after protein synthesis. The results are consistent with the concepts that the growing membrane protein is extruded across the endoplasmic reticulum membrane amino terminus first and that glycosylation is restricted to the lumenal surface of the membrane. The cell-free system reported here should prove valuable for studying these processes.**

Many membrane proteins asymmetrically span the lipid bilayer (1–3). Frequently, a large proportion of the polypeptide chain, including any carbohydrate, is on the extracytoplasmic side of the membrane. Protein synthesis, in contrast, is restricted to the cytoplasm. This observation focuses attention on a fundamental aspect of membrane assembly: how do the extracytoplasmic portions of these proteins [ectoproteins (3)] reach the extracytoplasmic side of the membrane when it is precisely the inability of these same portions of the completed ectoproteins to cross the lipid bilayer that is responsible for the maintenance of protein asymmetry (3)?

To resolve this question, to understand how specific membrane proteins are inserted into only the appropriate cytoplasmic membranes, and to elucidate the basis of membrane protein asymmetry, cell-free systems capable of membrane protein synthesis and asymmetric insertion are necessary. This requires a membrane protein whose overall topography in the membrane is known and whose mRNA is available in sufficient quantity and purity to permit translation *in vitro*.

The glycoprotein (G protein) of vesicular stomatitis virus (VSV) is well suited for such studies. The G protein is found in the plasma membrane of the infected cell, and after budding of the virus from the cell becomes the spike of the mature virion (4–7). Most of the G protein is located external to the viral (8) or plasma membrane (9) bilayer, where it is accessible to proteases. It is synthesized in the rough endoplasmic reticulum (10–12) by membrane-bound polyribosomes (13, 14). It is partially glycosylated in the endoplasmic reticulum (10–12, §) and is further glycosylated as it is transported in a membrane-bound form from the endoplasmic reticulum to, ultimately, the plasma membrane (9–12). The G protein is an asymmetric transmembrane protein in the endoplasmic reticulum.§ Most of the polypeptide chain appears to be within the bilayer or lumen of endoplasmic reticulum vesicles. However, the extreme carboxyl-terminal region is exposed on that surface of endoplasmic reticulum-derived vesicles which corresponds to the cytoplasmic face in cells.§ Thus, the overall orientation of G protein in the endoplasmic reticulum is the inverse of that in the plasma membrane and the mature virion. mRNA for the G protein can be obtained from virus-infected cells (13, 15) and has been translated in cell-free extracts of wheat germ (13, 16).

Blobel and Dobberstein (17, 18) have constructed cell-free systems in which secreted proteins such as immunoglobulin light chains are specifically transferred into the lumen of vesicles derived from pancreatic rough endoplasmic reticulum. This segregation process requires that light chains be synthesized in the presence of the membrane vesicles.

On the hypothesis (3, 19) that secreted proteins and the external portions of ectoproteins such as the G protein of VSV cross the endoplasmic reticulum membrane by similar mechanisms, an analogous cell-free system for the assembly and synthesis of membrane proteins has now been devised. We report experiments here in which mRNA for the VSV G protein is translated by extracts of wheat germ in the presence of vesicles derived from pancreatic rough endoplasmic reticulum. The G protein thus produced is incorporated into the membrane during or immediately after its synthesis, spans the lipid bilayer asymmetrically, and is glycosylated.

MATERIALS AND METHODS

Wheat Germ Extracts. Extracts of wheat germ for cell-free protein synthesis were prepared as described (20), with the following modifications: wheat germ was ground in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) adjusted to pH 7.2 with KOH and containing KCl (100 mM),

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; VSV, vesicular stomatitis virus; G protein, glycoprotein of VSV; G_0, G_1, and G_2, form of G protein synthesized in extracts of wheat germ, found in intracellular membranes of infected cells, and found in plasma membranes of infected cells and in virions, respectively; Con A, concanavalin A.

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§ F. N. Katz and H. F. Lodish, unpublished data.

magnesium acetate (1 mM), $CaCl_2$ (2 mM), and dithiothreitol (1 mM). The extract was preincubated at 25° for 15 min, as described (20), except with different concentrations of GTP (0.2 mM) and creatine phosphokinase (10 μg/ml). The Sephadex G-25 column was eluted with buffer containing dithiothreitol (1 mM) in place of 2-mercaptoethanol. Extracts were stored in aliquots at −70°; the precipitate that formed upon thawing was removed by low speed centrifugation before use.

