ABSTRACT

Previous work has shown that the mRNA encoding the vesicular stomatitis virus (VSV) glycoprotein (G) is bound to the rough endoplasmic reticulum (RER) and that newly made G protein is localized to the RER. In this paper, we have investigated the topology and processing of the newly synthesized G protein in microsomal vesicles. G was labeled with $[^{35}S]$methionine ($[^{35}S]$met), either by pulse-labeling infected cells or by allowing membrane-bound polysomes containing nascent G polypeptides to complete G synthesis in vitro. In either case, digestion of microsomal vesicles with any of several proteases removes ~5% (30 amino acids) from each G molecule. These proteases will digest the entire G protein if detergents are present during digestion. Using the method of Dintzis (1961, Proc. Natl. Acad. Sci. U. S. A. 47:247-261) to order tryptic peptides, we show that peptides lost from G protein by protease treatment of closed vesicles are derived from the carboxyterminus of the molecule.

The newly made VSV G in microsomal membranes is glycosylated. If carbohydrate is removed by glycosidases, the resultant peptide migrates more rapidly on polyacrylamide gels than the unglycosylated, Go, form synthesized in cell-free systems in the absence of membranes. We infer that some proteolytic cleavage of the polypeptide backbone is associated with membrane insertion of G. Further, our findings demonstrate that, soon after synthesis, G is found in a transmembrane, asymmetric orientation in microsomal membranes, with its carboxyterminus exposed to the extracisternal, or cytoplasmic, face of the vesicles, and with most or all of its amino-terminal peptides and its carbohydrate sequestered within the bilayer and lumen of the microsomes.

KEY WORDS vesicular stomatitis virus • glycoprotein • membrane (synthesis) • endoplasmic reticulum • transmembrane protein

All membrane proteins that have been investigated are found to maintain a fixed orientational asymmetry with respect to their host membrane (35). Of these proteins, those species that maintain a close association with the phospholipid bilayer such that they can be removed from the bilayer only by detergents are considered integral membrane proteins. Glycophorin and the glycoproteins of vesicular stomatitis virus (VSV), Sindbis virus, and Semliki Forest virus are examples of this group. The majority of their peptide mass and all their carbohydrate portions are located on the extracytoplasmic side of the membrane. Glycophorin is known to span the lipid bilayer, with its carboxyterminal residues on the cytoplasmic side.
Little is known of the site of synthesis of these surface glycoproteins, of the origin of transmembrane asymmetry, or of the events that intervene between synthesis and deposition of the protein on the plasma membrane.

The glycoprotein (G) of VSV is a good model to study these processes. It is synthesized on membrane-bound polysomes (2, 12, 30, 47). Glycosylation occurs on the nascent chain during insertion of the protein into the rough endoplasmic reticulum (RER) (36). G is then transported from the RER to the smooth membrane fractions where glycosylation is completed and from there to the cell surface of the host cell, from which the virus buds (14, 16, 22, 25, 34, 49). The G protein then comprises the external spikes of the virion (28).

It has been suggested (1, 35, 37) that transmembrane proteins, like secretory proteins, are directed to microsomal membranes early in their synthesis by signals located at the amino-termini. These signals are envisioned to participate in organizing functional membrane-ribosome junctions through which the protein, in the process of integration or secretion into the cisternal lumen, passes amino-terminus first. The signal peptide is then cleaved (1). The topology and processing of the VSV glycoprotein in microsomes is shown to be consistent with this hypothesis.

Isolated microsomes from VSV-infected cells, containing nascent G polypeptides, will complete synthesis of G in cell-free extracts (11,47). Combining information from this system with experiments in intact cells, we show that immediately after its synthesis, G spans the ER bilayer asymmetrically. Approximately 30 amino residues at the carboxyterminus of the protein remain exposed on the cytoplasmic face of the ER vesicles; the remainder of the polypeptide, including some carbohydrate residues, is localized in the lumen of the ER or embedded in the lipid bilayer. During or soon after translation, a portion of the polypeptide backbone of G is cleaved.

**MATERIALS AND METHODS**

**Materials**

Trypsin-TPCK, α-chymotrypsin, and bovine pancreatic protease were purchased from Worthington Biochemicals (Freehold, N. J.). Papain, thermolysin, soybean trypsin inhibitor, emetine-HCl, actinomycin D, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, Mo.). Sodium deoxycholate (DOC) was a Fisher Scientific product (Pittsburgh, Pa.). Jack bean α-mannosidase was purchased from Boehringer-Mannheim (Indianapolis, Ind.). Endo-β-N-acetyl-glycosaminidase H (hereafter referred to as Endo H) was the kind gift of Dr. P. W. Robbins (Massachusetts Institute of Technology). This enzyme was purified to homogeneity according to the procedure of Tarentino and Maley (45) and had a sp act of 25 U/mg. [3S]Methionine ([3S]met) was purchased from New England Nuclear (Boston, Mass.) and from Amersham-Searle Corp. (Arlington Heights, Ill.).

**Virus and Cells**

Standard B particles of VSV (Indiana Serotype) were grown in Chinese hamster ovary (CHO) cells and were purified as previously described (22). CHO cells were grown in suspension culture at 12–40 x 10⁶ cells/ml in Joklik's modified minimal essential medium (MEM) supplemented with 7% fetal calf serum (FCS) and 1% nonessential amino acids. A monolayer line of CHO and a ricin-resistant clone, 15B, derived from this line were the kind gift of Dr. S. Kornfeld. They were maintained as monolayers in MEM/10% FCS from Grand Island Biological Co. (Grand Island, N. Y.).

