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Association of Vesicular Stomatitis Virus Glycoprotein with Virion Membrane: Characterization of the Lipophilic Tail Fragment

ROBERT H. SCHLOEMER AND ROBERT R. WAGNER*

Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22901

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The proteolytic enzyme, thermolysin, degraded the external segment of the membrane glycoprotein of intact vesicular stomatitis (VS) virions but left behind a small nonglycosylated fragment, presumably embedded in the virion membrane. Other proteases generated membrane-associated glycoprotein fragments differing somewhat in molecular weight. The thermolysin-resistant, virion-associated fragment, which can be selectively solubilized by either Triton X-100 or chloroform/methanol, has a molecular weight of 5,200. Amino acid analysis of the glycoprotein fragment reveals a preponderance of hydrophobic amino acids (64% of the residues); the amino-terminal amino acid is alanine as determined by dansylation. Cyanogen bromide digestion of the tail fragment generated two peptides, confirming the presence of one methionine residue per thermolysin-resistant glycoprotein fragment. The secondary structure of this glycoprotein tail peptide is maintained by at least one disulfide bridge. Thermolysin treatment of isolated VS viral glycoprotein in the presence of Triton X-100 also generated a hydrophobic peptide fragment which is very similar to the virion-associated glycoprotein fragment. The amino acid terminus of intact glycoprotein was also found to be alanine as was its dansylated Triton-micellar fragment that resisted thermolytic degradation; this finding suggests that the amino-terminal end of the VS viral glycoprotein is embedded in the virion membrane. These results suggest that the VS viral glycoprotein is an amphipathic molecule, the hydrophilic portion of which contains all the carbohydrate and a lipophilic tail segment which forms lipid or detergent micelles, thus rendering it resistant to proteolysis.

Vesicular stomatitis (VS) virus is an RNA-containing enveloped virus which consists of an helical ribonucleocapsid core and a lipoprotein membrane (16). The virion membrane contains cell-derived lipids and glycolipids, in addition to two viral proteins: the membrane or matrix (M) protein and the glycoprotein (G) (11, 14, 26, 27). The G protein comprises the spike-like projections which emanate from the external surface of the lipid bilayer (2, 3, 28) and is selectively degraded by incubation of VS virions with proteolytic enzymes (2, 13, 18). The isolated glycoprotein is the major antigen which induces and binds to VS viral neutralizing antibody (10). It seems probable that the G protein provides the attachment site of VS virions to cellular surfaces since removal of glycoprotein from intact VS virions by trypsin results in the inability of these virions to adsorb to L cells (19).

In view of the biological importance of the VS viral glycoprotein in the infectivity of this virus, the question arises as to the nature of the association of the G protein with other constituents of the viral membrane. Recently, Mudd (15) has demonstrated a virion-associated glycoprotein fragment after proteolytic digestion of intact VS virus. We have confirmed and extended this finding. Specifically, the isolation and characterization of a lipophilic peptide segment of the VS viral glycoprotein are described herein. Our data indicate that this segment of the glycoprotein represents the amino terminus of the G protein and is responsible for the association of the glycoprotein with the viral membrane.

MATERIALS AND METHODS

Chemicals and radiochemicals. Trypsin (3× crystallized) and chymotrypsin (2× crystallized) were purchased from Worthington Biochemical Corp., Freehold, N.J. Pronase (B grade, nuclease free) was procured from Calbiochem, La Jolla, Calif. Sigma Chemical Co., St. Louis, Mo. provided the following: thermolysin, bromelain, subtilisin, dansyl chloride,

marker dansylated amino acids, bacitracin, cytochrome *c*, and pepsin. Cyanogen bromide (CNBr) was from Eastman Kodak Co., Rochester, N.Y., and polyamide-6 plastic sheets were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. [^{3}H]leucine (60 Ci/mmol) and [^{14}C]methionine (59 mCi/mmol) were purchased from Schwarz/Mann, Orangeburg, N.Y. [^{3}H]valine (15 Ci/mmol), [^{3}H]glycine (1 Ci/mmol), and [^{14}C]protein hydrolysate (51 mCi/matom of carbon) were supplied by Amersham/Searle, Arlington Heights, Ill.; D-[6-^{3}H]glycosamine hydrochloride (7.3 Ci/mmol) was from New England Nuclear Corp., Boston, Mass.