Cell-Free Protein Synthesis. Proteins were synthesized (20) in reactions that contained, per 50 μl, 15 μl of wheat germ extract, 2.5 μl of VSV mRNA (where specified), and the following components at the stated final concentrations: Hepes, pH 7.2 (27 mM), KCl (98 mM), magnesium acetate (1.5 mM), dithiothreitol (5.6 mM), ATP (2.6 mM), GTP (260 μM), creatine phosphate (16 mM), rabbit skeletal muscle creatine phosphokinase (42 μg/ml, Calbiochem), spermidine (730 μM), 19 amino acids, except methionine (130 μM each), and [^{35}S]methionine (300 μCi/ml, Amersham-Searle, specific activity 580 Ci/mmol). Membranes were included where specified. Incubation was at 23°, usually for 1 hr.

Gel Electrophoresis. Proteins were analyzed by electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate, as described (21). Proteins were precipitated from cell-free reactions (50 μl) by added 10% trichloroacetic acid (50 μl). The samples were centrifuged for 20 sec at 4° in an Eppendorf 3200 centrifuge, and the pellets were suspended in 50 μl of sample buffer (21). The dye turned yellow due to the acid pH. The sample was then neutralized by the addition of Tris free base until the blue color returned (19). After incubation at 37° for 1 hr and 100° for 1 min, 20 μl of sample was loaded on each lane 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 gels were exposed to Kodak SB54 single-side emulsion, blue sensitive x-ray film for 1 day to make autoradiographs.

Pancreatic Membranes. Canine pancreas was kindly provided by the Surgical Cardiovascular Unit at the Massachusetts General Hospital, Boston, MA. All manipulations were done at 0–4°. One pancreas was rinsed with a buffer containing triethanolamine (50 mM, adjusted to pH 7.5 with HCl), sucrose (0.25 M), KCl (50 mM), $MgCl_2$ (5 mM), and dithiothreitol (2 mM). The fat, major blood vessels, and connective tissue were removed, and the pancreas was minced. The minced tissue was then forced through stainless steel window screen (mesh size approximately 1 × 1 mm), and the resulting pulp was adjusted to 100 ml with the above buffer. A homogenate was prepared with three strokes of a motor-driven Teflon and glass homogenizer (A. H. Thomas, Inc., catalogue no. 3431-E25), and the homogenate was centrifuged for 10 min at 1000 × *g*. This postnuclear supernatant was centrifuged at 10,000 × *g* in the Sorvall SS34 rotor for 10 min, and the resulting supernatant was centrifuged in the Beckman 30 rotor at 30,000 rpm for 2 hr. The microsomal pellet was suspended by homogenization in 20 ml of a buffer containing triethanolamine (pH 7.5, 25 mM), sucrose (0.25 M), and dithiothreitol (4 mM). The volume was adjusted with this buffer until the A_{280} was 85. Then, ribosomes were removed from the rough microsomes by treatment with EDTA. One volume of a buffer containing disodium EDTA (20 mM) and triethanolamine (pH 7.5, 100 mM) was added and mixed. Ten milliliters of this mixture were layered over 1 ml of a buffer containing triethanolamine (25 mM, pH 7.5), sucrose (0.5 M), NaCl (25 mM), $MgCl_2$ (2 mM), and dithiothreitol (4 mM), and centrifuged in the Beckman 40 rotor at 40,000 rpm for 1 hr. The pellet, consisting of "stripped" microsomes (17), was resuspended by homogenization with 1 ml of 20 mM Hepes (adjusted to pH 7.5 with KOH). The volume was adjusted with Hepes buffer so that the A_{280} was 50. Then, one volume of glycerol was added to prevent the formation of ice crystals, and the membrane suspension was stored at −20°. No loss in either the glycosylation or protection activities of membrane preparations has been observed over a period of 5 months.

VSV mRNA. RNA was extracted from BHK-21 cells infected with VSV as described (15), but fractionation by binding to and elution from oligo(dT)-cellulose was omitted. RNA obtained from 1 × 10^8 cells was stored in 0.5 ml of water at −70°.