**Preparation of Microsomes for In Vitro Protein Synthesis**

CHO suspension cells (10⁷ cells/ml MEM) were infected with VSV (3 plaque-forming units/cell) in the presence of actinomycin D (5 μg/ml), supplemented with 2 vol of MEM at 30 min, and harvested at 4 h postinfection. The cells were swelled in a hypotonic buffer (0.025 M HEPES, pH 7.4; 0.05 M KCI; 0.002 M MgAc₂) for 20 min on ice, then broken by 40 strokes of a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). Nuclei were removed by low-speed centrifugation (1,000 g for 5 min). The postnuclear supernate was centrifuged at 20,000 g for 30 min, and the microsomal pellet resuspended in buffer II (50 mM KCl; 50 mM Tris-HCl, pH 7.6; 5 mM MgCl₂) for further analysis.

**Cell-Free Protein Synthesis**

The microsomal fraction from 4 x 10⁷ unlabeled suspension CHO cells was resuspended in 250 μl of buffer II and incubated for 30 min at 37°C with 250 μl of a high-speed supernatant fraction (S100) from uninfected cells and 125 μl of a protein synthesis mix containing [3S]met (sp act, 100 Ci/nmol, New England Nuclear), as previously described (50).
Preparation of Labeled Microsomes

Cells were infected with VSV and incubated as above. At 4 h postinfection, cells were harvested, resuspended in MEM supplemented with 1% dialyzed calf serum but lacking methionine at 10^6 cells/ml, and labeled for 10 min with [35S]met (sp act, 100 Ci/mmole, New England Nuclear) at 24 μCi/ml. Cells were then harvested, lysed, and a 20,000 g pellet was prepared as above. The microsomal fraction from 2.5 × 10^7 cells was resuspended in 1 ml of buffer II.

Digestion of G with Endo H

G protein and its protease-resistant derivative (see Fig. 1) were prepared from microsomes labeled with [35S]met both in vivo and in vitro. In addition, proteins were synthesized in a cell-free protein synthesis reaction including VSV mRNA, wheat germ extract, and protein synthesis components as previously described (19) to generate the unglycosylated form of G protein, G0. 25-μl aliquots from the wheat germ reaction, and 20-μl aliquots from all other reactions, were used for Endo H analysis.

All aliquots were precipitated with 0.1 vol of 50% (wt/vol) trichloroacetic acid and resuspended in 100 μl of 0.1 M Tris-HCl at pH 8.0 containing 1% (wt/vol) sodium dodecyl sulfate (SDS) and 1% (vol/vol) 2-mercaptoethanol. After boiling for 2 min, 30 μl of 0.1 M Tris-HCl, pH 8.0, containing 1 M iodoacetamide was added and the samples were kept at room temperature for 30 min. The reduced and alkylated proteins were again precipitated by adding 0.1 vol of 50% (wt/vol) trichloroacetic acid. Pellets were air-dried and dissolved in 20 μl of 0.3 M sodium citrate (adjusted to pH 6.0 with acetic acid) containing 0.09% (wt/vol) SDS (relyrisolated). Each sample was divided into two portions containing 20 μl each. To one portion, 1 μl of 0.1 M KH₂PO₄ was added. To the remaining portion, 1 μl of Endo H (7.4 × 10⁻⁴ U) in 0.1 M KH₂PO₄ was added. Incubation was allowed to proceed for 2.5 h at 37°C.

α-Mannosidase Digestion of G

G protein and its protease-resistant derivative were prepared from microsomes labeled with [35S]met both in vivo and in vitro and from a wheat germ cell-free protein synthesis reaction including VSV mRNA, wheat germ extract, and protein synthesis components as previously described (19) to generate the unglycosylated form of G protein, G0. 25-μl aliquots of the microsomal in vitro synthesis products, and 25-μl aliquots of all other samples, were used in these digestions. 5 μl per sample of α-mannosidase in ammonium sulfate suspension was spun for 2 s at 20,000 g in an Eppendorf Tabletop Centrifuge 3200 and resuspended in 50 μl/sample H₂O. 50 μl of α-mannosidase was added to each sample which was then supplemented to a total volume of 200 μl with 0.1 M sodium citrate buffer (adjusted to pH 4.5 with NaOH). Samples were incubated at room temperature overnight.

Pulse Labeling of the G and Analysis of Tryptic Peptides

Cells were infected with VSV and incubated as described above. At 4 h postinfection, the cells were harvested, washed two times with Earle's saline, and resuspended at 4.3 × 10⁶ cells/ml in MEM-methionine. Cells were permitted to equilibrate for 15 min at 23°C.
then labeled with 83 μCi/ml [35S]met (sp act, 440 Ci/ mmol, Amersham/Searle Corp.). 1-ml aliquots were removed at 30 s, 1 min, 2 min, 3 min, 15 min, and 60 min and pipetted immediately into a 10-fold excess of ice-cold Earle’s containing 200 μg/ml emetine and were left on ice. When all aliquots had been collected, the cells were harvested, dissolved in 2 × SDS sample buffer, and analyzed on a total of three preparative SDS polyacrylamide slab gels. The G band was located by autoradiography, excised, and digested in situ with trypsin (30). The peptides were analyzed by high-voltage paper electrophoresis at pH 3.5 (23).

