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In vitro synthesis of vesicular stomatitis virus membrane glycoprotein and insertion into membranes

(transmembrane glycoprotein mRNA/*in vitro* translation and glycosylation/reconstruction of functional membrane-bound polysomes/stripped microsomal membranes/segregation of glycoproteins)

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ABSTRACT Translation *in vitro* of the mRNA coding for the vesicular stomatitis virus membrane glycoprotein G in a membrane-free ribosomal extract from HeLa cells allowed the synthesis of only the unglycosylated protein G_1 (molecular weight, 63,000). Addition of stripped crude microsomal membranes from HeLa cells resulted in the conversion of G_1 to the glycosylated protein G_2 (molecular weight, 67,000). The G_2 protein synthesized by the reconstructed microsomal membrane/ribosome system was found to be segregated inside the microsomal membrane vesicles and was thus protected from the proteolytic action of trypsin and chymotrypsin. Stripped membranes were required at an early stage of protein synthesis for the synthesized protein to be inserted into the membrane vesicles and to be glycosylated. The segregated protein G_2, however, was not completely protected from proteolytic digestion, showing that a portion of the polypeptide chain of about 3000 daltons was present on the cytoplasmic side of the membrane vesicle. Our data thus suggest that, unlike the secretory proteins, the membrane glycoproteins are not completely discharged across the microsomal membranes.

In eukaryotic cells the synthesis of proteins that are destined for secretion occurs on membrane-bound polyribosomes (1, 2). The attachment of the polyribosomes to the membrane is believed to be mediated by the association between a unique sequence of amino acids, present at the amino terminus of the nascent polypeptide chain, and the endoplasmic reticulum membrane (3, 4). It has been proposed that formation of this ribosome–membrane junction allows the discharge of the nascent protein chain through the membrane and into the lumen of the endoplasmic reticulum (1, 3). The vectorially discharged protein is then transported through the cell and is secreted (1). Recently it has been established that membrane glycoproteins are synthesized on membrane-bound polyribosomes (5–8). However, the events involved in the glycosylation of the synthesized protein, its insertion into membranes, and ultimate migration to the plasma membrane are not yet understood.

Vesicular stomatitis virus (VSV) contains a single glycosylated membrane protein G (molecular weight, 69,000) (9) that is synthesized exclusively by membrane-bound polyribosomes in infected cells (5–7). The synthesized protein G migrates through the intracellular membrane system and is ultimately inserted into the host cell plasma membrane (9–11). Protein G therefore can be used as a model to study the biosynthesis, glycosylation, and intracellular migration of membrane glycoproteins *in vitro*.

We have previously shown (12) that, in the presence of membranes, a glycosylated precursor (protein G_2; molecular weight, 67,000) of protein G is synthesized *in vitro*. In the absence of membranes, the G-specific mRNA was translated on free polysomes, but the translation product was an unglycosylated protein G_1 (molecular weight, 63,000) that was the protein moiety of both protein G_2 and protein G.

In order to examine the steps involved in the synthesis, glycosylation, and insertion into membranes of the glycoprotein, by adding stripped microsomal membrane from HeLa cells to a homologous protein synthesizing system containing free ribosomes we have reconstructed a system that synthesizes glycosylated protein G_2. The *in vitro* synthesized and glycosylated protein G_2 is protected from proteolytic digestion by being inserted into the microsomal membrane vesicles. We further show that protein G_2 is not completely discharged across the microsomal membrane but a portion of the polypeptide chain remains on the cytoplasmic face of the membrane.

MATERIALS AND METHODS

Plaque-purified VSV (Indiana HR-LT) and HeLa S_3 cells were grown as described (12).

