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Synthesis and glycosylation *in vitro* of glycoprotein of vesicular stomatitis virus

(coupled transcription and translation/vesicular stomatitis virus-specific ribonucleoprotein particles/membranes/concanavalin A/exoglycosidase)

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ABSTRACT **Coupling of ribonucleoprotein particles from L cells infected with vesicular stomatitis virus to a pre-incubated ribosomal system obtained from uninfected HeLa cells allowed synthesis of two proteins, G_1 (molecular weight 63,000) and G_2 (molecular weight 67,000), and all other proteins of vesicular stomatitis virus except the spike protein G (molecular weight 69,000). Analyses of the tryptic peptides showed that G_1, G_2, and G had identical peptide sequences. The synthesis of G_2 required the presence of membranes; only G_1 was synthesized in the absence of any membranes. G_2 but not G_1 was shown to be a glycoprotein by affinity chromatography on a concanavalin A-Sepharose column. Removal of sialic acid residues from G by neuraminidase resulted in a product having an identical mobility to G_2. Digestion of G_2 or G with a mixture of neuraminidase (EC 3.2.1.18), β-galactosidase (EC 3.2.1.23), and β-*N*-acetylglucosaminidase (EC 3.2.1.30), however, produced a protein of molecular weight 65,000. These data suggest that G_2 is the desialated G and is formed by glycosylation of G_1, which is the unglycosylated polypeptide backbone of G.**

Vesicular stomatitis virus (VSV) consists of a ribonucleoprotein (RNP) core composed of the negative-stranded genomic RNA and the three viral proteins, L, N, and NS, surrounded by an envelope containing the two viral proteins, G and M (1). The G protein (molecular weight 69,000) is the only glycosylated protein present in the vesicular stomatitis virion (1) and contains about 10% carbohydrate by weight (2).

Studies from this laboratory and from other laboratories have shown that the mRNA coding for G is localized exclusively on membrane-bound polysomes in infected cells (3–6). Translation of membrane-bound polysomal RNA in cell-free systems, however, results in the synthesis of a protein G_1, which has a tryptic peptide pattern identical to that of virion G but a lower molecular weight of 63,000 and, therefore, is believed to be an unglycosylated precursor of virion G (4, 5, 7). Translation by different cell-free systems of VSV-specific mRNA species synthesized *in vitro* by RNP particles also resulted in the synthesis of G_1 (8, 9).

Although several viral glycoprotein mRNAs have been translated *in vitro* into the unglycosylated protein precursors (3–10), the synthesis of a glycosylated protein in a cell-free system by glycosylation of the synthesized polypeptide *in vitro* has not been reported. Here we report the synthesis *in vitro* of a glycosylated precursor of G in a cell-free system that synthesizes VSV mRNA species and translates them into VSV-specific proteins. Our results also show that the translation of the glycoprotein mRNA can occur on free polysomes and that the presence of membranes is required for glycosylation of the polypeptide chain.

Abbreviations: VSV, vesicular stomatitis virus; Con A-Sepharose, concanavalin A bound to Sepharose matrix; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; RNP, ribonucleoprotein.

MATERIALS AND METHODS

Plaque-purified VSV (Indiana, HR-LT) was grown in L cells as described (8). HeLa S_3 suspension cultures were grown in Joklik modified minimum essential medium containing 6% fetal calf serum.

Coupled Transcription-Translation System. The coupled transcription-translation system contained RNP particles, isolated from VSV-infected L cells (8) and further purified by sucrose gradient centrifugation, and preincubated ribosomal extracts obtained from uninfected HeLa cells (11). HeLa S-4, S-10, and S-20 extracts were prepared by centrifuging the disrupted cell suspension at 4000 × *g* for 10 min, 10,000 × *g* for 10 min, and 20,000 × *g* for 30 min, respectively. Protein synthesis was followed as described (8) under conditions allowing both RNA and protein synthesis. The reaction mixture contained 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.6), 75 mM KCl, 4.5 mM magnesium acetate, 1 mM dithiothreitol, 0.8 mM each of CTP, UTP, and GTP, 2 mM ATP, 10 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 80 μM spermine, 20 μM of each of 19 unlabeled amino acids, 200 μCi of [^{3}H]leucine or 400 μCi of [^{35}S]methionine per ml, 0.7 mg of the purified RNP particles per ml, and 400 μl of the preincubated HeLa extract per ml. The mixture was incubated at 30° for 60 min.