**SDS-Polyacrylamide Gel Electrophoresis of Proteins**

Labeled viral proteins were subjected to electrophoresis in 10% polyacrylamide slab gels by the method of Laemmli (24). Cells were stained, fixed, dried, and exposed as previously described (21).

**RESULTS**

**G Synthesis In Vitro by Microsomal Membranes**

Microsomal membranes containing membrane-bound polyribosomes were isolated from CHO cells 4 h after infection by VSV, at the time of peak virus protein synthesis. When supplemented with a ribosome-free high-speed supernate fraction (S100) from uninfected cells, these will direct synthesis, in vitro, of VSV, G, N, NS, and M polypeptides (Fig. 1c). In this system, ~80% of the incorporation of [35S]met into polypeptides is completed within 10 min (data not shown).

Tryptic peptide analysis of the in vitro G protein (Fig. 2) demonstrates that most of the major peptides present in the cytoplasmic G protein are also present in the in vitro product. The fastest migrating peptide (57.5 cm), however, is absent.

As will be shown later, (Fig. 6), this peptide is located closer to the amino-terminus than is any other [35S]met-containing peptide in G1. The absence of radioactivity in this peptide is presumably due to the fact that no re-initiation of new chains occurs in this system. This peptide, near the NH2-terminus, is present in virion G protein (not shown), in the cellular G1 form of the protein (Fig. 5), and in the G0 or G1 protein synthesized in wheat germ lysates in the presence or absence of pancreatic membranes (19). Thus, it cannot be part of any “signal” sequence removed during processing of G.

**FIGURE 2** Comparison of tryptic digests of [35S]met-labeled G protein made in vivo and in vitro. Tryptic peptides of G protein synthesized by membrane-bound polyribosomes in vitro (B) and tryptic peptides of G protein isolated from cells pulse-labeled with [35S]met for 60 min at 23°C as in Fig. 7 (A) were prepared and iontophoresed separately at pH 3.5, as previously described (23). Microdensitometer tracings were made from autoradiographs of the electrophoretograms.

The G protein synthesized in this system is glycosylated. It co-migrates (Fig. 1) with the partially glycosylated (G1) form of the G which is the only radioactive form of G found in the infected cell after a brief period of labeling with [35S]met; this form migrates on SDS-polyacrylamide gels more slowly than the unglycosylated form of G (G0) which is produced by translation of G mRNA in a wheat germ cell-free system (22). Moreover, it is a substrate for both Endo H and α-mannosidase. Endo H cleaves between the two glcNAc residues of the proximal chitobiose unit in serum-type glycoproteins (45). When G, synthesized in vitro by microsomes (Fig. 3a and b), or isolated with microsomes derived from cells pulse-labeled for 10 min with [35S]met in vivo (Fig. 3g and h) is treated with Endo H, it migrates faster on gels. However, the enzyme has no effect on G0 (Fig. 3e and f), supporting the conclusion that the change in the rate of migration is due to removal of carbohydrate residues and not to extraneous protease activity. Similarly, treatment with α-mannosidase, a glycosidase which digests α-linked mannose residues from the nonreducing termini of carbohydrate chains, also causes an increase in the rate of migration of the G1 form (Fig. 4a, b, e, and f) but has no effect on G0 (Fig. 4c and d).

We conclude that partial glycosylation of G occurs in the rough microsomal fraction concomitant with or immediately after synthesis of G. The carbohydrate side chains are mannose-terminal at this stage. Because in these experiments we can study only the completed G protein, we cannot determine whether glycosylation occurred on the
Endo H digestions of G protein and its proteolytic derivative. [35S]met-labeled proteins derived from the indicated sources, after reduction plus alkylation, were incubated in the presence (+) or absence (−) of Endo H. Lanes a and b: proteins synthesized in vitro by microsomes isolated from VSV-infected CHO cells. Lanes c and d: products of trypsin digestion of these microsomal membranes labeled in vitro. Lanes e and f: products of cell-free protein synthesis by VSV mRNA in a wheat germ extract supplemented with a protein synthesis mix as previously described (19). Lanes g and h: proteins associated with microsomes derived from VSV-infected CHO cells pulse-labeled for 10 min with [35S]met in vivo. Lanes i and j: products of trypsin digestion of these microsomal membranes labeled in vivo. Shown is an autoradiograph of the dried gel, 2-wk exposure.

nascent G polypeptide before disruption of the cells.

Association of G Protein with Membrane Vesicles

Subcellular fractionation studies have shown that all cellular forms of G protein are found associated with membranes (15, 22). To probe the nature of this association, membrane vesicles which had synthesized G protein in vitro were treated with trypsin. In parallel, microsomes from infected cells pulse-labeled with [35S]met were also subjected to protease digestion. It was reasoned that proteins adhering exclusively to the exterior (cytoplasmic face) of these vesicles should be digested by externally added protease, while those proteins inserted in or through the lipid bilayer should be wholly or partially protected from digestion (3, 41).

Trypsin digestion of microsomal membranes labeled either in vitro (Fig. 1, lane d) or in vivo (lane i) results in the disappearance of radioactive G protein and the appearance of a new polypeptide which migrates slightly faster. Peptide mapping studies (see below) establish that this is indeed a fragment of G. Quantitation of scans of autoradiographs from nine independent in vitro experiments has shown that, on the average, 66.6% of the radioactivity present in G before digestion is preserved in the protected, but partially cleaved, form of the G. Neither the size nor the amount of this fragment is changed if from 5 to 100 μg/ml trypsin is utilized over a time-course of 60 min. Thus, this is a limit digestion product (data not shown).

