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Biochemistry

Synthesis and assembly of membrane glycoproteins: Presence of leader peptide in nonglycosylated precursor of membrane glycoprotein of vesicular stomatitis virus

(NH$_2$-terminal sequence/processing *in vitro*/insertion into membranes *in vitro*/glycosylation *in vitro*/radiosequencing)

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ABSTRACT **Translation of mRNA encoding vesicular stomatitis virus envelope glycoprotein G by a membrane-free ribosomal extract obtained from HeLa cells yielded a nonglycosylated protein G_1 (M_r 63,000). In the presence of added microsomal membranes, G_1 was converted to the glycosylated protein G_2 (M_r 67,000) which is inserted in the membrane vesicles as a transmembrane protein. Labeling with methionine donated by wheat germ initiator $tRNA_1^{Met}$ showed that G_1 but not G_2 contains methionine in the NH$_2$-terminal position. Determination of the NH$_2$-terminal sequence of G_1, G_2, and G showed that a leader peptide of 16 amino acids is present in G_1 but absent from the glycosylated proteins G_2 and G. This leader peptide contains at least 62% hydrophobic amino acids and is removed presumably during insertion of G_1 into the membrane.**

An important aspect of membrane biogenesis is the mechanism by which integral membrane proteins are transported from the site of synthesis and are assembled in the membrane. The enveloped viruses that serve as models to study membrane structure and function also are excellent systems for study of membrane biogenesis (1). A study of the mechanisms of synthesis and glycosylation of the viral membrane glycoproteins and of their insertion into the membrane, transport along cellular membrane systems, and ultimate migration to the plasma membrane of the infected cell can help to elucidate the sequence of events involved in membrane biogenesis. Because cell surface glycoprotein molecules are believed to be involved in cellular recognition and information exchange processes occurring on the cell membrane (2), insight into these processes may be gained from studies on membrane glycoprotein synthesis and assembly. Studies from this (3, 4) and other laboratories (5, 6) have shown that translation *in vitro* of the mRNA coding for the membrane glycoprotein G (M_r 69,000) of the enveloped vesicular stomatitis virus (VSV) in the absence of microsomal membranes results in the synthesis of the nonglycosylated protein G_1 (M_r 63,000). In the presence of added microsomal membrane vesicles, however, G_1 is converted to the glycosylated protein G_2 (M_r 67,000) which is inserted into the vesicle membrane. It was also shown that insertion into the membrane and glycosylation are cotranslational events. Unlike secretory proteins, which are also synthesized by membrane-bound ribosomes and are discharged vectorially across the microsomal membrane (7), glycoprotein G_2 is not completely discharged but spans the membrane (4–6).

It has been postulated that the transport of secretory proteins across membranes is initiated by the association of a signal or leader sequence at the NH$_2$ terminus of the nascent polypeptide chain with the endoplasmic reticulum (8, 9). The formation of this ribosome–membrane junction results in the passage of the nascent polypeptide chain through the membrane, the discharge of the completed protein into the lumen of the endoplasmic reticulum, and the proteolytic cleavage of the signal sequence (7–9). The vectorially discharged and processed protein is then transported through the cell and secreted (7). The existence, at the NH$_2$ terminus, of a signal or leader sequence containing 15–30 amino acid residues has been demonstrated recently for a number of secretory proteins (9–16).

Because of our interest in the synthesis and assembly of membrane glycoproteins, we looked for the presence of a leader sequence in the nonglycosylated precursor of the transmembrane glycoprotein G of VSV. In this communication we provide direct evidence that the nonglycosylated precursor protein G_1 contains a leader peptide of 16 amino acids that, in the presence of membranes, is cleaved during the conversion of G_1 to G_2. A preliminary report of this work has been presented (17).

MATERIALS AND METHODS

Plaque-purified VSV (Indiana HR-LT) and HeLa S3 cells were grown as described (3, 4). Radioactive amino acids were obtained from New England Nuclear or Amersham/Searle. Hen egg-white lysozyme was from Worthington.