Separation and Translation of Membrane-Bound and Free Polysomes. Membrane-bound polysomes formed in the coupled reaction mixture containing nonradioactive precursors were centrifuged of at 20,000 × *g* for 30 min (6). The free polysomes were pelleted from the supernatant fraction by centrifugation at 150,000 × *g* for 1 hr. Both pellets were resuspended to their original volumes in 20 mM Hepes (pH 7.6)/80 mM KCl/4 mM magnesium acetate/6 mM 2-mercaptoethanol. Protein synthesis was carried out with the isolated polysome fraction as described (4) except that 100 μl of a preincubated HeLa S-10 extract per ml was included in reaction mixtures containing the membrane-bound polysomes (5). The addition of the preincubated HeLa S-10 extracts increased the amount of protein synthesized by the membrane-bound polysomes (5).

Concanavalin A-Sepharose Column Chromatography. The [^{35}S]methionine-labeled translation products from coupled reaction mixtures were made 1% in Nonidet P-40 (Shell Chemical Co.) in a final volume of 0.5 ml, sonicated for 1 min, and incubated at 37° for 30 min. Affinity chromatography of glycoproteins on concanavalin A (Con A)-Sepharose columns was done according to Stohlman *et al.* (12), except that the column was equilibrated with 20 mM Hepes (pH 7.6)/120 mM KCl/5 mM magnesium acetate/6 mM 2-mercaptoethanol/0.1% Nonidet P-40. The adsorbed protein was eluted with the equilibrating buffer containing 0.2 M α-methylmannoside.

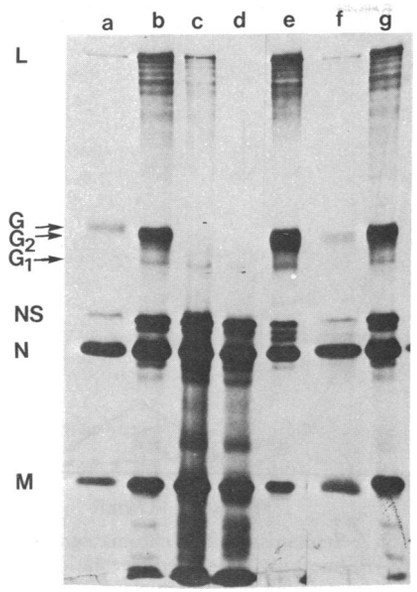

FIG. 1. Autoradiogram of proteins synthesized *in vitro* by the coupled system and by ribosomal extracts from VSV-infected HeLa cells. Purified RNP particles were prepared by treating a VSV-infected L cell extract (8) with 0.5% Triton N101/20 mM EDTA and centrifuging it through a 15–30% sucrose gradient in 0.01 M Tris·HCl (pH 7.8)/5 mM EDTA/2 mM dithiothreitol at 100,000 × *g* for 5 hr. The polymerase activity sedimenting in the 140–100S region was recovered by centrifugation and the RNP particles were resuspended in 0.01 M Tris·HCl (pH 7.8)/10% glycerol/2 mM dithiothreitol. Free and membrane-bound polysomes were isolated from a coupled reaction mixture and from VSV-infected cells as described in *Materials and Methods*. The reaction mixtures for protein synthesis by free and membrane-bound polysome contained 20 mM Hepes (pH 7.6), 100 mM KCl, 3.2 mM magnesium acetate, 1 mM ATP, 20 μM GTP, 10 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 80 μM spermine, 20 μM of each of the 19 unlabeled amino acids, 200 μCi of [^{3}H]leucine or 400 μCi of [^{35}S]methionine per ml, and 300 μl of the resuspended polysome pellet per ml. The mixtures were incubated at 30° for 1 hr. The labeled translation products were electrophoresed on 10% polyacrylamide slab gels as described earlier (8), except that the acrylamide:bis-acrylamide ratio was changed to 30:0.4. (*a*) VS virion proteins; (*b* and *g*) proteins synthesized by an S-4 extract prepared from VSV-infected HeLa cells and by a coupled reaction mixture containing an uninfected HeLa S-4 extract, respectively; (*c* and *d*) proteins synthesized by free polysomes isolated from VSV-infected HeLa cells and from a coupled reaction mixture, respectively; (*e* and *f*) proteins synthesized by membrane-bound polysomes isolated from VSV-infected HeLa cells and from a coupled reaction mixture, respectively.