The generation of this protease-resistant G fragment (henceforth referred to as Gf) is not peculiar to trypsin and is a result of the inaccessibility of trypsin-sensitive sites in the native conformation of the molecule. A similar polypeptide band is observed after treatment of vesicles with any of several proteases of various specificities: chymotrypsin, thermolysin, or papain (data not shown). Thus, only a very limited number of sites in G are accessible to protease acting from the cytoplasmic face.
side of the membrane. From migration on polyacrylamide gels, we estimate that a minimum of 3,000 daltons of G is digested to form the derivative, or ~5% of each molecule is exposed on the exterior of the vesicle. This corresponds to ~30 amino acid residues.

That G\textsubscript{i} retains at least some of its carbohydrate residues is indicated by its susceptibility to Endo H (Fig. 3c, d, i, and j).

This fragment of G is specifically protected by the membrane permeability barrier. When either DOC or Triton X-100, at concentrations sufficient to disrupt the membrane, was added together with trypsin, G protein was completely digested and G\textsubscript{i} was no longer observed (Fig. 3b and h and Fig. 5a and b). Triton X-100 is a nondenaturing detergent which disrupts membranes by displacing native lipid molecules from hydrophobic-binding sites which exist on membrane proteins in the native conformation (13, 48). It is unlikely, therefore, that the increased susceptibility of G to protease in the presence of this detergent can be accounted for by conformational alteration of the G molecule. Rather, it is more likely that the membrane is sequestering a large portion of each molecule.

This conclusion is supported by experiments in which microsomes isolated from infected cells pulse-labeled with [\textsuperscript{35}S]met in vivo were treated with protease during and after sonication. In this case, no conformational alteration of the G protein is expected, but sonication should disrupt the integrity of the vesicles, exposing their lumenal space to protease. All G molecules are completely digested by this treatment (data not shown).

That additional G polypeptide becomes accessible upon disruption of the integrity of the vesicles also indicates that the polypeptide is unlikely to be wholly sequestered within the bilayer itself. Instead, we find these observations to be consistent with a model of a transmembrane configuration for the G protein in these microsomal membranes.

**Tryptic Peptide Analysis of G\textsubscript{i}**

To establish that G\textsubscript{i} is in fact a derivative of the G protein, an analysis of [\textsuperscript{35}S]met-containing tryptic peptides was undertaken. Fig. 5 shows a comparison of the tryptic peptides of G protein synthesized in vivo (A) with those of its putative membrane-sequestered derivative (B). All peptides are in common with the exception of three, indicated by arrows, which are present only in undigested G. The peptides at 4 and 18 cm are found in G protein isolated from whole virions and consequently are authentic G peptides and are not derived from a co-migrating contaminant protein (data not shown). The peptide at 6.5 cm was not present in sufficient yield for unambiguous identification in virions and will consequently be disregarded in the following discussion. We conclude that G\textsubscript{i} is indeed a derivative of G. The two peptides absent in the derivative are presumed to be exposed on the exterior of the vesicles and hence to be susceptible to trypsin, while the remaining peptides belong to that portion of the protein embedded in the bilayer and are thereby protected.

**Orientation of the G in Microsomal Membranes**

To determine the orientation of the G protein in microsomal membranes, the relative NH\textsubscript{2} – COOH order of the [\textsuperscript{35}S]met-containing tryptic peptides of G was established. We employed a technique developed by Dintzis (8) in which cells are given a pulse of radioactive amino acids for periods much less than is required for ribosomes to synthesize a particular protein. Completed, released protein chains are isolated and analyzed by peptide mapping. The first chains to be completed will contain radioactivity only in those peptides closest to the COOH-terminus of the protein; the last to be labeled in completed chains will be those closest to the NH\textsubscript{2}-terminus.

Cells at 4 h postinfection were placed at 23°C, to slow the rate of protein synthesis, and then labeled with [\textsuperscript{35}S]met for various times (Fig. 6). Completed G proteins were isolated by SDS gel
Tryptic peptide analysis of G protein labeled in vivo with \[^{[aS]}\text{met}\] for varying lengths of time. VSV-infected CHO cells were incubated with \[^{[aS]}\text{met}\] at 23°C for the periods of time indicated in the figure, and G was isolated. Tryptic digests were prepared and paper iontophoresis at pH 3.5 performed as in Fig. 6. (A) Photograph of autoradiograph of the electrophoretogram. (B) Microdensitometer tracings of autoradiograph of the electrophoretograms. A microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England) was utilized with full-scale pen deflection set at 1.16 OD units for 1- and 2-min scans, and at 1.49 OD units for 5-, 15-, and 50-min scans.

Electrophoresis, and tryptic maps were prepared from them.

\[^{[aS]}\text{met}\] is first found in two peptides (arrowed, at 5 and 18 cm). These are precisely the same two peptides that are lost when microsomal vesicles containing G are digested with protease (Fig. 5). We conclude that the peptides exposed on the external face of the vesicles, corresponding to the cytoplasmic face of the ER, are in the carboxyterminal 5% of the molecule.