RESULTS

Forms of the VSV G Protein. When RNA isolated from VSV-infected cells is translated in extracts of wheat germ, synthesis of virus-coded proteins is observed (13, 16). Except for the G protein, all of the proteins synthesized *in vitro* comigrate with their *in vivo* counterparts (13, 16). A polypeptide with the methionine-containing tryptic peptides of G protein is produced *in vitro*, but it has a lower apparent molecular weight than the virion G protein, as revealed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. This difference has been explained by assuming (13, 16) that the *in vitro* form of the G protein, referred to as G_0, is an unglycosylated precursor of the virion glycoprotein, referred to as G_2. Another form of the glycoprotein has been detected by pulse-labeling experiments with intact cells (9, 10). This form, termed G_1, is the kinetic precursor of G_2 and migrates between G_0 and G_2 on gels. G_1 is found in intracellular membranes but not in the plasma membrane. The G_1 form is glycosylated but lacks the sialic acid which is contained in the G_2 form (9, 10). The inability to detect G_0 in infected cells even with short labeling periods (9, 10) supports the view that the G_1 form is the direct product of G protein biosynthesis in the rough endoplasmic reticulum, and that partial glycosylation occurs within that organelle, perhaps during protein biosynthesis.

Synthesis of VSV Glycoprotein in the Presence of Pancreatic Microsomal Membranes. Fig. 1*A* shows that when VSV mRNA is translated in the wheat germ extract in the presence of pancreatic microsomes, a polypeptide (labeled G_1) appears (lane 3) which is absent when membranes are not included during protein synthesis (lane 1). This new polypeptide comigrates with the cytoplasmic form of G, called G_1 (data not shown), and is indistinguishable from this *in vivo* form. The *in vivo* and *in vitro* G_1 forms have similar methionine-containing tryptic peptides (Fig. 2 *A* and *C*). Increasing concentrations of membrane (Fig. 1*A*, lanes 3, 5, and 7) result in increased yields of G_1 as compared to G_0, and at sufficiently high membrane concentrations, only G_1 is observed (Fig. 1A, lane 7).

Pancreatic membranes contain no detectable mRNA activity that results in synthesis of discrete proteins (Fig. 1*B*, lane 2). Furthermore, the *in vitro* G_1 form is not synthesized by ribosomes endogenous to pancreatic rough microsomes since the microsome preparations used here were treated with 20 mM EDTA to remove most endogenous ribosomes (22). More importantly, when protein synthesis was initiated by wheat germ ribosomes in the absence of pancreatic microsomes and then further initiations were inhibited with 7-methylguanosine-5′-phosphate and microsomes were added, the subsequently completed glycoprotein chains were all in the G_1 form (J. E. Rothman and H. F. Lodish, unpublished data). The production of G_1 *in vitro* requires synthesis in the presence of membranes. When membranes were added after protein biosynthesis was complete (Fig. 1*B*, lane 6), only G_0 was observed.

The mobility of G_1 made in the presence of microsomes suggests that it is partially glycosylated. To confirm this, the

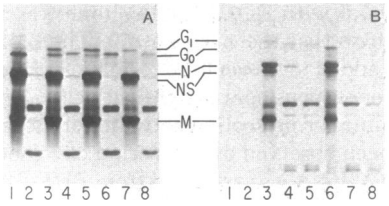

FIG. 1. Synthesis of the VSV glycoprotein in the presence of pancreatic microsomal membranes. N, NS, and M are other viral proteins. (*A*) Effect of increasing concentrations of membranes and of trypsin treatment. Proteins were synthesized in 100-μl incubation mixtures for 1 hr in the absence or presence of increasing amounts of freshly prepared pancreatic membranes before dilution with glycerol and storage at −20°. Lanes 1 and 2, no membrane; lanes 3 and 4, 1 μl; lanes 5 and 6, 2 μl; lanes 7 and 8, 5 μl. After 1 hr of incubation, pancreatic ribonuclease A (10 μg/ml) was added to inhibit further protein synthesis, and each incubation mixture was divided in half. To one half of each mixture (lanes 2, 4, 6, and 8), trypsin (0.5 mg/ml final concentration) was added in a 5-μl volume. After 20 min of subsequent incubation at 23°, proteins were analyzed by gel electrophoresis. (*B*) In this experiment, membranes were used after dilution (1:1) with glycerol and storage. Lane 1: VSV mRNA and membranes were omitted. Lane 2: One microliter of membranes was present in 50 μl of incubation mixture; VSV mRNA was omitted. Lanes 3–5: A 150-μl incubation mixture containing 15 μl of membranes and 7.5 μl of VSV mRNA was incubated for 60 min at 23°. Then, pancreatic ribonuclease (10 μg/ml) was added. The mixture was divided into three portions, which were incubated for 30 min more without trypsin (lane 3), 15 min with trypsin (0.5 mg/ml) (lane 4), or 30 min with trypsin (0.5 mg/ml) (lane 5). Lanes 6 and 7: Proteins were synthesized for 30 min in an incubation mixture (100 μl) to which no membranes were added. At 30 min, pancreatic ribonuclease (10 μg/ml) was added. Five minutes later, membranes (2 μl) were added, and incubation was continued for another 30 min. Then, half of the reaction was incubated with trypsin (0.5 mg/ml) 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 1 μl of membranes in a total volume of 50 μl for 60 min. Then, pancreatic ribonuclease, Triton X-100 (1%, wt/vol), and trypsin were added, in that order, and incubation was continued for 15 min more.