Exoglycosidase Digestion of Glycoproteins. A partially purified preparation of G from [^{35}S]methionine-labeled VSV or a reaction mixture containing [^{35}S]methionine-labeled G_2 was digested with 0.06 unit/ml of neuraminidase or with a mixture of 0.06 unit/ml of neuraminidase (EC 3.2.1.18), 0.17 unit/μl of β-galactosidase (EC 3.2.1.23), and 0.35 unit/ml of β-*N*-acetylglucosaminidase (EC 3.2.1.30) in 0.10 M phosphate buffer (pH 6.0) at 37° for 1½ hr and electrophoresed on 10% polyacrylamide gels. One enzyme unit released 1 μmol of the glycosidic residue in 15 min.

RESULTS

Coupled Transcription-Translation System. A detailed account of the characterization and conditions for the coupled transcription-translation system will be presented elsewhere. RNA synthesis by the RNP was linear for at least 3 hr under these conditions and resulted in the production of all four VSV-specific mRNA species. Addition of RNP particles to HeLa extract S-10 resulted in a 20- to 25-fold stimulation in protein synthesis, which was also linear for at least 3 hr.

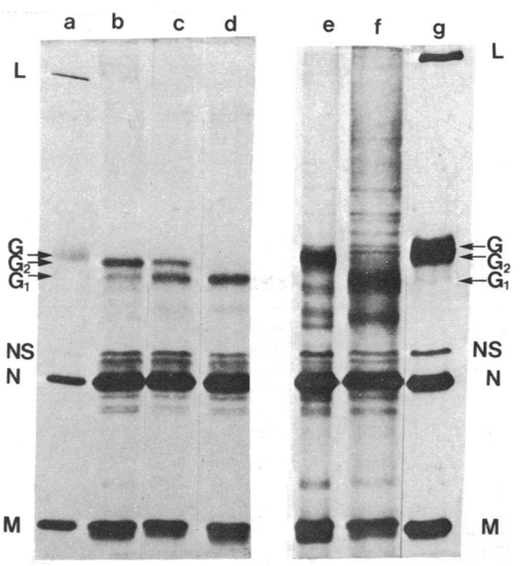

FIG. 2. Autoradiogram of proteins synthesized *in vitro* in the presence and absence of membranes. The [^{35}S]methionine-labeled proteins synthesized in the coupled system containing HeLa S-4, S-10, and S-20 extracts were electrophoresed on 10% polyacrylamide slab gels. (*a*) VS virion proteins; (*b*, *c*, and *d*) proteins synthesized by coupled reaction mixtures containing HeLa S-4, S-10, and S-20 extracts, respectively. Membrane-bound polysomes isolated from a coupled reaction mixture containing HeLa S-4 extract were treated with 0.5% deoxycholate and recovered by centrifugation through a 1 M sucrose cushion. The [^{35}S]methionine-labeled translation products were analyzed on polyacrylamide slab gels. (*e* and *f*) Proteins synthesized by untreated and by deoxycholate-treated membrane-bound polysomes, respectively; (*g*) VS virion proteins.

Isolation of Membrane-Bound and Free Polysomes from the Reaction Mixture *In Vitro*. Polysomes were formed *in vitro* in the coupled reaction mixture containing an S-4 extract and nonradioactive nucleosidetriphosphates and amino acids. The free and membrane-bound polysomes were then isolated by differential centrifugation and protein synthesis was continued in the presence of [^{3}H]leucine, under conditions that did not allow RNA synthesis. The proteins synthesized by the polysome fractions formed *in vitro* were compared to those synthesized *in vitro* by free and membrane-bound polysomes isolated from VSV-infected HeLa cells (4) (Fig. 1). The coupled system containing HeLa S-4 allowed the synthesis of the same five VSV proteins (Fig. 1*g*) as were synthesized by an S-4 extract prepared from VSV-infected HeLa cells (4) (Fig. 1*b*). Free polysomes isolated either from the coupled reaction mixture or from VSV-infected HeLa cells synthesized all the viral proteins except for glycoprotein G (Fig. 1*c* and *d*). Protein G_2 was synthesized *in vitro* only by the membrane-bound polysomes isolated either from the VSV-infected HeLa cell extract or from the coupled reaction mixture (Fig. 1*e* and *f*).