Preparation of Stripped Microsomal Membranes. A ribosomal extract containing membranes (S-4) was obtained as a 4000 × *g* supernatant fraction from uninfected HeLa cell extract as described (12). The extract was preincubated for 1 hr at 30° in the presence of 30 mM NaCl, which inhibits initiation of translation of nonviral eukaryotic mRNAs (13). The preincubated extract was made 30% (wt/vol) in sucrose by addition of solid sucrose and then 80% (wt/vol) sucrose in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5/50 mM KCl/5 mM Mg acetate (HKM buffer) was added to make the final concentration of sucrose 70% (wt/vol). It was overlayered with 10 ml of 60% (wt/vol) sucrose and 10 ml of 20% (wt/vol) sucrose in the same buffer. The discontinuous gradient was centrifuged at 100,000 × *g* for 18 hr at 4°. The membrane band at the 60%/20% sucrose interphase was collected, diluted with HKM buffer, and centrifuged at 150,000 × *g* for 1 hr at 4°. The pellet was resuspended by brief sonication in 20 mM Hepes, pH 7.5/120 mM KCl/15 mM Mg acetate/6 mM mercaptoethanol/0.25 M sucrose, to a concentration of 40–50 A_{260} units/ml and stored in liquid nitrogen. Microsomal membranes stripped by this method showed an A_{280}/A_{260} ratio of 0.8. Stripped membranes obtained either by the KCl/puromycin (14) or the EDTA procedure (15) had an identical A_{280}/A_{260} ratio.

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Abbreviations: VSV, vesicular stomatitis virus; protein G, fully glycosylated virion glycoprotein of molecular weight 69,000; protein G_2, nonsialated glycoprotein of molecular weight 67,000 synthesized *in vitro*; protein G_1, nonglycosylated precursor protein of molecular weight 63,000 synthesized *in vitro*; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