The last peptide to receive radioactivity (48 cm) is presumably located closest to the amino-terminus of G. This assignment is consistent with the observation that when G protein, labeled with \[^{[aS]}\text{met}\] in vitro on isolated microsomes, is analyzed, this peptide is absent (Fig. 2). Because these polysomes have initiated protein synthesis in the whole cell, the more amino-terminal peptides will not receive label in such an in vitro incorporation which does not reinitiate new chains. The short time-course of radioactive incorporation consistently observed in these in vitro reactions supports the notion that no reinitiation is taking place.

**Endo H Analysis of G Synthesized in Monolayer CHO Lines**

It was of interest to determine whether the polypeptide backbone of the G protein, at any time during its intracellular migration, undergoes proteolytic processing, particularly NH\(_2\)-terminal cleavage analogous to the signal peptide cleavage of secretory proteins (1). When G protein is inserted into membranes, it is simultaneously glycosylated (36), and the anomalously high molecular weight of the \(G_1\) form which results from its carbohydrate constituents could easily obscure any proteolytic processing of the amino acid backbone. Therefore, we were interested in eliminating most of the carbohydrate moieties of the intracellular G forms so that the resultant peptides could be compared directly with the unglycosylated form of G, \(G_0\), presumed to represent the complete polypeptide product of the G mRNA.

Endo H will readily hydrolyze carbohydrate units of the form Asn-(GlcNAc)\(_n\)-(Man)\(_m\) but will not cleave complex oligosaccharides, i.e., carbohydrate chains possessing terminal sugars attached to the chitobiose-mannose "core." Consequently, the intracellular forms of the G found at late stages of radioactive chase are not substrates for Endo H.

15B is a ricin-resistant clone of a monolayer line of CHO cells. It lacks UDP-GlcNAc: glycoprotein N-acetylglycosaminyltransferase activity which results in the synthesis of membrane oligosaccharides which are deficient in sialic acid, galactose, and N-acetylgalcosamine (10). When VSV is grown in these cells, transport of G to the surface occurs in a normal manner, and G is incorporated into virions (38). However, as a result of its altered carbohydrate structure, G remains susceptible to Endo H digestion throughout its residency in the cell. 15B and its parent CHO monolayer line (CHO\(^0\)) were grown to confluence in T-75 flasks. At 4 h postinfection, samples of each cell line were pulse-labeled with \[^{[aS]}\text{met}\] for 7 min or pulse-labeled for 7 min and chased for 45 min in cold medium. The products of these incubations were then analyzed by Endo
At 45 min of chase, the CHO line produces a G molecule resistant to Endo H but with the approximate electrophoretic mobility of G1 (Fig. 7a and b). The 15B line produces a G with a mobility somewhat faster than G1 (lane c). This has been characterized by Tabas et al. (44) and represents molecules with oligosaccharide structures Asn-(GlcNAc)2-(Man)2, as compared to the "high mannose" G1 structure Asn-GlcNAc2-(Man)10. These molecules are, however, susceptible to Endo H (lane d) and upon digestion yield a form with a mobility slightly greater than that of G0 (Fig. 7d and e). After a 7-min pulse, both lines produce G molecules which have identical electrophoretic mobilities (lanes g and h) and yield identical Endo H products (lanes f and i) which migrate, like the 15B chase product, faster than G0. At times down to a 2-min pulse, this is the only Endo H product observed (data not shown).

These observations suggest that the unglycosylated polypeptide (G0) produced in a cell-free system, and the polypeptide synthesized in these monolayer lines with the bulk of its carbohydrate removed, are not identical. The faster migration of the latter product may indicate the absence of some amino acid residues from the protein backbone that are present in G0. Sequencing data, in fact, confirm this interpretation (see Discussion).

The G synthesized by these monolayer lines after a short radioactive pulse migrates slightly faster than the G produced in the suspension line of CHO cells after short pulse label (Fig. 7k and l). This is discussed below.

**DISCUSSION**

We have shown that, immediately after synthesis, the glycoprotein of VSV is found in a transmembrane, asymmetric orientation in the microsomal membranes of the host cell, with its carboxyterminus exposed to the extracisternal face of the vesicles and with most or all of its amino-terminal peptides, and all or some of its carbohydrate, sequestered within the bilayer or lumen of the microsomes.

Preliminary sequencing data confirm these orientational assignments (V. Lingappa, F. Katz, H. F. Lodish, and G. Blobel, manuscript in preparation). The NH2-terminus of the intact, membrane-inserted G0 molecule is preserved in its proteolytic derivative, G1. This further suggests that carboxyterminal residues exclusively are digested by trypsin from the exterior of membrane vesicles.

It is important to establish that the orientation of the isolated microsomal vesicles is the same as in the undisrupted cell. Several investigators have concluded, on the basis of ribosome (1) and enzyme localization (6), and freeze-fracture electron microscopy (9), that the original cytoplasmic-lumenal relationship is preserved after the conversion of the ER into microsomal vesicles. In experiments utilizing in vitro protein synthesis, this sidedness was established operationally by adding protein synthesis components exclusively to the exterior of the vesicles. As these vesicles are...
impermeable to protease (17), they are likely to be similarly impermeable to molecules of the same or greater molecular weight necessary to protein synthesis. This should mimic the behavior of the ER in whole cells which has ribosomes and components for protein synthesis exclusively on the cytoplasmic side. Moreover, the accessibility of the M and NS proteins made in vitro to externally added protease indicates that no synthesis is taking place inside the vesicles. The congruity of the orientation of G molecules in microsomes isolated from pulse-labeled cells with those molecules synthesized in vitro indicates that no inversion in sidedness has occurred during isolation.