Requirement of Membranes for Synthesis of G_2. The mobility of protein G_2 synthesized *in vitro* by membrane-bound polysomes was slightly greater than that of virion G and corresponded to a molecular weight of 67,000 (Fig. 1). Translation of VSV mRNA synthesized *in vitro* in a wheat embryo cell-free system (8), however, produced a protein G_1 that moved faster than G and G_2 and had a molecular weight of 63,000. To determine whether the presence of membranes was required for the synthesis of G_2, we prepared extracts containing different amounts of membranes from HeLa cells and used them in the coupled system. Thus, a membrane-rich HeLa extract S-4

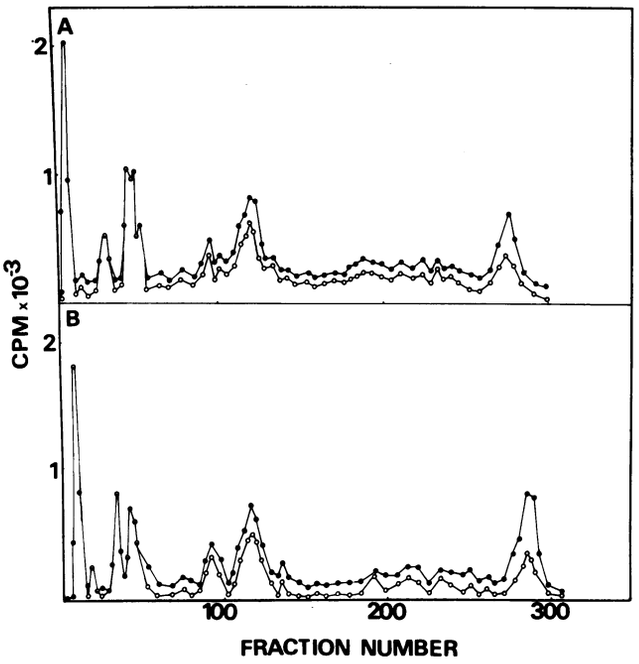

FIG. 3. Comparison of the tryptic peptides of G_1, G_2, and virion G. [^{35}S]Methionine-labeled G_1 and G_2 and [^{3}H]methionine-labeled G were isolated from 10% polyacrylamide slab gels by cutting out the appropriate band and were digested by incubating the gel slice with 100 μg of trypsin per ml for 16 hr at 37°. An additional 100 μg of trypsin per ml was added and incubation was continued for 4 hr. The digests were run on a cation exchange column (Aminex A-5) as described (8). (*A*) Comparison of [^{3}H]methionine-labeled tryptic peptides of virion G and [^{35}S]methionine-labeled tryptic peptides of G_2. (*B*) Comparison of [^{3}H]methionine-labeled tryptic peptides of virion G and [^{35}S]methionine-labeled tryptic peptides of G_1. (○) ^{3}H radioactivity; (●) ^{35}S radioactivity.