Virus and cell cultures. Cultivation of BHK-21 cells and of the Indiana serotype of VS virus has been previously described (18). Cell cultures of BHK-21 cells were infected with plaque-purified VS virus at a multiplicity of ~1 PFU/cell which resulted in yields of only VS-B virions. For the production of labeled virus, radioactive isotopes ([^{14}C]protein hydrolysate at 1 μCi/ml; [^{14}C]methionine or [^{3}H]glucosamine at 2 μCi/ml; or [^{3}H]leucine, [^{3}H]glucine, or [^{3}H]valine at 5 μCi/ml) were added along with the viral growth medium after the initial adsorption of virus to cells. Virus that had been released into the medium 18 to 20 h postinfection was purified by differential, rate zonal, and equilibrium centrifugations (4, 14). Purified virions were stored in 10 mM Tris-hydrochloride buffer (pH 7.0) at −80 C.

Infectivity of B particles was determined by plaque assay on monolayers of L cells and expressed as PFU (25). Hemagglutination titrations were performed as described earlier (19).

Isolation of VS viral glycoprotein. Glycoprotein was selectively liberated from purified VS virions which had been suspended in 10 mM Tris-hydrochloride buffer (pH 7.0) by exposure to 2% Triton X-100 for 1 h at room temperature (10). The Triton-virus mixture was centrifuged at 125,000 × *g* for 90 min, and the supernatant fraction, which contains the glycoprotein, was lyophilized. Triton X-100 was removed from the lyophilized residue by acetone extraction. Lipids were solubilized from the acetone powder by treatment with chloroform/methanol (2:1 vol/vol). The delipidated glycoprotein was extensively lyophilized to remove all organic solvents and then resuspended in the appropriate buffer.

Enzyme treatment of intact virions. Purified VS virions were incubated in a reaction mixture which contained, in a final volume of 0.5 ml, 5 μmol of Tris-hydrochloride buffer (pH 7.0), 50 μmol of NaCl, and either Pronase, subtilisin, or bromelain (viral protein:enzyme protein ratio of 10:1). For digestion of VS virions with either thermolysin, trypsin, or chymotrypsin, an enzyme protein:viral protein ratio of 1:5 was used, and 5 μmol of $CaCl_2$ was included in the reaction mixture. After incubation at 37 C for 30 min, the reaction was terminated by centrifugation through a 0.5-ml pad of 50% glycerol at 125,000 × *g* for 90 min; the pellet and supernatant fraction were partitioned. The pellet was washed twice with 30 ml of phosphate-buffered saline, and the virions were pelleted by centrifugation at 70,000 × *g* through a 50% glycerol pad.

SDS-polyacrylamide gel electrophoresis. Protein samples to be examined were resuspended in 10 mM sodium phosphate buffer (pH 7.4) containing 8 M urea, 1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 1% 2-mercaptoethanol (2-ME) and were placed in a boiling water bath for 2 min prior to transfer to gels. Electrophoresis was usually on 7.5% SDS-polyacrylamide gels, and the determination of incorporated isotopes by scintillation counting was as described previously (28).

Molecular weight determination. Protein standards and viral peptides were dissolved in 10 mM Tris-phosphate buffer (pH 6.8) containing 1% SDS, 1% Triton X-100, 1% 2-ME, and 8 M urea. Samples were layered onto 12.5% polyacrylamide gels (10:1 acrylamide:bis-acrylamide) as described by Swank and Munkres (23) and electrophoresed for 17 to 20 h at 2.5 mA/gel. Gels were stained and destained as described (23) and scanned at 620 nm using a Gilford spectrophotometer. Radioactivity was determined as previously described (28). Molecular weights were determined by comparison with protein standards which included pepsin (mol wt, 33,000), cytochrome *c* (mol wt, 12,300), and bacitracin (mol wt, 1,400).