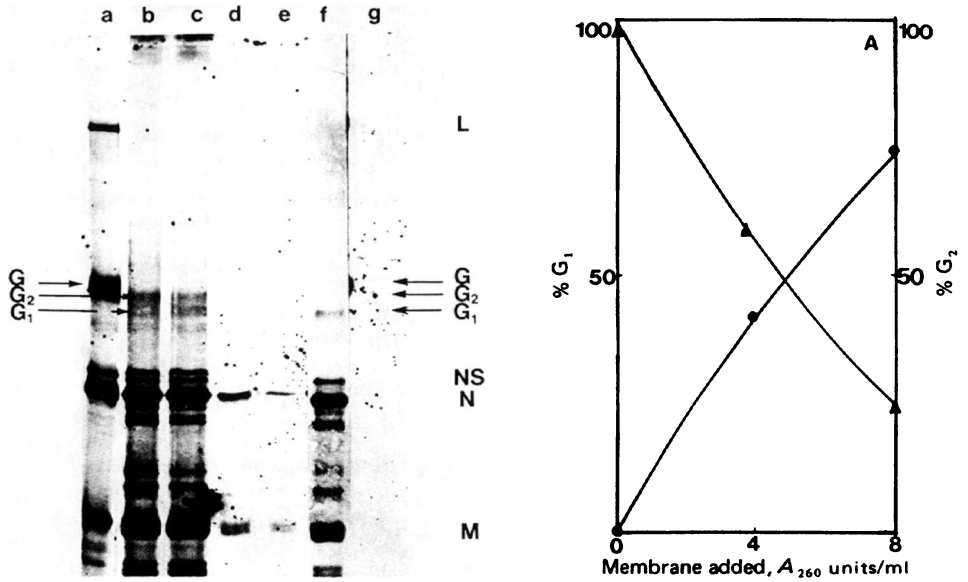

FIG. 1. Synthesis of G_1 and G_2 by reconstituted system containing free ribosomes and stripped membranes. (*Left*) Autoradiogram of proteins synthesized by free ribosomes in the presence and absence of stripped microsomal membranes. The reaction mixtures for protein synthesis contained 40 mM Hepes (pH 7.6), 75 mM KCl, 4.5 mM Mg acetate, 1 mM dithiothreitol, CTP, GTP, UTP each at 0.8 mM, 2 mM ATP, 10 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 80 μM spermine, 19 unlabeled amino acids each at 20 μM, 200 μCi of [^{35}S]methionine per ml, 0.7 μg of the purified ribonucleoprotein particles from VSV-infected L cells (12) per ml, and 300 μl of the preincubated HeLa S-27 extract per ml. Stripped microsomal membranes were added in the amounts indicated at the beginning of the incubation. All incubations were at 30° for 90 min. The [^{35}S]methionine-labeled proteins synthesized were electrophoresed on 10% polyacrylamide slab gels (12). The direction of migration was from the top (−ve). Lanes: (a) VS-virion proteins; (b) and (c) proteins synthesized by a HeLa S-27 extract in the presence of 8 A_{260} units and 4 A_{260} units of membranes per ml of reaction mixture, respectively; (d and e) proteins synthesized in a reaction containing stripped membranes and a HeLa S-100 fraction in the presence and absence of ribonucleoprotein particles, respectively; (f) proteins synthesized in a reaction containing HeLa S-27 extract and ribonucleoprotein particles; (g) proteins synthesized in a reaction containing HeLa S-27 extract. (*Right*) Quantitation of G_1 and G_2 synthesized. The G_1 and G_2 bands present in the autoradiogram were scanned by using a Joyce–Loebl densitometer (7) and the areas under the peaks were calculated and plotted as the percentage of G_1 (▲) and the percentage of G_2 (●) synthesized relative to the total amount of G_1 and G_2 synthesized. L, NS, N, and M, nonglycosylated proteins present in VS virion.

Reconstruction of Membrane-Bound Polysomes. The conditions for the coupled transcription/translation system were as described (12). A preincubated HeLa cell S-27 extract prepared by centrifuging the homogenized cell suspension at 27,000 × *g* for 25 min was used as a source of membrane-free ribosomes in these experiments. Stripped microsomal membranes were added in varying amounts at the indicated times and the incubation was carried out at 30° for 90 min.

Protease Digestion of Reaction Products. Reaction mixtures were treated with proteolytic enzymes as described by Dobberstein and Blobel (16). Twenty-five microliters of the reaction mixture was incubated at 27° for 1 hr with 5 μl of a solution containing trypsin and chymotrypsin (3 mg of each per ml in 0.2 M NH_4HCO_3, pH 8.5). Proteolysis was terminated by addition of 1 volume of 20% trichloroacetic acid. The precipitated material was pelleted, washed with acetone, and analyzed by sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis (12). In incubation mixtures containing deoxycholate, 0.5% deoxycholate was added to dissolve the membrane and the proteolytic reaction was carried out as described above.

RESULTS

Synthesis of Glycoprotein by a Reconstructed System Containing Ribosomes and Stripped Microsomal Membranes. Microsomes prepared from uninfected HeLa cells were stripped of their ribosomes by preincubation in the presence of NaCl followed by equilibrium centrifugation. The stripped microsomal membranes retained little protein-synthesizing activity and, in the presence of VSV mRNA, synthesized about 12% of the protein synthesized by the HeLa S-27 extract. Also, addition of stripped microsomal membranes (up to a concentration of about 10 A_{260} units/ml) to a coupled system containing a HeLa S-27 extract resulted in only a slight inhibition of protein synthesis (data not presented).

Slab gel analysis (Fig. 1 *left*) of the [^{35}S]methionine-labeled proteins synthesized *in vitro* showed that the coupled system containing HeLa S-27 extract synthesized only the unglycosylated protein G_1 (lane f). No protein G_2 or G_1 was detected in the proteins synthesized by the stripped membranes in the presence of mRNA (lane d). Addition of 4 A_{260} units of stripped microsomal membrane per ml to the HeLa S-27 extract, however, resulted in the synthesis of the glycosylated protein G_2 as well as G_1 (lane c). Addition of twice the amount of stripped membranes resulted in an increased synthesis of G_2 and a decrease in G_1 (lane b).

The amounts of protein G_1 and G_2 bands were quantitated and the relative percentages of G_1 and G_2 were plotted (Fig. 1 *right*). The results show that, with increasing amounts of membranes, the amount of G_2 synthesized increased with a concomitant decrease in the amount of G_1. These results suggest that G_1 is the precursor of G_2.