Preparation of Formylated and Nonformylated Methionyl-$tRNA_1^{Met}$. Initiator $tRNA_1^{Met}$ was purified from wheat germ as described (18) and was free from $tRNA_2^{Met}$ species which donates methionine into internal positions of a polypeptide chain (18). [^{35}S]Met-$tRNA_1^{Met}$ was obtained by charging $tRNA_1^{Met}$ with methionine, using *Escherichia coli* methionyl-tRNA synthetase which can aminoacylate only $tRNA_1^{Met}$ (18). The charged [^{35}S]Met-$tRNA_1^{Met}$ was recovered (18) and chemically formylated as described (19).

Synthesis of VSV-Specific Proteins. Ribosomal systems, containing either membranes (S-4 extract) or free from membranes (S-27 extract), as well as stripped microsomal membranes were isolated from uninfected HeLa cells as described (3, 4). The coupled transcription-translation system containing ribosomes from HeLa cells and purified ribonucleoprotein particles from VSV-infected L cells has been described (3, 4). VSV-specific mRNA was prepared from VSV-infected HeLa cells by extraction with phenol/$CHCl_3$/sodium dodecyl sulfate followed by precipitation with LiCl/ethanol as described (20). The isolated RNA was used to direct VSV-specific protein

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Abbreviations: VSV, vesicular stomatitis virus; protein G, fully glycosylated virion glycoprotein of M_r 69,000; protein G_2, nonsialated glycoprotein (M_r 67,000) synthesized *in vitro*; protein G_1, nonglycosylated precursor protein (M_r 63,000) synthesized *in vitro*.

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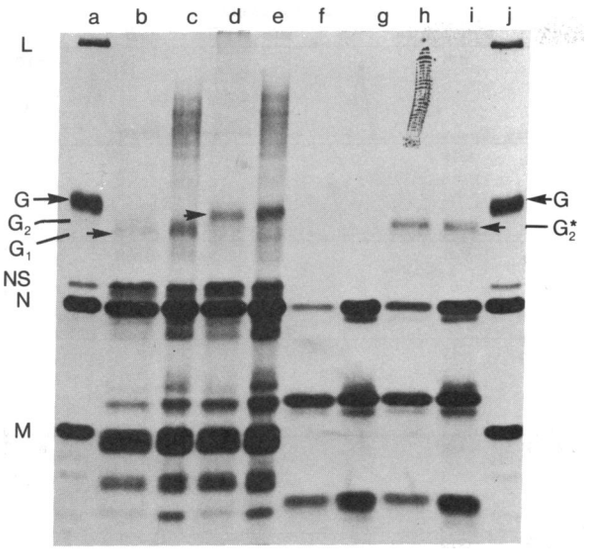

FIG. 1. Autoradiogram of proteins synthesized *in vitro* by direct translation of VSV mRNA and by the coupled system. The reaction mixtures for protein synthesis by the direct translation system contained 40 mM Hepes (pH 7.6), 90 mM KCl, 3.2 mM Mg acetate, 1 mM ATP, 0.2 mM GTP, 1 mM dithiothreitol, 10 mM creatine phosphate, 40 μg of creatinekinase per ml, 80 μM spermine, 19 unlabeled amino acids each at 20 μM, 40 μg of unfractionated VSV mRNA per ml, 200 μCi of [^{35}S]methionine per ml, and 300 μl of the preincubated HeLa extract (3, 4). The coupled system contained the same components except for the following modification: 75 mM KCl, 4.5 mM Mg acetate, 0.8 mM each of CTP, UTP, and GTP, 2 mM ATP, and 0.7 mg of the purified ribonucleoprotein particles from VSV-infected L cells instead of the VSV mRNA (3, 4). All incubations were at 30°C for 90 min. The [^{35}S]methionine-labeled proteins were electrophoresed on 10% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate (3, 4). Lanes: a and j, VS-virion proteins; b and c, proteins synthesized in the absence of membranes in a direct translation and in a coupled reaction mixture, respectively; d and e, proteins synthesized in the presence of membranes in a direct translation and in a coupled reaction mixture, respectively; f–i, proteolytic digestion of the proteins synthesized in reaction mixtures shown in b–e, respectively.