CNBr cleavage. Lyophilized viral peptides which had been labeled with [^{14}C]methionine and [^{3}H]amino acids were dissolved in 0.1 ml of 70% formic acid, and a 50-fold excess (wt/wt) of CNBr was added. The reaction mixture was then processed as described earlier (22). The final lyophilized powder was dissolved in 10 mM Tris-phosphate (pH 6.8) containing 8 M urea, 1% SDS, 1% Triton X-100 with or without 1% 2-ME. The samples were boiled for 2 min and electrophoresed on 12.5% polyacrylamide gels (23) as described above.

NH_2-terminal analysis. Amino end-group analysis was carried out by the dansylation procedure of Gray (5) using 10 to 20 nmol of material dissolved in 0.2 M sodium bicarbonate buffer (pH 8.5) containing 0.5% SDS. Dansyl chloride (25 μl of acetone at 10 mg/ml) was then added, and the reaction proceeded for 6 h at room temperature with occasional shaking to ensure adequate mixing. After hydrolysis in 6.7 N HCl for 12 to 14 h, the dansylated amino acids were extracted from the hydrolysate by adsorption to acid-washed charcoal and by subsequent elution with pyridine. Dansylated amino acids were separated by either thin-layer ascending chromatography using polyamide sheets or high-voltage paper electrophoresis at pH 4.4. Thin-layer chromatography was accomplished using a two-dimensional chromatographic system in which the plates were developed in a water-90% formic acid (200:3 vol/vol) solvent system, dried for 1 h, and then placed at a 90 degree angle in a benzene-acetic acid (9:1 vol/vol) solvent system. High-voltage paper electrophoresis using Whatman no. 3 paper at pH 4.4 (buffer = pyridine/acetic acid/water, 1/2/250 by volume) was conducted for 2.5 h at 80 V/cm. Dansylated amino acids were visualized under UV light and identified by migration relative to dansyl hydroxide and by comparison of R_f volumes with those of marker dansylated amino acids.

Percent labeling of N-terminal amino acids was determined by quantitating the amount of dansylated

amino acids recovered from the polyamide plates. Dansylated amino acids were solubilized from sections of polyamide plates which emitted fluorescence at 366 nm by exposure to 2 ml of acetone-90% formic acid (9:1 vol/vol) for 6 h. Residues of polyamide plates were removed by centrifugation at 800 × *g* for 30 min, and the solubilized dansylated amino acid was evaporated and dissolved in acetone. The amount of dansylated amino acid was determined by absorbancy at 366 nm and by comparison to elution of a known quantity of marker dansylated amino acid. Efficiency of labeling of N-terminal amino acids was greater than 80% in all cases.

Other assys. Protein concentrations were determined according to the Lowry procedure (12) using crystalline bovine plasma albumin as a standard. Amino acid analysis was carried out with a Beckman amino acid analyzer on samples which were hydrolyzed in vacuo for 24 h in 6.7 N HCl at 106 C.

RESULTS

Location and isolation of a glycoprotein fragment associated with VS virions after thermolysin digestion. As described in Materials and Methods, purified VS virions were incubated with the proteolytic enzyme, thermolysin, and examined by electron microscopy as well as for infectivity and hemagglutination of goose erythrocytes. Electron micrographs of thermolysin-treated virions revealed absence of surface spikes (data not shown). The thermolysin-treated VS virions also exhibited a 5-log decrease in infectivity (from a titer of 5×10^9 PFU to 3×10^4 PFU) and completely lost the capacity to hemagglutinate goose erythrocytes. These data suggest that thermolysin removes the external portion of the VS viral glycoprotein.

It was of interest to determine whether a peptide fragment remains associated with VS virions following thermolysin digestion of protruding glycoprotein spikes. Therefore, purified VS virions which had been labeled with [^{3}H]leucine were incubated with thermolysin or buffer for 30 min. Subsequently, the virions were pelleted by centrifugation and washed, and the viral proteins were analyzed by electrophoresis on 7.5% polyacrylamide gels. Initially, it was exceedingly difficult to consistently detect in association with VS virions a low-molecular-weight degradation product of the G protein which was similar to that reported by Mudd (15). However, the addition of Triton X-100 (1% final concentration) following thermolysin treatment but prior to disruption of the virus by SDS for gel electrophoresis resulted in consistent recovery of a virion-associated glycoprotein peptide segment.