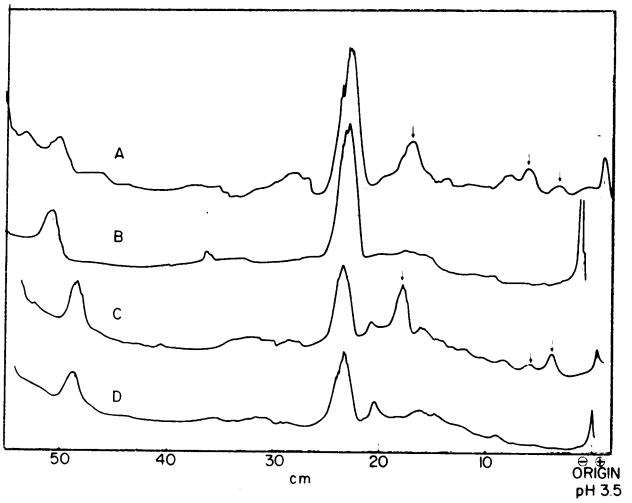

FIG. 2. High-voltage paper electrophoresis of [^{35}S]methionine-labeled tryptic peptides derived from G_1 protein and the fragments of G protein protected by membranes from trypsin digestion. (*A*) Presumed glycosylated form of G protein (G_1) synthesized in the wheat germ extract in the presence of pancreatic microsomes. (*B*) The fragment of G_1 protected by membranes from trypsin digestion. (*C*) G_1 isolated from microsomes of VSV-infected CHO cells that had been labeled for 10 min with [^{35}S]methionine. (*D*) Fragment of this cytoplasmic G_1 protected by membranes from trypsin digestion. The *in vitro* G_1 form (*A*) and its membrane-protected derivative (*B*) were synthesized in a manner similar to that detailed in the legend of Fig. 1. Preparation of microsomes from VSV-infected cells, of cytoplasmic G_1, and of the fragment of G_1 protected by the membranes from protease digestion will be published elsewhere (F. N. Katz and H. F. Lodish, unpublished). All polypeptides were prepared by gel electrophoresis. The appropriate band from the dried gel was located from the autoradiogram, excised, and incubated with trypsin (13). The resulting tryptic peptides elute from the gel. The lyophilized eluates were then subjected to electrophoresis at pH 3.5, 40 V/cm, for 3 hr, as described (13). Each peak apparently represents a unique peptide since no additional peaks were observed upon separation by chromatography in a second dimension (data not shown). Arrows indicate peptides present in G_1 but absent in the protected fragment.

ability of the various forms of the glycoprotein to bind specifically to columns of concanavalin A (Con A)-Sepharose was tested. Con A is a plant lectin which binds to mannose residues present in oligosaccharides (23). Binding is specifically prevented by competition with α-methylmannoside (23). The glycoprotein of vesicular stomatitis virions (G_2) is substituted at two Asn residues (24) with apparently identical oligosaccharides whose "core" (25) contains four mannose residues (12); thus, both G_2 and any partially glycosylated forms that contain the core region of the mature oligosaccharide would be expected to bind specifically to columns of Con A.

Fig. 3 shows that the G_1 form produced in the cell-free system binds to Con A, whereas G_0 does not (Fig. 3, lanes 1 and 2). The binding of G_1 is specifically prevented by α-methylmannoside (lane 3). Thus, both G_1 and G_0 are found in the effluent when α-methylmannoside is present during chromatography (lane 3), but the binding of G_1 is not prevented by galactose (lane 4). Similarly, G_2, isolated from virions, and G_1, isolated from cytoplasmic membranes of infected cells, bind specifically to columns of Con A (data not shown).