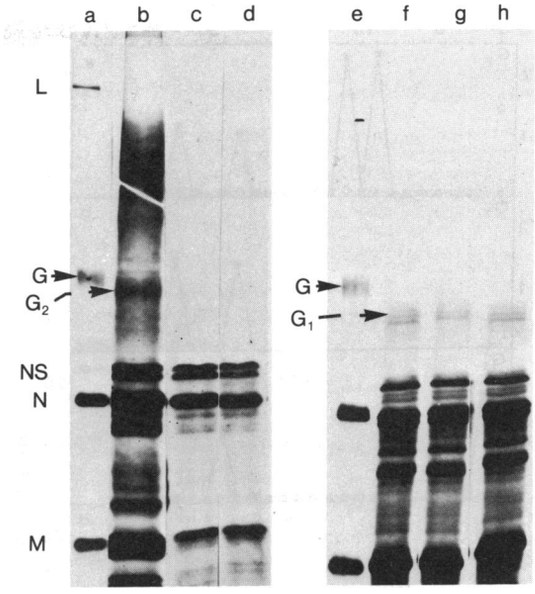

FIG. 2. Autoradiograms of proteins synthesized *in vitro* and containing formyl[^{35}S]methionine and [^{35}S]methionine transferred from formyl[^{35}S]Met-tRNA$_1^{Met}$ and [^{35}S]Met-tRNA$_1^{Met}$, respectively. The conditions of the reaction mixtures were the same as in the direct translation system in Fig. 1 with the following modifications: reaction mixtures containing charged tRNA$_1^{Met}$ also contained 200 μM nonlabeled methionine and the amounts of formyl[^{35}S]Met-tRNA$_1^{Met}$ and [^{35}S]Met-tRNA$_1^{Met}$ used were 1×10^6 cpm/ml and 1.2×10^6 cpm/ml, respectively. Lanes: a and e, VS-virion proteins; b–d, proteins synthesized in the presence of membranes and in reaction mixtures containing [^{35}S]methionine, formyl[^{35}S]Met-tRNA$_1^{Met}$, and [^{35}S]-Met-tRNA$_1^{Met}$, respectively; f–h, proteins synthesized in the absence of membranes and in reaction mixtures containing [^{35}S]methionine, formyl[^{35}S]Met-tRNA$_1^{Met}$, and [^{35}S]Met-tRNA$_1^{Met}$, respectively. The presence of additional peptides migrating between N and M in the reaction mixtures containing membranes (lanes f, g, and h) could be due to premature termination or proteolysis of the synthesized proteins.

synthesis in HeLa cell ribosomal systems in the absence and in the presence of microsomal membranes. The reaction conditions were identical to those described for the coupled system (3, 4) except for the following changes: UTP and CTP were omitted and the concentrations of KCl, magnesium acetate, ATP, and GTP were changed to 90 mM, 3.2 mM, 1 mM, and 0.2 mM, respectively.

Partial Sequence Determination. The volumes of the reaction mixtures for protein synthesis were increased 20-fold, and the mixtures contained one tritiated amino acid and [^{35}S]methionine. The reaction products were separated by electrophoresis in a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and identified by autoradiography (3, 4). The G_1 and G_2 bands were excised from the dried gel and eluted electrophoretically into dialysis casing in the presence of 0.5 mg of hen egg-white lysozyme. The radioactive proteins were recovered by precipitation with 9 vol of acetone at −20°C in the presence of 5 mg of lysozyme and dissolved in 5 mM Tris·HCl (pH 7.5). The eluted samples were checked for purity by reelectrophoresis of a small aliquot and by digestion with Pronase and identification of the released labeled amino acids. In all cases only one radioactive protein band containing [^{35}S]methionine and the specific tritiated amino acid was obtained.