Figure 1A shows the electrophoretic peptide profile of VS virions which were incubated with either thermolysin or buffer. As with other proteolytic enzymes (2, 13, 15, 18), thermolysin selectively degraded only the G protein. Moreover, a small peak of ^{3}H radioactivity, which accounts for 16% of the [^{3}H]leucine label found in the G protein, migrated slightly behind the tracking dye. This small peptide which is not found in the control virus preparation is probably derived from the G protein since no degradation of the four remaining VS viral proteins (L, N, NS, and M) could be detected (Fig. 1A).

The fact that thermolysin does not appear to penetrate the viral membrane, as determined by the lack of degradation of internal viral proteins, indicates that the small glycoprotein fragment is located in the viral membrane. Since treatment of intact VS virions with Triton X-100 solubilizes only the glycoprotein (10), we reasoned that the virion-associated glycoprotein fragment of virions which were exposed to thermolysin should also be solubilized by Triton X-100. To investigate this possibility, [^{3}H]-protein-labeled VS virions which had been incubated with thermolysin or buffer alone were exposed to 2% Triton X-100. After stirring for 1 h at room temperature, the Triton-virus mixtures were centrifuged at 125,000 × *g*, and the supernatant fraction and the pellet were partitioned.

Polyacrylamide gel electropherograms of the material solubilized by Triton X-100 from thermolysin-treated and untreated VS virions revealed that the glycoprotein segment which is generated by thermolytic attack of VS virions is liberated by detergent treatment in the same way as the whole glycoprotein of virions which were not treated with thermolysin (Fig. 1B). Absence of glycoprotein or a glycoprotein fragment in the pelleted virions revealed that this Triton solubilization procedure is essentially 100% efficient (data not shown). These data indicate that thermolysin cleaves the external portion of the VS viral glycoprotein and leaves a fragment of the G protein associated with the viral membrane.

Comparative molecular weights of virion-associated glycoprotein fragments generated by different proteases. The virion-associated glycoprotein fragment resulting from thermolysin treatment of VS virions migrates as a single, homogeneous peptide in 12.5% SDS-polyacrylamide gel system described by Swank and Munkres (23); the molecular weight was estimated to be 5,200 (Table 1). Longer incubation times of virions with thermolysin did not result in further degradation of this peptide, indicating that the viral membrane protects

this peptide from extensive degradation. Table 1 also shows that chymotrypsin, which has the same amino acid specificity as thermolysin but cleaves at the carboxyl side of the peptide bond (7), yields a peptide of only slightly greater molecular weight. Treatment of VS virions with the less specific proteases, such as bromelain, Pronase, and subtilisin, resulted in heterogeneous fragments ranging in molecular weights between 3,500 and 11,000. Prolonged incubation