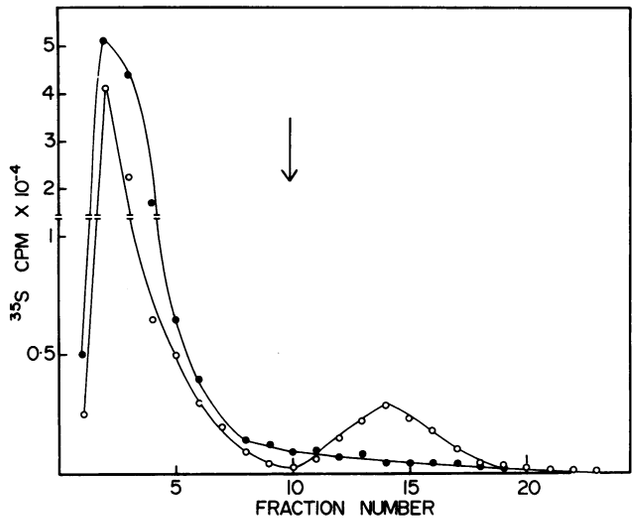

FIG. 4. Con A-Sepharose column chromatography of translation products. The [^{35}S]methionine-labeled translation products (about 300,000 cpm) from coupled reaction mixtures containing HeLa S-4 or S-20 extracts were chromatographed on a Con A-Sepharose column as described in *Materials and Methods*. The 5% Cl_3CCOOH-insoluble radioactivity of each fraction was determined. Seventy to 80% of the total counts applied on the column were recovered in either case. The arrow denotes elution with buffer containing 0.2 M α-methylmannoside. (○) Reaction mixture containing HeLa S-4 extract; (●) reaction mixture containing HeLa S-20 extract.

synthesized major amounts of G_2 and minor amounts of G_1 (Fig. 2*b*). An S-10 extract, in which some of the membranes were removed by centrifugation, allowed the synthesis of equal amounts of G_1 and G_2 (Fig. 2*c*). An S-20 extract, which contained very little membrane, synthesized predominantly G_1 when coupled to RNP particles (Fig. 2*d*). The requirement of membranes for the synthesis of G_2 was further showed by dissolving the membranes of membrane-bound polysomes isolated from *in vitro* coupled reaction mixtures with deoxycholate and analyzing the proteins synthesized by these polysomes. In contrast to the synthesis of G_2 by membrane-bound polysomes, translation of deoxycholate-treated membrane-bound polysomes resulted in the synthesis of G_1 (Fig. 2*e* and *f*). The synthesis of G_2 was, therefore, dependent on the presence of intact membranes.

Tryptic Peptide Patterns of G, G_1, and G_2. The differences in molecular weights among G_1, G_2, and virion G could be due to their different carbohydrate contents. Thus, if virion G represents the fully glycosylated proteins, then G_2 and G_1 could represent partially glycosylated and carbohydrate-free species, respectively. Comparison of the tryptic peptide patterns of G, G_2, and G_1 would indicate if G_1 represents the common protein backbone. The [^{35}S]methionine-labeled tryptic peptides of G_1 or G_2 were cochromatographed on a cation exchange column with the [^{3}H]methionine-containing tryptic peptides of virion G. The elution pattern showed that G_1, G_2, and G all contained identical tryptic peptides (Fig. 3). The absence of any extra methionine-containing peptides in either chromatogram suggested that all three proteins had the same amino-acid sequence and that the increase in molecular weight of G_2 was not due to the presence of any additional polypeptide residue.

Presence of Glycosidic Residue(s) on G_2. Since Con A binds specifically to the glycosidic residues of glycoproteins (13), affinity chromatography on Con A-Sepharose columns can be used to separate glycosylated and nonglycosylated proteins (12). Analyses on Con A-Sepharose columns were, therefore, used to determine whether G_1 and G_2 contained glycosidic residues. The glycoprotein G isolated from VSV-infected L cells could be specifically adsorbed to a Con A-Sepharose column and could be eluted with 0.2 M α-methylmannoside. Only 7% of the total protein synthesized in the coupled system containing HeLa S-4 was retained by Con A-Sepharose and was eluted with α-methylmannoside (Fig. 4). No detectable radioactivity from the reaction mixture containing HeLa S-20 was retained on the Con A-Sepharose column (Fig. 4). Slab gel electrophoresis of the proteins synthesized by the coupled system containing HeLa S-4 and retained by Con A-Sepharose showed the presence of G_2 (Fig. 5*c*). The protein G_1 synthesized by the coupled system containing HeLa S-20 was, however, not bound on the Con A-Sepharose column (Fig. 5*f*). The retention and consequent elution by α-methylmannoside of both G and G_2 showed that only these two proteins contained carbohydrate moieties. The apparent retention of the nonglycosylated protein N by Con A-Sepharose might be due to the incomplete removal of N, which was present in a large excess amount.