Furthermore, endoglycosidase-H (32), which cleaves between the two *N*-acetylglucosamine units of a mannose-rich core region in serum-type glycoproteins, will cleave the G_1 produced in this cell-free system to a form that migrates more rapidly on polyacrylamide gels, but has no effect on G_0. No proteolytic activity has been detected in this enzyme preparation (F. N. Katz, unpublished data).

These observations indicate that G_1 contains the mannose-containing core region of the mature G_2 form of the glycoprotein. They do not conclusively prove, however, that G_0 is unglycosylated, but do indicate that few, if any, mannose residues are contained in G_0.

Glycoprotein Is Inserted into the Membrane Asymmetrically and Spans the Lipid Bilayer. The topographical relationship of the *in vitro* G_1 product to the pancreatic membranes has been investigated by using trypsin to degrade external portions of the glycoprotein. Proteolytic enzymes are reliable probes of membrane topography (1, 2).

Fig. 1*A*, lane 2, shows that the G_0 form of the glycoprotein, synthesized in the absence of membranes, is completely degraded by trypsin. The N, NS, and M proteins are similarly degraded, although it has been consistently observed that N protein is degraded more slowly than other viral coded proteins. Two prominent new bands result from trypsin treatment. One migrates slightly more slowly than M, the other considerably faster. Neither appears to contain peptides of G (data not shown). These may represent cores or partial degradation products of N and/or M proteins.

In contrast, the G_1 form of the glycoprotein, produced by synthesis in the presence of membranes, is protected from proteolysis (Fig. 1*A*, lanes 4, 6, and 8). However, a discrete portion of G_1 is removed by trypsin, resulting in a small increase in electrophoretic mobility, corresponding to a loss of approximately 3000 in molecular weight. Peptide mapping (Fig. 2*B*) has shown that this polypeptide is derived from G_1. This

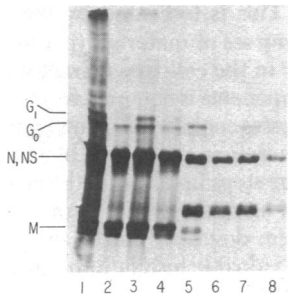

FIG. 3. Binding of VSV glycoprotein forms to columns of Con A-Sepharose. Columns of Con A-linked Sepharose 4B (Pharmacia), 2 cm in length, were formed in pasteur pipettes. All manipulations were at room temperature. Each column was washed with 5 ml of a buffer containing Hepes (25 mM, pH 7.5), $MnCl_2$ (1 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), bovine serum albumin (0.1 mg/ml), and Triton X-100 (1%, wt/vol). 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-methylmannoside (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 addition of 8 ml of acetone. After 15 min at −20°, the precipitates were collected by low-speed centrifugation, lyophilized, and subjected to gel electrophoresis. *Gel represents proteins not bound to column.* Lanes 1–4: Proteins were synthesized in a total volume of 100 μl which contained 2 μl 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 Con 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: Proteins were synthesized in a total volume of 100 μl which contained 10 μl 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.

partial digestion product of G_1 represents the endproduct of digestion since this product does not diminish in amount or become smaller upon prolonged incubation with trypsin (Fig. 1*B*, lanes 4 and 5). Quantitation of scans of autoradiograms from 15 independent experiments has shown that 79 ± 10% (one standard deviation) of the radioactivity present in G_1 before digestion is preserved in the protected, but partially cleaved, form of the glycoprotein. Most, if not all, copies of the G_1 polypeptide are therefore retained in the large tryptic fragment. Some loss of radioactivity would be expected because three or more methionine-containing tryptic peptides are lost in this conversion (Fig. 2). Because the methionine content of G_1 is not yet established, it is not possible to calculate the recovery of G_1 in a more quantitative way.

The protection afforded more of the G_1 polypeptide results from the permeability barrier of the pancreatic membranes. Thus, G_1 is completely hydrolyzed by trypsin in the presence of 1% Triton X-100 (Fig. 1*B*, lane 8) or 1% deoxycholate (data not shown). The effect of detergents is highly specific since the rate of degradation of other proteins is not affected (Fig. 1). Indeed, Triton X-100 is a nondenaturing detergent which appears to disrupt membranes simply by displacing native lipid molecules from hydrophobic binding sites that exist on membrane proteins in the native conformation (26, 27). Therefore, it is unlikely that this detergent makes portions of the glycoprotein that reside on the outside of vesicles susceptible to trypsin because it denatures or otherwise affects the conformation of G.