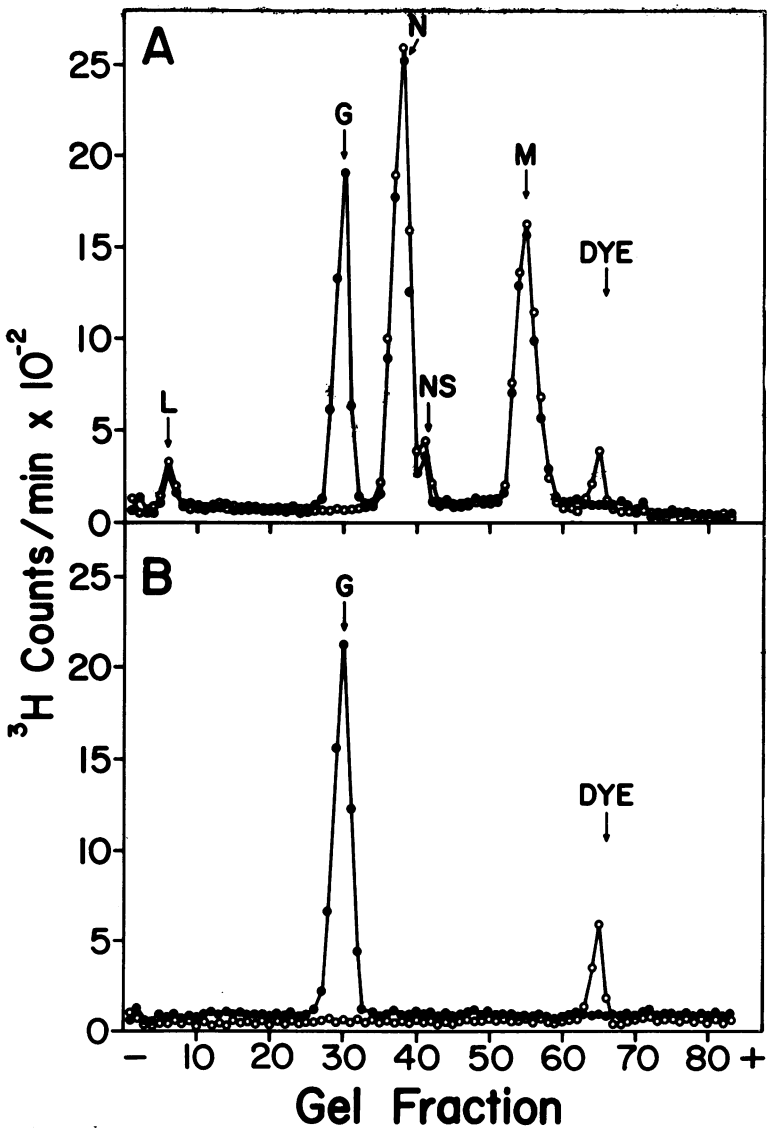

FIG. 1. *Polyacrylamide gel electrophoresis of VS virions incubated with or without thermolysin (A) and of nonsedimentable proteins of virions treated with Triton X-100 (B). VS virions that had been labeled with [^{3}H]leucine were incubated with either thermolysin (○) or buffer (●) for 30 min at 37 C. Reactions were terminated by centrifugation through a pad of 50% glycerol at 125,000 × g for 90 min, and the pellets were washed twice with phosphate-buffered saline. Proteins of a portion of the pelleted virions were extracted for electrophoresis with 10 mM sodium phosphate (pH 7.4) containing 8 M urea, 1% SDS, 1% 2-ME, and 1% Triton X-100. The remaining virions were resuspended in 10 mM Tris-hydrochloride (pH 7.0) and exposed to 2% Triton X-100 for 1 h at room temperature. The Triton-virus mixture was then centrifuged at 125,000 × g for 90 min, and the supernatant fraction was partitioned. The proteins in the supernatant were extracted with 1% SDS, 8 M urea, and 1% 2-ME prior to electrophoresis carried out employing SDS-7.5% polyacrylamide gels for 9 h at 5 mA/gel. Arrows mark the position of VS viral proteins L, G, N, NS, and M and of the tracking dye.*

TABLE 1. *Molecular weight estimations of virion-associated glycoprotein fragments remaining after exposure of VS virions to various proteolytic enzymes*[a]

Enzyme	Incubation time (min)	G-peptide fragments ~mol wt
Thermolysin	30	5,100
	60	5,200
	120	5,100
Chymotrypsin	30	6,100
	60	5,500
	120	5,700
Subtilisin	30	6,300–10,500
	60	5,600–8,700
	120	5,600–6,900
Bromelain	30	7,500–9,600
	60	3,500–8,400
Pronase	30	6,500–11,000
	60	5,300–7,900
	120	3,500–5,300

[a] Purified VS virions that had been labeled with [^{3}H]amino acids were incubated at 37 C for various time intervals with the specified enzyme. Thermolysin and chymotrypsin were used at an enzyme: viral protein ratio of 1:10, whereas a ratio of 1:5 was employed for subtilisin, bromelain, and Pronase digestions. After digestion, the reaction mixtures were centrifuged at 125,000 × *g* for 90 min, and the pellets were washed extensively with buffer. The virions were resuspended in 10 mM Tris-phosphate buffer (pH 6.8), containing 8 M urea, 1% Triton X-100, 1% SDS, and 1% 2-ME. Samples were boiled for 2 min and electrophoresed on 12.5% polyacrylamide gels (1:10 bis-acrylamide:acrylamide). VS viral polypeptides, cytochrome *c*, and bacitracin were used as molecular weight markers.

with bromelain and Pronase led to further degradation of the G protein tail peptide as well as some digestion of the internal VS viral proteins. Treatment of VS virions with trypsin resulted in variable production of heterogeneous peptides, ranging from a single peptide whose molecular weight was ~7,000 to complete absence of any virion glycopeptide fragment (data not shown). This inconsistent effect was present in trypsin obtained from various sources, as well as within the same preparation of trypsin. Therefore, either thermolysin or chymotrypsin was used in all the following experiments.