Microsequence analysis of the radiolabeled proteins was carried out as described (21) except that hen egg-white lysozyme was used as a carrier protein in place of apomyoglobin. The proteins were sequentially degraded for 12 or 24 cycles in a Beckman 890C sequencer with a volatile buffer containing *N*-dimethylaminobenzylamine (22). The phenylthiohydantoin derivative of norleucine was added as an internal standard to each tube in the fraction collector. About 25% of each fraction was used for identification of the amino acid derivatives arising from the lysozyme. After being dried in a nitrogen stream, the residues were converted to the phenylthiohydantoin derivatives. These were silylated with *N,O*-bis(trimethylsilyl) acetamide and identified by gas/liquid chromatography in a Hewlett–Packard HP 5700A gas chromatograph with automatic sampler using a glass column with SP400 (Beckman) packing as described (22). The remaining 75% of each fraction containing the thiazolinones was dried in the presence of N_2 and dissolved in dioxane, and an aliquot was assayed for radioactivity. Partial amino acid sequences of G_1, G_2, and G were also determined by automated Edman degradation of proteins labeled with [^{35}S]methionine and a mixture of ^{3}H-labeled amino acids whose phenylthiohydantoin derivatives are separated clearly on thin-layer chromatography plates (16, 21).

RESULTS

Synthesis of G_1 and G_2. For the synthesis and translation of VSV-specific mRNAs we previously used a coupled transcription-translation system containing ribonucleoprotein particles from VSV-infected L cells and ribosomes from unin-