These observations indicate that G_1, synthesized in the presence of membranes *in vitro*, is incorporated into and, indeed, spans, the membrane of vesicles. Essentially all copies of G_1 have the same orientation in the membrane since most or all copies of G_1 are preserved in the slightly smaller derivative of the glycoprotein that results from trypsin treatment.

The glycoprotein is not protected from proteolysis by membranes if membranes are added after protein biosynthesis (Fig. 1*B*, lanes 6 and 7). Thus, asymmetric transmembrane incorporation of the glycoprotein as well as glycosylation (see above) require the presence of membranes during or immediately after polypeptide chain synthesis.

Carboxyl-Terminal Region of the Glycoprotein Is Outside the Vesicles. Three methionine-containing tryptic peptides are lost from G_1 when vesicles containing this glycoprotein are incubated with trypsin (Fig. 2*B*). The same three peptides are lost from the *in vivo* G_1 contained in microsomal vesicles isolated from [^{35}S]methionine-labeled VSV-infected cells upon trypsinization (Fig. 2*D* and §). These peptides are located closer to the carboxyl terminus of the G protein than are any other methionine-containing peptides,§ as shown by the order in which peptides of completed G protein acquire radioactivity when infected cells are pulse-labeled (28). Since only about 3000 daltons of about 60,000 are lost upon trypsinization, it is the extreme carboxyl-terminal region of the glycoprotein that is located on the outside of these vesicles, and most or all of the amino-terminal 95% (approximately) of the glycoprotein is protected from proteolysis in vesicles. Most or all of the protected fragment may therefore be tentatively assigned to the inside of the vesicle and within the lipid bilayer. Further documentation of this point will require specific digestion of the glycoprotein by proteases trapped inside vesicles.

The amino-terminal portion of the G_1 glycoprotein, which is protected by membranes from trypsin, is completely retained by columns of Con A (Fig. 3, lanes 5 and 6). As was the case with G_1, binding is prevented by α-methylmannoside (lane 7) but not by galactose (lane 8). These results are consistent with the notion that the oligosaccharide moieties are located within the membrane vesicles.

DISCUSSION

When the VSV glycoprotein is synthesized *in vitro* in the presence of pancreatic microsomal membranes, a form of the glycoprotein is produced which is indistinguishable from the G_1 form found in association with intracytoplasmic membranes from infected cells (9, 10). Furthermore, the product of the cell-free system has the same topographical relationship to the pancreatic microsomal vesicles as does the G_1 form to intracytoplasmic membranes.§ Specifically, both cell-free and *in vivo* G_1 forms span the membrane asymmetrically with their carboxyl termini outside the vesicles, are glycosylated, and appear to have most, and possibly all, of their amino-terminal portion inside the vesicle permeability barrier. Since ribosomes are added to the outside of vesicles for protein synthesis *in vitro*, it is this side which is operationally the equivalent of the cytoplasmic side of the rough endoplasmic reticulum membrane in cells.

The glycosylation of an identified membrane protein in a cell-free system may potentially be of value in elucidating the

temporal sequence, biochemical mechanisms, and the basis for specificity in protein glycosylations. It is presumed that membrane-bound, energy-rich intermediates such as the dolichol-linked sugar carrier (25) exist in the membrane preparation from pancreas.

The observation that synthesis of the glycoprotein in the presence of membranes is required for protection of intravesicular portions of the glycoprotein from external proteolysis strongly suggests that the intravesicular portions of the glycoprotein can only cross the membrane during or immediately after protein synthesis. Together with the fact that the extreme carboxyl-terminal portion of the protein is exposed on the surface of vesicles after synthesis, these observations support a model (3, 19) in which the intravesicular portions of the glycoprotein pass amino 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° 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 data).

All of the sugar in the G protein in virions is readily released with external proteases (8), yet sites for binding to Con A are retained in the large fragment of G_1 protected by membranes from trypsin *in vitro*. This, and the observation that synthesis of the glycosylated form of G requires the presence of membranes during protein synthesis, are consistent with the notion that glycosylation is restricted to the intravesicular surface (1) 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 can be concluded that the plasma membrane glycoprotein is derived from glycoprotein of intracellular vesicles by a membrane fusion process (29, 30).