Solubility of the VS virion glycoprotein segment in chloroform/methanol. The above experiments suggest that the protease-generated fragment of the G protein may represent that portion of the glycoprotein which is associated with the lipids of the viral membrane. Utermann and Simons (24) demonstrated that a peptide fragment which remains attached to Semliki Forest virus after proteolytic digestion is soluble in organic solvents. We sought to determine if the glycoprotein tail segment associated with the VS virion could likewise be extracted by lipid solvents.

To this end, chloroform/methanol (2:1 vol/vol) was added to thermolysin-treated VS virions which had been labeled with [^{3}H]glucosamine and ^{14}C-labeled amino acids. The insoluble residue which formed was removed by centrifugation at 800 × *g*, and water was added to the organic phase. The two phases were separated, and the chloroform/methanol layer was washed twice with water and dried. The aqueous phases were pooled, lyophilized, and resuspended in buffer containing 1% Triton X-100; no radioactivity could be detected in the water layer.

Figure 2A depicts the electrophoretic profile of proteins extracted from VS virions that had been exposed to thermolysin. Only one peak of [^{3}H]carbohydrate label is detected in thermolysin-treated virions, which is present in a position where glycolipids are found (17); no sugar label is observed in the glycoprotein fragment. The material insoluble in the organic solvent consisted of the L, N, NS, and M proteins (Fig. 2B), whereas the glycoprotein fragment had been extracted into the chloroform phase, together with the glycolipids (Fig. 2C). Similar results were obtained when chymotrypsin was employed or if VS virions were labeled with [^{3}H]fucose, [^{3}H]galactose, or [^{3}H]mannose.

These data indicate the lipophilic nature of the glycoprotein fragment and lend credence to the assumption that the glycoprotein fragment obtained by proteolytic digestion of VS virus is responsible for the association of the glycoprotein with the viral membrane.

Amino acid composition of the virion-associated glycoprotein fragment. Membrane proteins which cannot be released from membranes without disruption of the lipid matrix are called integral proteins (20). These proteins are postulated to interact with constituents of membranes via regions which are rich in hydrophobic amino acids and poor in carbohydrates. Since the thermolysin-resistant glycoprotein fragment is devoid of sugar residues, it was of interest to determine the nature of the amino acid composition of this virion-associated peptide.

For amino acid analysis, the virion-associated glycoprotein fragment was isolated in the fol-