Effect of Addition of Membranes at Different Times on the Synthesis of Protein G_2. We have shown that the synthesis *in vitro* of protein G_2 occurs only on membrane-bound polysomes (12). Because the G-specific mRNA can be translated by ribosomes in the absence of membranes, the formation of the membrane–ribosome complex could occur after the initiation of G synthesis. Addition of membranes at different times to a reaction mixture containing free polysomes should indicate at what stage of protein synthesis the formation of membrane-bound polysomes occurs. Slab gel analysis (Fig. 2) of the proteins synthesized in a reaction containing HeLa S-27 extract to which

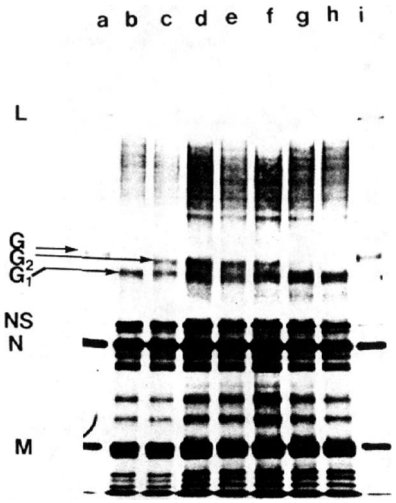

FIG. 2. Effect of addition of membranes at different times on proteins synthesized by free ribosomes. Stripped membranes (8 A_{260} units/ml) were added, at different times of incubation, to a reaction mixture containing HeLa S-27 extract and the incubation was continued for a total of 90 min at 30°. The [^{35}S]methionine-labeled proteins were analyzed on polyacrylamide slab gels. Lanes: (a and i) VS-virion proteins; (b) proteins synthesized by a HeLa S-27 extract in absence of membranes; (c–h) proteins synthesized in a HeLa S-27 reaction to which membranes were added at 0, 5, 10, 15, 30, and 60 min, respectively.

stripped microsomal membranes were added at different times of incubation showed that maximal G_2 synthesis occurred when the membranes were present within the first 10 min of the incubation period (lanes c–e). Addition of membranes after 10 min of incubation resulted in a decrease in G_2 synthesis; after 30 min of incubation, addition of membranes no longer led to G_2 synthesis (lanes f–h).

To determine the relative amounts of G_1 and G_2 synthesized at different times of addition of membranes, we quantitated the G_1 and G_2 bands by densitometric scanning of the autoradiogram (Fig. 3). The data show that, when membranes were added between 0 and 10 min of the incubation period, 45% of

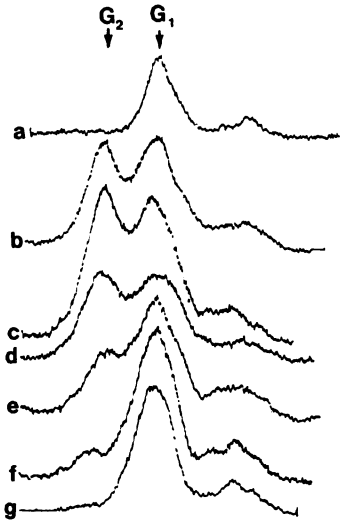

FIG. 3. Quantitation of G_1 and G_2 synthesized in presence of membranes added to a reaction mixture at different times of incubation. The G_1 and G_2 bands shown in lanes b–h of Fig. 2 were scanned. Scans: (a), no membranes; (b–g), membranes added at 0, 5, 10, 15, 30, and 60 min, respectively. The percentage of G_2 relative to the total amount of G_1 and G_2 synthesized in each case was as follows: (a), 0%; (b), 45%; (c), 45%; (d), 43%; (e), 24%; (f), 10%; (g), 0%.