The presence of carbohydrate residues in G_2 was further confirmed by digestion of G, G_2, and G_1 with exoglycosidases. A reaction mixture containing [^{35}S]methionine-labeled G_2 was digested with a mixture of neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase, and the digested material was analyzed on sodium dodecyl sulfate/polyacrylamide gels (Fig. 6). As a control, [^{35}S]methionine-labeled virion G digested with either neuraminidase or a mixture of neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase, was also electrophoresed. Results presented in Fig. 6 showed that digestion of G with neuraminidase increased its mobility, and the product

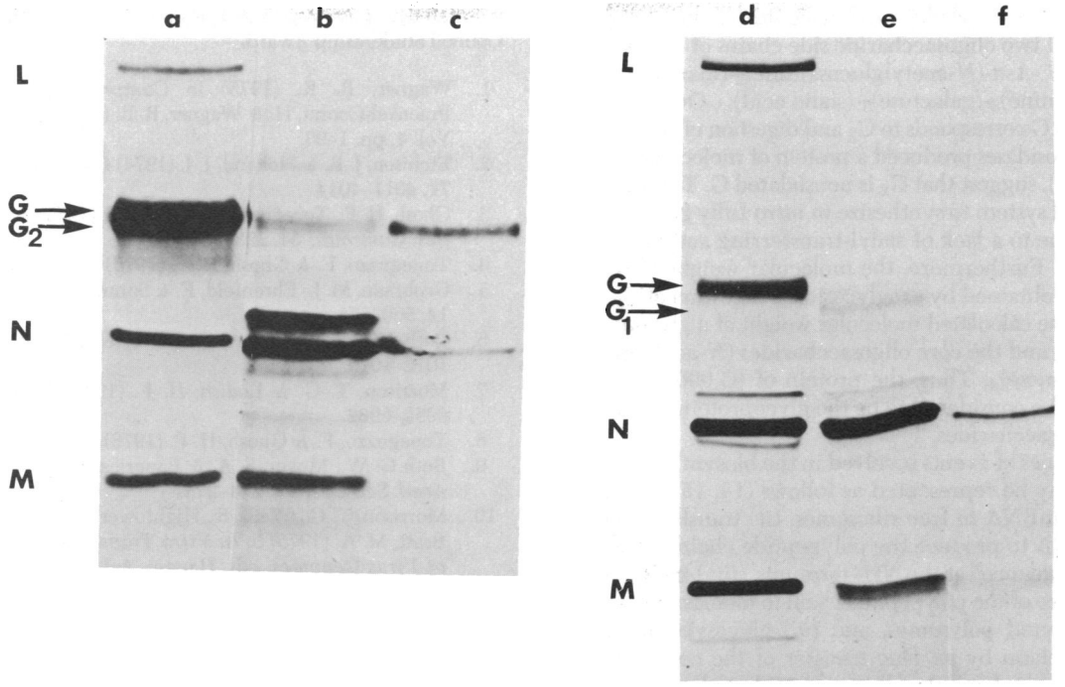

FIG. 5. Sodium dodecyl sulfate/polyacrylamide slab gel analysis of the proteins separated by Con A-Sepharose column chromatography. The material retained on a Con A-Sepharose column and eluted with 0.2 M α-methylmannoside (fractions 11–15 of Fig. 4) was precipitated with acetone, redissolved in sample buffer, and electrophoresed on 10% polyacrylamide slab gels. (*Left*) Slab gel analysis of the protein synthesized in a coupled reaction mixture containing a HeLa S-4 extract and separated by Con A-Sepharose chromatography. (*a*) VS virion proteins; (*b*) proteins synthesized in a coupled reaction mixture containing HeLa S-4 extract; (*c*) proteins retained by Con A-Sepharose. (*Right*) Slab gel analysis of the proteins synthesized in a coupled reaction mixture containing a HeLa S-20 extract and separated by Con A-Sepharose column chromatography. (*d*) VS virion proteins; (*e*) proteins synthesized in a coupled reaction mixture containing HeLa S-20 extract; (*f*) proteins retained by Con A-Sepharose.

had the same mobility as G_2 (Fig. 6*c* and *e*). The mobilities of the proteins obtained after digestion of either G or G_2 with a mixture of the three glycosidases were identical and corresponded to a molecular weight of approximately 65,000. The results showed that G_2 was desialated G and contained galactose and *N*-acetylglucosamine residues.