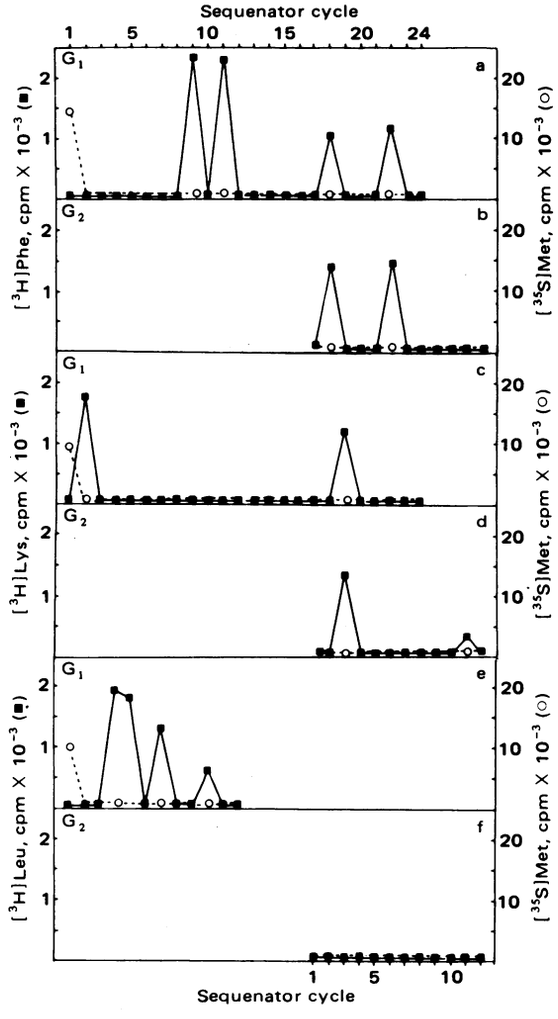

FIG. 3. Partial NH_2-terminal sequence analyses of G_1 and G_2 synthesized *in vitro*. VSV mRNA from infected HeLa cells was translated in HeLa extracts in the absence and in the presence of membranes. The reaction mixture was the same as in Fig. 1 except that it contained: (*a* and *b*) 100 μCi of [^{35}S]methionine (about 100 Ci/mmol) and 100 μCi of [^{3}H]phenylalanine (48 Ci/mmol) per ml and 18 nonlabeled amino acids; (*c* and *d*) 100 μCi of [^{3}H]lysine (90 Ci/mmol) and 100 μCi of [^{35}S]methionine per ml; and in (*e* and *f*) 100 μCi of [^{3}H]leucine (58 Ci/mmol) and 100 μCi of [^{35}S]methionine per ml. The reaction volume was 0.5 ml. After 90-min incubation at 30°C the reaction mixtures were analyzed on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and the appropriate bands of G_1 or G_2 were excised. The radioactive G_1 or G_2 was eluted electrophoretically with a recovery of at least 85%. The recovered proteins were precipitated with 5 mg of carrier lysozyme by the addition of 9 vol of acetone and the precipitate was dissolved in 5 mM Tris·HCl (pH 7.5). Sequence analysis was performed and the samples were assayed for both ^{35}S and ^{3}H; the normalized counts are plotted.

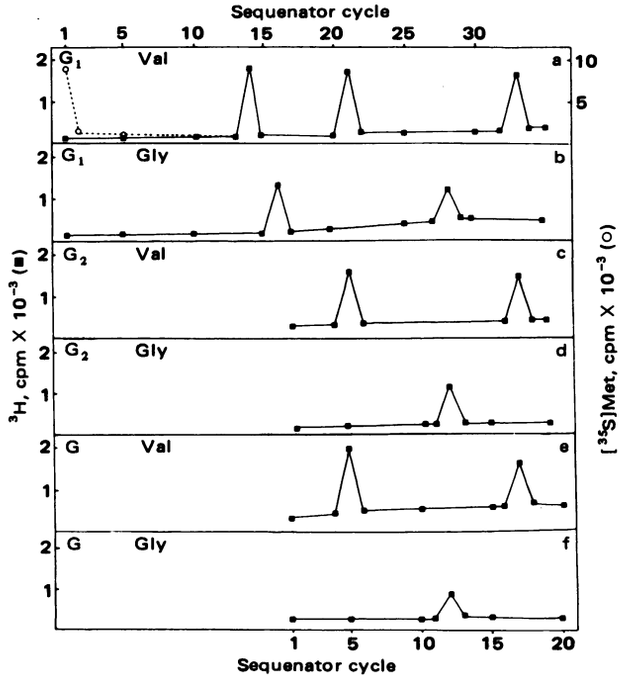

FIG. 4. Partial NH_2-terminal sequence analyses of G_1 and G_2 synthesized *in vitro* and G isolated from VSV-infected HeLa cells. The conditions for *in vitro* synthesis of G_1 and G_2 are described under Fig. 3 except for the following modifications: In the case of G_1 the reaction mixture contained 100 μCi of [^{35}S]methionine (100 Ci/mmol) and 200 μCi of each of [^{3}H]valine (16 Ci/mmol), [^{3}H]glycine (13 Ci/mmol), and [^{3}H]proline (13 Ci/mmol) per ml and 16 nonradioactive amino acids. In the case of G_2 the reaction mixture contained only [^{35}S]methionine, [^{3}H]valine, and [^{3}H]glycine. Labeled G protein was isolated from VSV-infected HeLa cells as follows. HeLa cells were infected with VSV at a multiplicity of infection of 30 (3) and at 4.5 hr after infection were labeled for 20 min with 40 μCi of each of [^{35}S]-methionine, [^{3}H]valine, [^{3}H]glycine, and [^{3}H]alanine (15 Ci/mmol) per ml. The radioactively labeled infected cells were harvested, and virus-specific proteins were separated by gel electrophoresis and isolated from the gel by electrophoresis as described under Fig. 3. Sequence analysis was performed, and aliquots were assayed for both ^{35}S and ^{3}H. The phenylthiohydantoin derivative of the labeled amino acid present at each cycle in the degradation, where a peak of radioactivity appeared, as well as the phenylthiohydantoin derivatives of the nonlabeled amino acids obtained from degradation of the carrier lysozyme and present in the degradative steps on either side of the peak of radioactivity were identified by chromatography on silica gel thin-layer plates (21). Release of radioactive amino acids was plotted against cycle of degradation. Only the data for [^{35}S]methionine, [^{3}H]valine, and [^{3}H]glycine are presented. Analysis of the degradation products of G_1 also showed the presence of proline residues in position 12 and 23. No alanine was detected in the first 24 cycles of G. The nonradioactive residues obtained from degradation of carrier lysozyme were identified by gas chromatography or amino acid analysis.