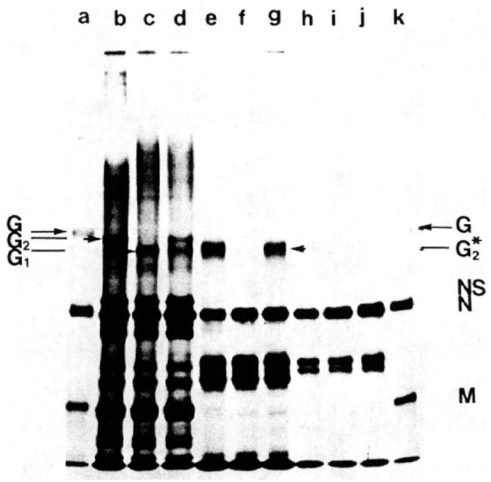

FIG. 4. Autoradiogram of protease-resistant proteins synthesized in the presence and absence of membranes. [^{35}S]Methionine-labeled proteins synthesized *in vitro* in separate reactions containing a HeLa S-4 extract, a HeLa S-27 extract, or a HeLa S-27 extract plus stripped microsomal membrane (4 A_{260} units/ml) added at the beginning of protein synthesis were digested with proteases. Protease digestion was also carried out in the presence of 0.5% deoxycholate to dissolve the membranes. The proteins obtained in each case were analyzed on 10% polyacrylamide slab gels. In the case of protease-treated samples 3 times as much radioactivity as used in the untreated samples was loaded on the gel. Lanes: (a and k) VS virion proteins; (b–d) proteins synthesized *in vitro* by a HeLa S-4 extract, a HeLa S-27 extract, and a HeLa S-27 extract plus membranes, respectively; (e–g) proteolytic digestion of the proteins synthesized by a HeLa S-4 extract, a HeLa S-27 extract, and a HeLa S-27 extract plus membranes, respectively; (h–j) proteolytic digestion in the presence of deoxycholate of the proteins synthesized by a HeLa S-4 extract, a HeLa S-27 extract, and a HeLa S-27 extract plus membranes, respectively. G_2*, new bond.

the total G_1 and G_2 synthesized was G_2. Addition of membranes at 15 or 30 min of incubation resulted in synthesis of 55% of 20%, respectively, of the G_2 synthesized when membranes were added at the start of the reaction. Synthesis of G_2 was not detected when membranes were added after 60 min of reaction.

Insertion into Membranes and Segregation of *In Vitro* Synthesized G_2. The vectorial discharge of nascent proteins synthesized by membrane-bound polysomes is obligatory for the intracellular movement of proteins that are to be secreted (1, 3). Because isolated rough microsomes exist as vesicles (1), vectorial discharge of the proteins synthesized *in vitro* would segregate the proteins into the intravesicular space. The surrounding membrane would then render the discharged protein resistant to mild proteolysis (3).

In order to determine whether membrane glycoproteins synthesized on membrane-bound polysomes are also vectorially discharged and segregated, we subjected our reaction mixtures to mild proteolysis. Reaction mixtures were treated with protease and then were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 4). Treatment of reaction mixtures with proteases resulted in the disappearance of the synthesized protein bands and the appearance of new bands. Protease-treated reaction mixtures containing HeLa S-4 extract or the reconstructed system containing HeLa S-27 extract and stripped microsomal membrane showed a new band (G_2*) moving slightly faster than G_2 (lanes e and g). When the proteolytic digestion was carried out in the presence of deoxycholate to dissolve the membrane, G_2* was no longer observed (lanes g and h). In contrast to these results, the G_1 protein made by the HeLa S-27 extract was completely digested by the protease treatment (lanes f and i).

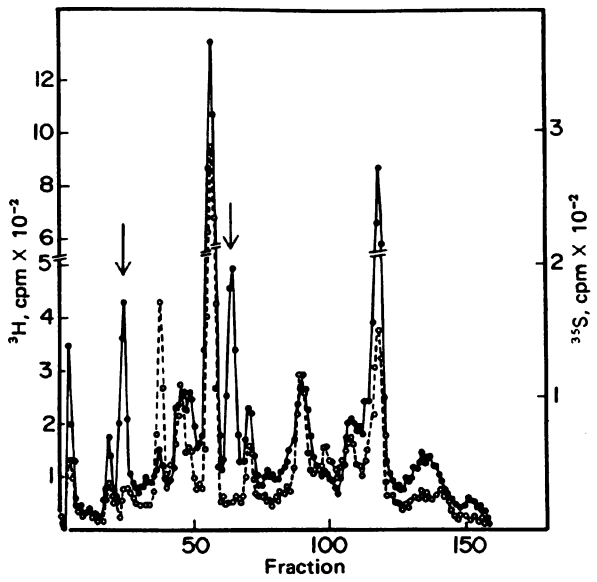

FIG. 5. Comparison of the tryptic peptides of G_2* and G. Conditions of digestion of the radioactive proteins isolated from slab gels and analysis of the tryptic peptides on a cation exchange column have been described (12). ●, ^{3}H radioactivity from G; ○, ^{35}S radioactivity from G_2*. ↓, Peptides present in G but not in G_2*.