The protease-resistant fragment of G retains ~67% of the original methionine radioactivity contained in G before digestion. Some considerable loss of radioactivity would be expected because two or more major [35S]met tryptic peptides are lost in the conversion to the proteolytic derivative. This suggests that most, if not all, molecules of G are oriented in the membranes in the same direction, and that proteases remove the same carboxyl fragment from all molecules, i.e., the G protein is oriented asymmetrically.

When microsomes are allowed to synthesize VSV proteins in vitro and are subsequently treated with protease, all VSV proteins are digested with the exception of the G derivative and a small amount of N protein. The persistence of N is not due to membrane protection since it is present even if detergents are added before the protease digestion. We believe that some N is bound in nucleocapsids and is configurationally inaccessible to digestion.

The persistence of some M protein in the presence of protease only in microsomes isolated from infected cells pulse-labeled in vivo (Fig. 1) may be due to either or both of two possibilities. First, M is a soluble protein, and it may have been included inside the vesicles during vesicularization. Alternatively, it may be enclosed by budding virions since previous work (21) has indicated that labeled M protein is found in virions after a 10-min pulse. The mRNA for M protein is found mainly on free polyribosomes, but a small amount of M (and N and NS) mRNAs co-purify with membranes (27). Indeed, microsomes will synthesize in vitro appreciably amounts of M, N, and NS proteins. However, all of these proteins, made by microsomes in vitro, are completely accessible to proteases, and thus, no large part of these proteins can be sequestered by the microsomal membrane. Most likely, the presence of N, NS, and M mRNAs in these microsomal preparations is an artifact.

If transmembrane proteins are indeed integrated into microsomal membranes by the same mechanisms that result in the secretion of secretory proteins into the microsomal lumen ("signal hypothesis") but the passage of the membrane protein is interrupted before the entire length of the molecule has passed through the membrane channel, one would predict that the amino-terminus would be found in the interior of the microsomal lumen, while the carboxyterminus would remain on the exterior (cytoplasmic) side of the membrane. The topology of G in microsomes is consistent with this model.

The signal hypothesis (1) also postulates that the NH2-terminal sequences that direct secretory proteins to membranes are cleaved during the process of secretion into the lumen of the endoplasmic reticulum. Many examples of secretory proteins that are synthesized as larger, precursor forms in cell-free extracts from which membranes, and their associated enzymes, are absent have been found (4, 5, 7, 18, 20, 29, 40, 42, 43). When G protein is inserted into membranes, it is simultaneously glycosylated (36), and the presence of carbohydrate constituents could easily obscure any proteolytic cleavage of the backbone. When G is treated with Endo H, however, the bulk of the carbohydrate is removed, leaving only two NAcglc residues per molecule. This enables us to compare the amino acid backbones of G1 and the unglycosylated form of G, G0, more directly. We make the assumption that G0 represents the complete polypeptide product of the G mRNA. Although it is possible that G0 represents a premature termination product and not the complete protein, the fact that G0 is the major product of G mRNA in several cell-free systems (2, 23, 30) and also contains (23) peptides now shown to be included in the carboxyterminal 5% of the molecule strongly argues against this possibility.

When G0 is coelectrophoresed on SDS-polyacrylamide gels with the Endo H-treated forms of G1 synthesized in vitro by microsomes (Fig. 3b) or isolated from the cytoplasm of infected cells grown in suspension culture (Fig. 3, lanes f and g), the two species appear to be of approximately the same molecular weight. However, when G is synthesized in a monolayer line of CHO, CHO*, or its ricin-resistant clone, 15B, and coelectropho-
reased with G₀, it migrates faster than G₀ (Fig. 7). It is possible that the presence of additional biochemical modifications, e.g., sulfation (33) in suspension cells which do not occur in the monolayer line, may account for this discrepancy. It is of interest, in this regard, that Pinter and Compans (33) found that the G protein of VSV was labeled with ³⁵S⁰₄⁻⁻⁻⁻ when virions were grown in monolayers of the MDBK line of bovine kidney cells, but not when they were grown in BHK21-F line of baby hamster kidney cells. The gel mobilities of the purified glycoproteins from these two lines are not identical. The sulfated, G appears to run slightly behind the nonsulfated glycoprotein. Indeed, the Endo H susceptible G₁ form synthesized in suspension cells migrates more slowly than the Endo H susceptible form of G produced after a short pulse-label in monolayer cells (Fig. 7k and l). We do not know, however, whether the glycoproteins produced in suspension and monolayer CHO cells are sulfated differently.

The observations on G₁ synthesized in the monolayer lines suggest that G₁ differs from G₀ in ways other than its carbohydrate structure. This difference might be due to biochemical modifications of G₀ (to increase its molecular weight) or, more likely, to proteolytic modification of G₁ (to decrease its molecular weight), which is obscured in suspension culture by additional biochemical processing. Preliminary sequencing of the amino-terminus of both G₀ and G₁ produced in a membrane-reassembly system (described in reference 19, using a reticuloocyte lysate in place of wheat germ) strongly supports the latter interpretation. The G₀ form has additional amino acids at its amino-terminus that are absent in the G₁ form, although when G₁ produced in this system is treated with Endo H, it appears to comigrate with G₀ (V. Lingappa, F. Katz, H. F. Lodish, and G. Blobel, manuscript in preparation).