fected HeLa cells (3, 4). In the present study we translated exogenously added VSV-specific mRNA by ribosomal extracts from HeLa cells. Ribosomes from HeLa cells translated added VSV mRNAs in either the absence or the presence of membranes to yield G_1 or G_2, respectively (Fig. 1, lanes b and d). However, the amounts of G_1 or G_2 synthesized in this system relative to N protein was less than that obtained from the coupled system. As in the coupled system (4), G_2 but not G_1 was protected from proteolytic cleavage by the added membrane vesicles (Fig. 1, lanes g and h). Furthermore, in both the systems, G_2 was reduced in size by 3000 daltons after digestion with protease (4).

Primary Translation Product of G mRNA. Analysis of tryptic peptides showed that the methionine-containing peptides in G_1 and G_2 were identical (3). It was also shown that G_2 is derived from G_1 by glycosylation (3, 4). The requirement of membranes at early stages of protein synthesis for glycosylation and segregation of G_2 (4, 6) suggests that the peptide sequence at the NH_2 terminus of nascent G_1, similar to the signal sequence of precursors of secretory proteins (9, 11, 13, 14), may not interact with the membrane once the peptide chain has elongated beyond a critical size. The possibility that G_1 contains an NH_2-terminal leader sequence that is cleaved off when G_1 is processed into the glycosylated protein G_2 was investigated

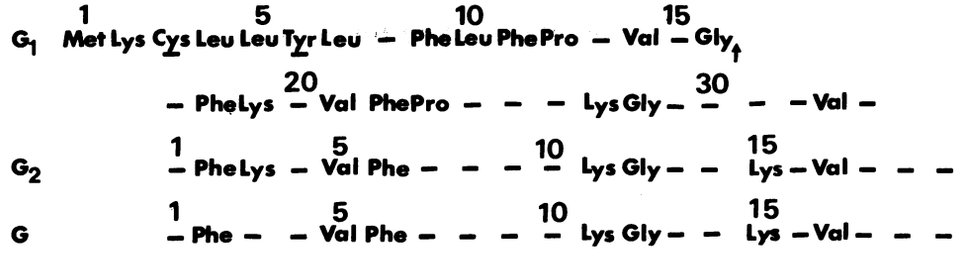

FIG. 5. Comparison of the partial amino acid sequences of G_1, G_2, and G. The Cys and Tyr residues at positions 3 and 6 of G_1 were assigned from the published results of Rose (24). The positions of proline in G_1 were obtained from results of an experiment described but not presented in Fig. 4. Sequence analysis for proline residues was not done for G_2 and intracellular G. However, proline residues were detected in positions 7 and 18 of G isolated from nonradioactive VSV. The amino acid sequence of radioactive G isolated from infected cells was in agreement with that obtained from the sequence analysis of nonradioactive virion G (unpublished data).

as follows. Because NH_2-terminal formylmethionine of proteins synthesized *in vitro* in eukaryotic systems is not removed by methionine aminopeptidase (23), we labeled VSV-specific polypeptides at the initiating methionines by synthesizing them *in vitro* in the presence of formyl[^{35}S]Met-tRNA$_1^{Met}$. The syntheses were carried out in the absence and in the presence of membranes. In the absence of membranes, G_1 and other viral proteins (N, NS, and M) were labeled, whereas in the presence of membranes N, NS, and M were labeled but G_2 was not (Fig. 2). The same results were obtained using non-formylated [^{35}S]Met-tRNA$_1^{Met}$. The multiple polypeptide bands seen in the region of G_1 were related to G_1 (data not presented) and possibly were formed by premature termination of G_1 polypeptide. These data show that polypeptides G_1, N, NS, and M are primary translation products of VSV-specific mRNAs. The fact that, in the presence of membranes, initiator methionine is absent from G_2 but not from viral proteins N, NS, and M suggests that G_2 is derived from G_1 by specific removal of the NH_2-terminal methionine either alone or as a part of a peptide sequence. To distinguish between these possibilities, the NH_2-terminal sequences of G_1 and G_2 were determined.