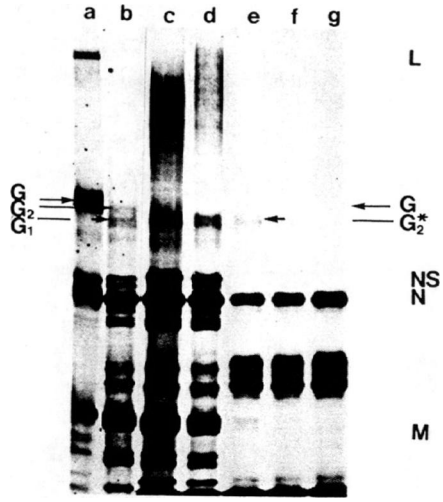

FIG. 6. Effect of addition of membranes at different times on the insertion of G_2 into membranes. Stripped microsomal membranes (8 A_{260} units/ml) were added, at 15, 30, and 90 min, to a reaction mixture containing a HeLa S-27 extract. The [^{35}S]methionine-labeled proteins synthesized at 90 min were digested with proteases and the products were analyzed on 10% polyacrylamide gels. Lanes: (a) VSV-virion proteins; (b–d) proteins synthesized by a HeLa S-27 extract to which membranes were added at 15, 30, and 90 min, respectively; (e–g) proteolytic digestion of proteins synthesized in a HeLa S-27 extract to which membranes were added at 15, 30, and 90 min, respectively.

The appearance of G_2* after protease treatment of reaction mixtures containing G_2 suggested that G_2* could be derived from G_2. Unambiguous proof that G_2* is derived from G_2 could be obtained by comparing tryptic peptide patterns of G_2* and G_2. Because G_2 and G contained identical methionine-containing tryptic peptides (12), we cochromatographed the [^{35}S]methionine-labeled tryptic peptides of G_2* on a cation exchange column with the [^{3}H]methionine-containing tryptic peptides of G. The elution pattern showed that, except for two peaks, G_2* contained all the tryptic peptides present in G (Fig. 5). Therefore, G_2* was derived from G_2.

Calculations of molecular weights based on electrophoretic mobilities showed that G_2* was smaller than G_2 by about 3000 daltons (Fig. 4). Therefore, the entire G_2 molecule was not inserted into the intravesicular space but a peptide of about 3000 daltons remained on the cytoplasmic side of the vesicle and was thus sensitive to proteolytic digestion. The 3000-dalton polypeptide remaining on the outside of the membrane vesicles would thus contain the two missing methionine-containing tryptic peptides. Quantitative analysis of the densitometric scans of the autoradiograms showed that about 80% of the G_2 synthesized *in vitro* in either HeLa S-4 extract or in the reconstituted system was protected against proteolytic digestion. The protected G_2* also contained the glycosidic residues that were present in G_2 (12) (data not presented). These data suggest that *in vitro* synthesis and glycosylation of G_2 by the ribosomal system in the presence of membranes is accompanied by insertion of the polypeptide chain into the intravesicular space. Our results consistently showed protection of a fraction (6%) of the nonglycosylated N protein synthesized against proteolytic digestion. This protection was insensitive to deoxycholate treatment (Fig. 4) and, therefore, could be due to the formation of ribonucleoprotein particles.