DISCUSSION

The observed requirement for membranes for the synthesis only of G_2 but not of G_1 shows that the translation of mRNA coding for membrane glycoprotein can proceed in the absence of membranes. Quantitative analysis shows that the amount of G_1 synthesized in a system lacking membranes is the same as the amount of G_2 synthesized when membranes are present (data not presented). Glycosylation of the protein moiety or the presence of membranes, therefore, appears to have no direct effect on the extent of translation of mRNA specific for glycoproteins.

It is now universally accepted that secretory proteins as well as proteins or glycoproteins that are localized on membranes are synthesized by polysomes attached to membranes (14). The formation of membrane-bound polysomes is believed to occur through the association of a unique sequence of amino acids present at the amino terminus of the nascent polypeptide chain to the membrane (15). This sequence of amino acids is referred to as the "signal" or "leader" sequence and has a high content of hydrophobic amino acid (16). Our findings, that G_2 has the same peptide sequence as G_1, is derived from backbone polypeptide G_1 by glycosylation, and is synthesized on membrane-bound polysomes, suggest that G_1 contains the "leader" polypeptide sequence that binds to membrane. Analysis of the amino-acid composition of the NH_2-terminal polypeptide fragment of the G protein that is buried in the virion envelope shows that it contains a very high percentage of hydrophobic amino acids (17). This fragment may thus represent the "signal" sequence of the G protein and, unlike the "leader" sequence of the immunoglobulins, is not removed from the completed protein (16).

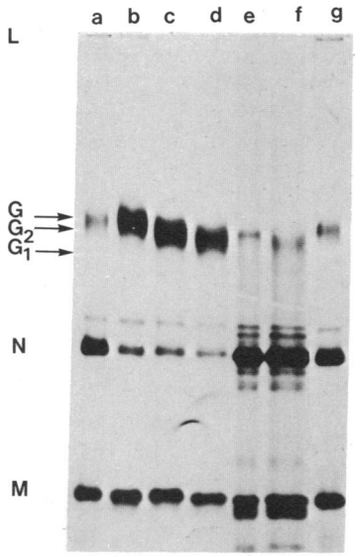

FIG. 6. Autoradiogram of exoglycosidase-digested G and G_2. (*a* and *g*) VS virion proteins; (*b*, *c*, and *d*) partially purified virion G, untreated, digested with neuraminidase, and digested with a mixture of neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase, respectively; (*e* and *f*) reaction mixture containing G_2, untreated and digested with a mixture of the three exoglycosidases, respectively.

Recent analyses of the oligosaccharide side chains of G show the presence of two oligosaccharide side chains of the possible structure (18): -Asn-(*N*-acetylglucosamine)$_2$-(mannose)$_4$-(*N*-acetylglucosamine)$_3$-(galactose)$_3$-(sialic acid)$_n$. Our findings, that desialated G corresponds to G_2 and digestion of both G and G_2 by exoglycosidases produced a protein of molecular weight 65,000 (Fig. 6), suggest that G_2 is nonsialated G. The inability of the coupled system to synthesize *in vitro* fully glycosylated G could be due to a lack of sialyl-transferring activity in the HeLa extract. Furthermore, the molecular weight of 65,000 of the protein obtained by exoglycosidase digestion of G_2 or G agreed with the calculated molecular weight of a glycoprotein containing G_1 and the core oligosaccharides (*N*-acetylglucosamine)$_2$-(mannose)$_4$. Thus, the protein of 65,000 molecular weight could be considered to be the glycoprotein containing the core oligosaccharides.

The sequences of events involved in the biosynthesis of glycoproteins may be represented as follows (14, 15, 19): (*i*) attachment of mRNA to free ribosomes, (*ii*) translation of the specific mRNA to produce the polypeptide chain containing the "leader sequence" at the NH_2 terminus, (*iii*) binding of the leader sequence of the polypeptide chain to membranes to form membrane-bound polysomes, and (*iv*) glycosylation of the polypeptide chain by *en bloc* transfer of the core oligosaccharide molecules from the oligosaccharide-polyprenol intermediate present in the membranes, followed by (*v*) addition of terminal carbohydrate residues to produce the completed glycoprotein. The reported *in vitro* synthesis of the unglycosylated and glycosylated precursors, G_1 and G_2, respectively, of VSV glycoprotein G by the coupled system as well as the availability of VSV mutants defective in G protein synthesis (20) allows us to study the sequences of events of glycoprotein biosynthesis using G as a model.

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