Partial NH_2-Terminal Sequence of G_1, G_2, and G. G_1 and G_2 labeled with [^{35}S]methionine and [^{3}H]leucine, [^{3}H]lysine, or [^{3}H]phenylalanine were isolated and the positions of the radioactive amino acids were determined by automatic sequence analysis (Fig. 3). The analyses were carried out for 24 cycles except for G_1 labeled with methionine and leucine and G_2 labeled with methionine and lysine, for which only 12 residues were sequentially analyzed. The results show that a methionine residue was present only in position 1 of G_1. Phenylalanine was released from positions 9, 11, 18, and 22 of G_1 and positions 2 and 6 of G_2. Lysine was found in G_1 at cycles 2 and 19 and in G_2 at cycles 3 and 11. The repetitive yield of lysine at position 11 of G_2 was, however, 83% compared to the normal repetitive yield of about 95%. Leucine was present in G_1 in positions 4, 5, 7, and 10. No methionine or leucine was detected in the first 24 positions of G_2.

To determine whether G_2 contained the same NH_2-terminal sequence as mature G protein, we analyzed partial sequences of G isolated from VSV-infected HeLa cells and labeled with [^{35}S]methionine and a mixture of three tritiated amino acids. Fig. 4 shows the results obtained from sequence analyses of G_1, G_2, and G, each labeled with a mixture of [^{35}S]methionine, [^{3}H]valine, and [^{3}H]glycine. The methionine residue was again present in position 1 of G_1 but was absent from the first 20 positions of either G_2 or G. Valine residues were present in positions 14, 21, and 33 of G_1 and in positions 5 and 17 of both G_2 and G. Glycine residues were present in positions 16 and 28 of G_1 and in position 12 of both G_2 and G. Sequence analyses of G_1, G_2, and G labeled with a mixture of [^{35}S]methionine, [^{3}H]leucine, [^{3}H]phenylalanine, and [^{3}H]lysine confirmed the positions of the four amino acids in G_1 and G_2 as deduced from Fig. 3 and further showed the presence of phenylalanine in positions 2 and 6 and lysine in positions 11 and 15 of G. As in G_2, leucine was absent from the first 24 residues of G.

Comparison of the partial amino acid sequence data of G_1, G_2, and G (Fig. 5) shows identity between the amino acids present in positions 18, 19, 21, 22, 27, 28, and 33 of G_1 and those in positions 2, 3, 5, 6, 11, 12, and 17 of G_2. An NH_2-terminal sequence of 16 amino acids present in G_1 is evidently removed in the presence of membranes and may, therefore, be considered as the leader sequence necessary for recognition of the nascent polypeptide by the endoplasmic reticulum. The results further show that G_2 and mature G contain identical amino acids in position 2, 5, 6, 11, 12, 15, and 17.