It cannot be concluded that there are not other forms of the glycoprotein that exist as transient intermediates in the process of incorporation into the membrane or in glycosylation. A broad spectrum of proteins secreted by pancreas possess short-lived hydrophobic amino-terminal sequences which are cleaved off during or immediately after protein synthesis (17, 19, 31). These sequences have been proposed (19) to function as "signals" which direct the ribosomes that 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 amino-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 which results from its carbohydrate constituents. If so, intermediates which, for example, are cleaved at the amino terminus but are 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 generating all known aspects of the topography and structure of an important class of membrane proteins, ectoproteins (3). 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 extracytoplasmic portions of the polypeptide (3). 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 by the vast evolutionary disparity of the sources of materials (plants and animals) that function together in the cell-free extract and by the fact that none of these components is obtained from virus-infected cells, so that these processes 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 in this communication can be expected to yield valuable insight into fundamental aspects of membrane assembly.

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1. Bretscher, M. S. (1973) *Science* **181,** 622–629.
2. Steck, T. L. (1974) *J. Cell Biol.* **62,** 1–19.
3. Rothman, J. E & Lenard, J. (1977) *Science* **195,** 743–753.
4. McSharry, J. J. & Wagner, R. R. (1971) *J. Virol.* **7,** 412–415.
5. McSharry, J. J., Compans, R. W. & Choppin, P. W. (1971) *J. Virol.* **8,** 722–729.
6. Cartwright, B., Talbot, P. & Brown, F. (1970) *J. Gen. Virol.* **7,** 267–272.
7. Howatson, A. F. & Whitmore, G. F. (1962) *Virology* **16,** 466–478.
8. Lenard, J. & Compans, R. W. (1974) *Biochim. Biophys. Acta* **344,** 51–94.
9. Knipe, D. M., Lodish, H. F. & Baltimore, D. (1977) *J. Virol.*, in press.
10. Knipe, D. M., Baltimore, D. & Lodish, H. F. (1977) *J. Virol.*, in press.
11. Hunt, L. A. & Summers, D. F. (1976) *J. Virol.* **20,** 637–645.
12. Hunt, L. A. & Summers, D. F. (1976) *J. Virol.* **20,** 646–657.
13. Morrison, T. G. & Lodish, H. F. (1975) *J. Biol. Chem.* **250,** 6955–6962.
14. Grubman, M. J., Moyer, S. A., Banerjee, A. K. & Ehrenfeld, E. (1975) *Biochem. Biophys. Res. Commum.* **62,** 531–538.
15. Rose, J. K. & Lodish, H. F. (1976) *Nature* **262,** 32–37.
16. Both, G. W., Moyer, S. A. & Banerjee, A. K. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 274–278.
17. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67,** 852–862.
18. Dobberstein, B. & Blobel, G. (1977) *Biochem. Biophys. Res. Commun.* **74,** 1675–1682.
19. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67,** 835–851.
20. Roberts, B. E. & Patterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2330–2334.
21. Laemmli, U. K. (1970) *Nature* **227,** 680–684.
22. Sabatini, D. D., Tashiro, Y. & Palade, G. E. (1966) *J. Mol. Biol.* **19,** 503–524.
23. Lis, H. & Sharon, N. (1973) *Annu. Rev. Biochem.* **42,** 541–574.
24. Robertson, J. S., Etchison, J. R. & Summers, D. F. (1976) *J. Virol.* **19,** 871–878.
25. Waechter, C. J. & Lennarz, W. J. (1976) *Annu. Rev. Biochem.* **45,** 95–112.
26. Helenius, A. & Simons, K. (1975) *Biochim. Biophys. Acta* **415,** 29–79.
27. Utermann, G. & Simons, K. (1974) *J. Mol. Biol.* **85,** 569–587.
28. Dintzis, H. M. (1961) *Proc. Natl. Acad. Sci. USA* **47,** 247–261.
29. Palade, G. E. (1975) *Science* **189,** 347–358.
30. Hirono, H., Parkhouse, B., Nicolson, G. L., Lennox, E. S. & Singer, S. J. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 2945–2949.
31. Devillers-Thiery, A., Kindt, T., Scheele, G. & Blobel, G. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 5016–5020.
32. Tarentino, A. L. & Maley, F. (1974) *J. Biol. Chem.* **249,** 811–817.