Requirement of Membranes at Early Stages of Protein Synthesis for Segregation of G_2. The results presented in Fig. 4 show that, even in the presence of membranes, unglycosylated protein G_1, which is synthesized by free polysomes, was sensitive to proteolytic digestion. The association of the nascent polypeptide chain with membranes to form membrane-bound polysomes may, therefore, be necessary for the insertion of G_2 into membranes. In order to investigate this possibility, we added membranes to a coupled reaction at different times of incubation and analyzed the proteins resistant to proteolytic digestion (Fig. 6). When membranes were added 15 min after the reaction was started, a decreased synthesis of G_2 was observed (lane b). However, the G_2 that was synthesized was protected against proteolytic digestion (lane e). Addition of membranes after 30 min of incubation or at the end of the incubation did not result in the synthesis of G_2 (lanes c and d). The synthesized G_1 was not protected from protease digestion in the presence of the added membranes (lanes f and g). It therefore appeared that insertion of the protein into the membrane vesicle was not possible after the polypeptide chain had been completed. These results strongly suggest that only the nascent polypeptide chain at an early stage of protein synthesis could recognize the membranes and could be inserted into the membrane and be glycosylated.

DISCUSSION

We have reconstructed an *in vitro* protein-synthesizing system by the addition of stripped microsomal membranes to free ribosomes, both obtained from HeLa cells. Translation of the membrane glycoprotein G mRNA by the reconstructed membrane–ribosome system leads to the insertion of the translation product into the membrane and its glycosylation to the glycoprotein G_2. Because G_2 is synthesized exclusively by membrane-bound polysomes (12), the synthesis of G_2 by the reconstructed system suggests the formation *in vitro* of a functional membrane–ribosome complex. The presence of membrane-bound polysomes synthesizing G_2 could also be detected by centrifuging the reconstructed reaction mixture at early stages of incubation (data not presented). Reconstitution of functional membrane–ribosome complexes synthesizing and vectorially discharging nonglycosylated secretory proteins has

also been found with membranes and free ribosomes from heterologous sources (15, 16).

The conversion of the nonglycosylated protein G_1 to the glycosylated protein G_2 as well as the protection of G_2 from proteolytic digestion occurs most efficiently when membranes are added in the first 10 min after start of the reaction. Because the completion of G_2 synthesis *in vitro* requires about 30 min (unpublished observation), it appears that the formation of G_2 as well as its insertion inside the vesicle requires the presence of membranes at the early stages of G_2 synthesis. Our observations agree with the results reported for the proteolytic processing of the signal sequence and intravesicular segregation of nonglycosylated secretory proteins (15, 16). It was shown that processing and segregation of the nonglycosylated secretory proteins were coupled and occurred as cotranslational but not post-translational events (15, 16). The protection of the glycosylated G_2 but not of the nonglycosylated G_1, even in the presence of membranes, suggests that insertion into the membrane and glycosylation of the protein may be coupled and occur while the polypeptide chain is still being synthesized. The insertion of the nascent polypeptide may serve to bring it into contact with the glycosyl transferases of the endoplasmic reticulum as well as allowing the transport of the protein from its site of synthesis.

The proteolytic digestion experiments show that the entire G_2 molecule is not inserted into the intravesicular space but a peptide of about 3000 daltons remains on the cytoplasmic side of the vesicle. Similar results were reported with the membrane glycoprotein PE_2 of Sindbis virus (8). Our finding that membranes are required at an early stage of protein synthesis for insertion of the polypeptide chain inside the vesicles suggests that the insertion into the membrane occurs while the protein is still nascent. If this is the case, the amino terminus of the peptide would enter the endoplasmic reticulum lumen first and, in the absence of any reorientation of the protein in the membrane, the carboxy terminus would remain exposed on the cytoplasmic face of the endoplasmic reticulum where it would be sensitive to proteolytic digestion. The situation with the membrane glycoprotein thus contrasts with that for secretory proteins for which it was found that the entire protein was protected from proteolytic digestion (15) and must, therefore, have crossed into the lumen of the endoplasmic reticulum.

Note Added in Proof. Digestion of G_2 and G with a mixture of carboxypeptidases A and B and tryptic peptide analyses of the digested proteins have shown that the carboxy terminus of G_2 remains exposed on the cytoplasmic face of the membrane vesicle.

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