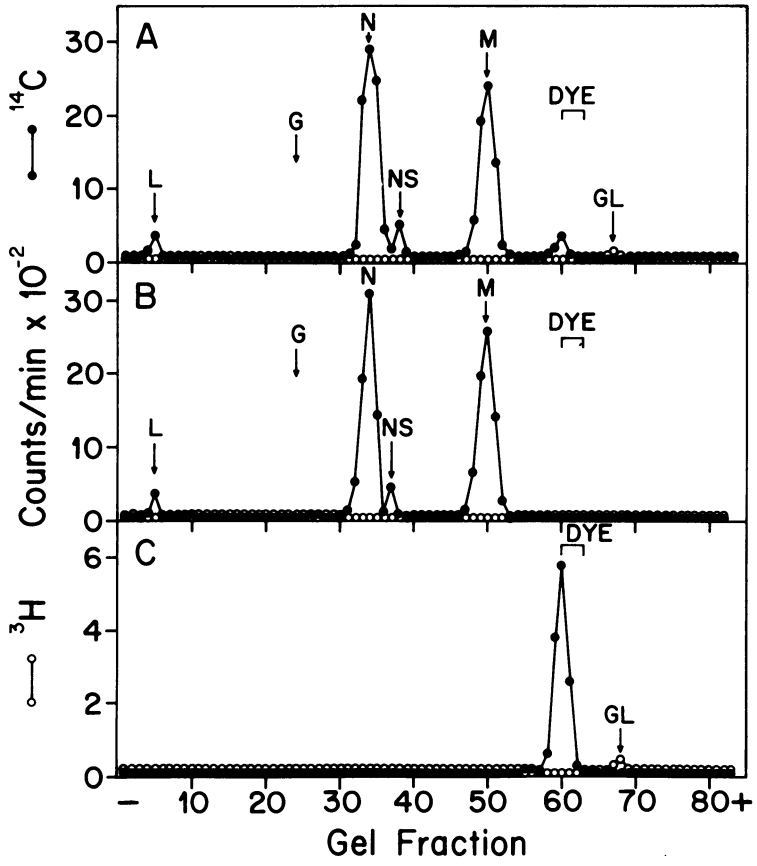

FIG. 2. *Electropherograms of thermolysin-treated VS virions and of fractions obtained by chloroform/methanol extraction of thermolysin-digested VS virions. VS virions that had been labeled with [^{3}H]glucosamine and ^{14}C-labeled amino acids were treated with thermolysin for 1 h at 37 C and then pelleted by centrifugation. Virions resuspended in 10 mM Tris-hydrochloride buffer (pH 7.0) were extracted with 30 volumes of chloroform/methanol (2:1 vol/vol) at room temperature for 1 h. Insoluble material was removed by low-speed centrifugation, and the supernatant was partitioned. The organic layer was separated and washed twice with an equal volume of water. Dried samples were reconstituted in buffer containing 1% Triton X-100, 1% SDS, 1% 2-ME, and 8 M urea and electrophoresed on 7.5% polyacrylamide gels for 8.5 h at 5 mA/gel. (A) Thermolysin-treated virions; (B) insoluble residue; (C) fraction soluble in chloroform/methanol. Arrows mark the position of VS viral proteins (L, G, N, NS, and M) and of the tracking dye and glycolipids (GL).*

lowing manner. VS virions which had been labeled with ^{14}C-labeled amino acids were treated with thermolysin, pelleted by centrifugation, and washed extensively with 0.1 Tris-hydrochloride, pH 7.0. The glycoprotein fragment was solubilized by exposure of thermolysin-treated virions to chloroform/methanol (2:1 vol/vol) as described in the legend to Fig. 2. The chloroform/methanol phase was evaporated to dryness, and the residue was washed to remove any water-soluble proteins. The purity of the glycoprotein fragment was ascertained by polyacrylamide gel electrophoresis; only one peak of radioactivity which migrated to the position of the glycoprotein tail segment was present.

Table 2 shows the amino acid composition of the VS viral glycoprotein and of the virion-associated glycoprotein fragment. The most striking features of the glycoprotein tail are the absence of tyrosine and the high proportion of hydrophobic amino acids (glycine, alanine, valine, leucine, isoleucine, and phenylalanine) representing 64% of the total amino acids of the glycoprotein fragment. This is in contrast to the fact that hydrophobic amino acids account for only 31% of amino acids in the viral G-protein. The minimum molecular weight based on assumption of 1 methionine per peptide is 5,281, a value which agrees well with the molecular weight determined by gel electrophoresis (see Table 1).

TABLE 2. *Amino acid analysis of the VS virion glycoprotein and the virion-associated glycoprotein fragment*[a]

Amino acid	Glycoprotein[b]		G-protein fragment	
	μmol	Ratio of amino acid to methionine	μmol	Ratio of amino acid to methionine
Lysine	7.11	6.3	3.16	1.2
Histidine	5.85	5.2	3.14	1.2
Arginine	5.74	5.1	4.96	1.8
Cystine	ND[c]	ND	ND	ND
Tryptophan	ND	ND	ND	ND
Aspartic acid	10.68	9.5	2.51	0.9
Threonine	7.59	6.7	10.63	3.9
Serine	10.49	9.3	13.01	4.8
Glutamic acid	9.63	8.5	7.08	2.6
Proline	3.85	3.4	5.98	2.2
Glycine	9.13	8.1	24.64	9.1
Alanine	6.98	6.2	24.08	8.9
Valine	7.29	6.5	13.67	5.1
Methionine	1.13	1.0	2.7	1.0
Isoleucine	6.65	5.9	7.58	2.8
Leucine	8.21	7.3	17.53	6.5
Tyrosine	4.75	4.2	—	—
Phenylalanine	5.8	5.1	6.21	2.3