DISCUSSION

We determined the NH_2-terminal sequence of the nonglycosylated precursor of G synthesized *in vitro* in a membrane-free translation system to establish whether a leader sequence is present in the precursor of a transmembrane glycoprotein. The partial sequences of G_1 and G_2 reveal that G_2 is derived from G_1 by removal of an NH_2-terminal peptide sequence containing 16 amino acids. Because G_2 is derived from G_1 by insertion into membranes, the removal of the NH_2 terminus appears to be analogous to the cleavage of the leader sequence from precursors of secretory proteins (9–16, 25). The partial NH_2-terminal sequence of the precursor protein G_1 is in agreement with the sequence Met-Lys-Cys-Leu-Leu-Tyr-Leu that can be deduced from the nucleotide sequence of the translation initiation site of G mRNA (24). Therefore, G_1 must be the primary translation product of G mRNA. The protein G_2 was shown to be synthesized as a transmembrane protein with the NH_2 terminus and the carbohydrate moieties in the inside of the membrane vesicle and the carboxy terminus on the cytoplasmic face of the membrane (4, 6). That virion G was also a transmembrane protein was shown by digestion of the VSV virion with carboxypeptidases, aminopeptidase, and exoglycosidases. Digestion of the carboxy and the NH_2 terminus of G by the exopeptidases required the solubilization of the envelope of VSV by a detergent. In contrast, the sugar residues present in G could be removed by exoglycosidases in the absence of a detergent. These results suggest that both the carboxy and the NH_2 terminus of the G protein are protected from peptidases by the lipid envelope (ref. 17; unpublished data). Our findings demonstrate that a transmembrane glycoprotein is synthesized as a nonglycosylated precursor containing an additional NH_2-terminal sequence, which is proteolytically removed during the insertion of the precursor into membranes.

The presence of a leader sequence at the NH_2 terminus of precursors of secretory proteins that are synthesized by mem-

brane-bound ribosomes was initially proposed in the signal hypothesis (8, 9), and subsequently a large number of secretory proteins that were synthesized on membrane-bound ribosomes were shown to contain a hydrophobic leader sequence (9–16). The leader sequence was removed from the nascent chain by endopeptidase(s) present in the endoplasmic reticulum (25). Recent reports show that bacterial proteins that are secreted outside the cell or are associated with membranes are synthesized on membrane-bound ribosomes and as precursors containing extra sequences that are removed (26–29). Thus, the role of membranes in the process of protein synthesis and export (7) and the mechanism of transport across membrane of proteins synthesized on membranes (7–9) may be similar in both eukaryotic and prokaryotic systems. Our present finding that transmembrane glycoproteins are also synthesized as a precursor containing a leader sequence that is removed in the presence of membrane extends the validity of the signal hypothesis to transmembrane proteins. Ovalbumin, a secretory glycoprotein synthesized in the hen oviduct, is an exception in that only the NH_2-terminal methionine is removed (30). Other glycoproteins synthesized and secreted from the same organ, however, do contain leader sequences that are removed (31).

The partial sequence data show that at least 62% of the leader sequence of G_1 consists of hydrophobic amino acids, which agrees with the high hydrophobicity of the leader sequences reported for a number of secretory proteins (9–16, 31). Analyses of the sequences of the leader peptides, however, have not yet revealed an extensive sequence homology that can account for specific association of the leader sequences with membranes (9–16, 31). The cleavage of the leader sequence of G_1 occurs next to a glycine residue. The presence of a glycine residue preceding the cleavage sites of leader sequences of a number of precursor proteins has also been reported (12, 16, 27, 31); however, no similarity in the sequence of the amino acids adjacent to the cleavage site has been observed (12, 16, 27, 31). In addition, other amino acids have also been shown to be present at the cleavage sites of leader sequences of a number of different precursor proteins (11–16, 31). It appears, therefore, that the mechanism of insertion into membranes and cleavage of the leader sequence does not involve a common primary structure but may be determined by secondary and tertiary structures.

Membrane glycoproteins would be expected to contain hydrophobic sequence(s) in addition to the NH_2-terminal hydrophobic sequence that serves as a signal. Other hydrophobic sequences must exist to provide for a permanent association with membranes as in hydrophobic domains on the membrane glycoprotein glycophorin (32, 33). The hydrophobic fragments of the envelope glycoproteins of Semliki Forest virus (34) and VSV (35) buried in the lipid membrane can be obtained by proteolytic digestion of the virion. Comparison of the amino acid sequence of the leader peptide and of the membrane-anchorage fragment may provide information on the structural basis of protein–lipid interactions in biological membranes.

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