The prolonged Endo H susceptibility of G grown in 15B can be utilized to ask whether there are additional "late" proteolytic processing events in the evolution of the virion spike. We do not observe any change in gel migration of the Endo H product up to 50 min of chase. Experiments are now in progress to analyze the G molecules extracted from virions grown in 15B.

A comparison of the topology of membrane proteins in microsomes with that at the cell surface may provide insight into the mechanism of migration of membrane proteins to the cell surface from their sites of synthesis. If, as has been suggested for secretory proteins (32), a vesicle-fusion event occurs during this transport, one would expect the formerly intraluminal aspect of proteins to be exposed on the exterior of cells. For the G protein this appears to be true, as suggested by the apparent inversion of mass distribution that occurs at the cell surface relative to the microsomes: virtually all G protein and all of the associated carbohydrate can be removed from the cell surface (22) or from mature virions (26, 31, 39) by the addition of externally added protease, while only 5% of the molecule and little, if any, of the carbohydrate, are digested from the exterior of microsomal vesicles. Although the precise orientation of G protein in plasma membranes is not yet known, it is of interest that the amino-terminus of glycoporphin, a transmembrane glycoprotein of the erythrocyte membrane, is on the exterior of the cell, while its carboxyterminus is located on the interior (cytoplasmic) face of the plasma membrane (46).

The characterization of the topology and immediate post-translational processing of a cell surface protein at its site of synthesis represents, we believe, novel and important observations which we hope will facilitate more detailed studies of membrane protein biogenesis.

We thank Mr. Martin Brock for expert technical assistance, and Drs. James Rothman and Dr. David Knipe for useful discussions. We also thank Ms. Marianne Robotham for quick and expert help in preparation of the manuscript. This work was supported by grant AI-08814 from the National Institutes of Health.

Received for publication 26 June 1978, and in revised form 25 September 1978.

REFERENCES

1. Blobel, G., and B. B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of pretranslocally processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of marine myeloma. J. Cell Biol. 71:335-351.
2. Born, G. W., S. A. Morse, and A. K. Balch. 1975. Translation and identification of the viral mRNA species isolated from subcellular fractions of vesicular stomatitis virus-infected cells. J. Virol. 15:1012-1019.
3. ERNST, M. S. 1973. Membrane structure: Some general principles. Science (Wash. D. C.) 181:425-429.
4. BURSTEIN, Y., and I. SCHNEIDER. 1977. Amino acid sequence of the N-terminal extra piece segments of the precursors of mouse immunoglobulin A-type and K-type light chains. Proc. Natl. Acad. Sci. U. S. A. 74:1716-1720.
5. CAIN, S. J., P. KEIM, and D. STERN. 1976. Cell-free synthesis of rat proerythropoietin: Characterization and partial amino acid sequence determination. Proc. Natl. Acad. Sci. U. S. A. 73:1964-1968.
6. DePESCHER, J. W., and G. DALLNER. 1975. Structural aspects of the membrane of the endoplasmic reticulum. Biochem. Biophys. Acta. 419: 411-412.
7. DERRY-THERY, A., T. KINDT, G. SCHERLE, and G. BLOBEL.
10. C. K. Amazawa and S. Komiya. 1975. Deficient asparagine-N-acetylglucosaminyl-transferase activity in a clone of Chinese hamster ovary cells with altered surface glycoproteins. J. Biol. Chem. 250:3103-3109.

11. Gurney, M. J., S. A. Mowry, A. K. Banerjee, and E. Ehrenfeld. 1975. Subcellular localization of vesicular stomatitis virus messenger RNAs. Biochem. Biophys. Res. Commun. 63:531-538.

12. Helinski, A., and K. Smolen. 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta. 415:20-80.

13. Houy, L. A., S. R. Ehrenberg, and D. F. Summers. 1978. Oligosaccharide chains are trimmed during synthesis of the envelope glycoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U. S. A. 75:754-758.

14. Houy, L. A., and D. F. Summers. 1976. Association of vesicular stomatitis virus proteins with HeLa cell membranes and released virus. J. Virol. 18:37-645.

15. Houy, L. A., and D. F. Summers. 1976. Glycoprotein of vesicular stomatitis virus glycoproteins in virus-infected HeLa cells. J. Virol. 18:646-657.

16. Ito, A., and R. Smith. 1969. Proteolytic microdissection of smooth-endoplasmic reticulum vesicles of liver microsomes. J. Cell Biol. 40:179-189.

17. Jorgensen, E. F., and S. Pfeffer. 1977. Amino acid sequence of the precursor region of MOPC-315 mouse immunoglobulin heavy chain. J. Exp. Med. 146:962-966.

18. Katz, F. N., J. E. Rothman, V. R. Lengyel, G. Blobel, and H. F. Lodish. 1977. Membrane assembly in vitro: Synthesis, glycosylation, and asymmetric insertion of a transmembrane protein. Proc. Natl. Acad. Sci. U. S. A. 74:3278-3282.

19. Kempner, B., J. F. Harper, M. D. Ezzy, J. T. Perria, and S. A. Gruenstein. 1976. Pepsinoporphine hormone: Analysis of radioactive tryptic peptides and amino acid sequence. Biochemistry. 15:15-19.

20. Kempner, D. M., D. Baltimore, and H. F. Lodish. 1977a. Separate intracellular biosynthetic pathways of the major structural proteins of vesicular stomatitis virus. J. Virol. 21:1128-1139.

21. Kempner, J. K. Ross, and H. F. Lodish. 1977b. Localization of two cellular forms of the vesicular stomatitis virus glycoprotein. J. Virol. 21:1121-1127.

22. Kempner, J. K., K. Ross, and H. F. Lodish. 1975. Translation of individual species of vesicular stomatitis virus mRNA. J. Virol. 16:1004-1011.

23. Klenow, U. K. 1970. Cleavage of structural proteins during the head of bacteriophage T4. Nature (Lond.). 227:680-684.

24. LaFay, F. 1974. Envelope proteins of vesicular stomatitis virus: Effect of temperature-sensitive mutations in complementation groups III and V. J. Virol. 14:1220-1228.

25. Laufer, J. and R. W. Compans. 1974. The membrane structure of lipoprotein viruses. Biochem. Biophys. Acta. 344:51-94.

26. Lodish, H. F., and S. Frohme. 1977. Binding of viral glycoprotein mRNA to endoplasmic reticulum membranes is disrupted by penicillin. J. Cell Biol. 74:358-364.

27. McSharry, J. J. R. Compans, and P. Coppel. 1971. Proteins of vesicular stomatitis virus-infected virus. J. Virol. 6:722-729.

28. Milsom, C. G. Brownlee, T. M. Harrison, and M. B. Mathews. 1972. A possible precursor of immunoglobulin light chains. Nat. New Biol. 239:117-120.

29. Mokrison, T. G., and H. F. Lodish. 1975. The site of synthesis of membrane and non-membrane proteins of vesicular stomatitis virus. J. Biol. Chem. 250:6955-6962.

30. Muold, J. A. 1974. Glycoprotein fragment associated with vesicular stomatitis virus after proteolytic digestion. Virology 63:3-577.

31. Palade, G. E. 1975. Intracellular aspects of the process of protein synthesis. Science (Wash. D. C.). 189:347-358.

32. Porter, A., and R. W. Compans. 1975. Sulfated components of enveloped viruses. J. Virol. 16:859-866.

33. Rambo, F. W., S. J. Hubert, S. J. Torde, and D. F. Winter. 1977. Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. Cell. 12:893-900.

34. Rohenek, J. E., and J. Lenard. 1977. Membrane asymmetry. Science (Wash. D. C.). 196:743-753.

35. Rothman, J. E., and H. F. Lodish. 1977. Synchronized transferase activity in a nascent membrane protein. Nature (Lond.). 266:775-780.

36. Sabatina, D. D., and G. Krebich. 1976. Functional specialization of membrane-bound ribosomes in eukaryotic cells. In The Enzymes of Biological Membranes, Vol. 2: Biosynthesis of Cell Components. A. Martinou, editor. Plenum Press, New York. 531-575.

37. Scheller, S. C. Gottlieb, P. Fiel, N. Oehl, and S. Kornfeld. 1976. Growth of enveloped RNA viruses in a line of CHO cells with deficient N-acetylglucosaminyl-transferase activity. J. Virol. 17:239-246.

38. Scheller, R. H., and R. W. Wagner. 1975. Association of VSV glycoprotein with viron membrane: Characterization of the lipoprotein tail fragment. J. Virol. 16:237-249.

39. Sheehy, D., and G. Blobel. 1977. Cell-free synthesis of fib proopiodynin and processing by heterologous mammalian microsomal membranes. Proc. Natl. Acad. Sci. U. S. A. 74:2069-2073.

40. Stick, T. L. 1974. The organisation of proteins in the human red blood cell membrane: A Review. J. Cell Biol. 62:1-19.

41. Stracher, A. M. A., C. R. Bensinger, J. A. Rockey, and A. W. Alberts. 1977. Rat liver proopiodynin: In vitro synthesis and partial amino acid sequence. Proc. Natl. Acad. Sci. U. S. A. 74:1358-1362.

42. Strubiner, G., G. Kreil, and M. A. Hermanson. 1978. Amino acid sequence of human proopiodynin synthesized in vitro. Proc. Natl. Acad. Sci. U. S. A. 75:701-704.

43. Tabar, I. S. Scheller, S. Kornfeld, 1978. Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly-synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. J. Biol. Chem. 253:716-722.

44. Tarentino, A. L., and F. Malley. 1974. Purification and properties of an Envelope viral glycoprotein from Streptomyces griseus. J. Biol. Chem. 249:811-817.

45. Totonich, M., and V. T. Marchesi. 1975. Amino acid sequences and oligosaccharide attachment sites of human erythrocyte glycoproteins. Proc. Natl. Acad. Sci. U. S. A. 72:2904-2908.

46. Toth-Buzas, E., and H. P. Giersch. 1975. Cell-free synthesis of vesicular stomatitis virus proteins: Translation of membrane-bound polyribosomal RNAs. FEBS (Fed. Eur. Biochem. Soc.) Lett. 59:369-373.

47. Uebermann, G., and K. Simon. 1974. Studies on the amphibian nature of the membrane proteins in Sennikl Forest virus. J. Mol. Biol. 86:566-567.

48. Wanger, R. R., M. F. Kiley, R. M. Snyder, and C. A. Schwartzman. 1972. Cytoplasmic compartmentalization of the proteins and lipochoic acid species of vesicular stomatitis virus. J. Virol. 6:782-783.

49. Winter, D. F., F. Katz, B. Small, and H. F. Lodish. 1977. How a single Sindbis virus mRNA directs the synthesis of one soluble protein and two integral membrane glycoproteins. Cell. 10:253-263.