[a] Purified VS virions which had been digested for 90 min at 37 C with thermolysin were exposed to chloroform/methanol (2:1 vol/vol). The chloroform/methanol phase containing the glycoprotein fragment was dried and washed with water to remove water-soluble contaminating proteins. The dried residue was hydrolyzed in 6.7 N HCl at 106 C for 24 h. After removal of HCl, the sample was dissolved in 0.2 mM sodium citrate buffer (pH 2.2) and analyzed in the Beckman amino acid analyzer.

[b] Data from Kelley and Emerson (personal communication, to be submitted for publication).

[c] ND, Not determined.

CNBr cleavage of the virion-associated glycoprotein fragment. If, as shown in Table 2, the thermolysin-resistant glycoprotein tail peptide has a single methionine, it should be cleaved by CNBr into two peptides, and the N-terminal peptide should contain homoserine after cleavage. Moreover, disulfide bonding plays an important role in the secondary structure of the intact VS viral glycoprotein (J. M. Kelley and S. U. Emerson, personal communication). Therefore, it was of interest to determine the presence, if any, of disulfide bonds in the virion-associated glycoprotein fragment extracted from thermolysin-treated VS virions.

VS virions, which had been labeled with [^{14}C]methionine and ^{3}H-labeled hydrophobic amino acids, were digested with thermolysin, and the glycoprotein fragment was solubilized by chloroform/methanol. The glycoprotein fragment was then subjected to CNBr cleavage in the presence of 70% formic acid (22). The lyophilized CNBr peptides were resuspended in 8 M urea, 1% SDS, and 1% Triton X-100 and divided into two aliquots. Samples were electrophoresed on parallel SDS-12.5% polyacrylamide gels with or without prior reduction by 2-ME (Fig. 3).

The electrophoretic pattern of the reduced CNBr peptides indicates the presence of two [^{3}H]protein-labeled peptides, only one of which contains the [^{14}C]methionine label (Fig. 3A). Molecular weights of these two peptides determined by migration relative to bacitracin were estimated to be 3,300 and 2,100. It is of considerable interest that the smaller, N-terminal peptide which contains the methionine label possesses three times the amount of label derived from the ^{3}H-labeled hydrophobic amino acids as compared to the larger peptide. If, on the other hand, the thermolysin-resistant glycoprotein fragment was isolated from VS virions that had been labeled with ^{14}C-labeled amino acids and then subjected to CNBr cleavage, 65% of the ^{14}C label was present in the 3,200-mol-wt peptide, and the smaller peptide contained only 35% of the [^{14}C]protein label (data not shown). These data confirm the presence of one methionine residue per glycoprotein fragment, as well as indicating the actual molecular weight of the thermolysin-resistant glycoprotein segment to be ~5,200. In addition, an asymmetrical distribution of hydrophobic amino acids occurs in the glycoprotein fragment.

Figure 3B strongly suggests the presence of at least one disulfide bond in the glycoprotein tail fragment by co-migration in the electropherogram of nonreduced CNBr peptides. Under nonreducing conditions, the two CNBr peptides migrate as a single peptide, exhibiting a molecular weight of 5,100. When the nonreduced CNBr peptide peak was eluted from the gel shown in Fig. 3B, reduced with 2-ME, and re-electrophoresed, two peptide peaks were observed. These two peptides were identical to those observed in Fig. 3A in the distribution of the [^{3}H]amino acid and [^{14}C]methionine label as well as in molecular weight (data not shown).

Resistance to thermolysin digestion of a lipophilic peptide segment of VS viral glycoprotein in the presence of Triton X-100. The above experiment suggests that the thermolysin-resistant, virion-associated glycoprotein tail segment is that portion of the G protein which is embedded in the VS viral membrane. Presumably, the viral membrane renders this peptide fragment inaccessible to thermolytic

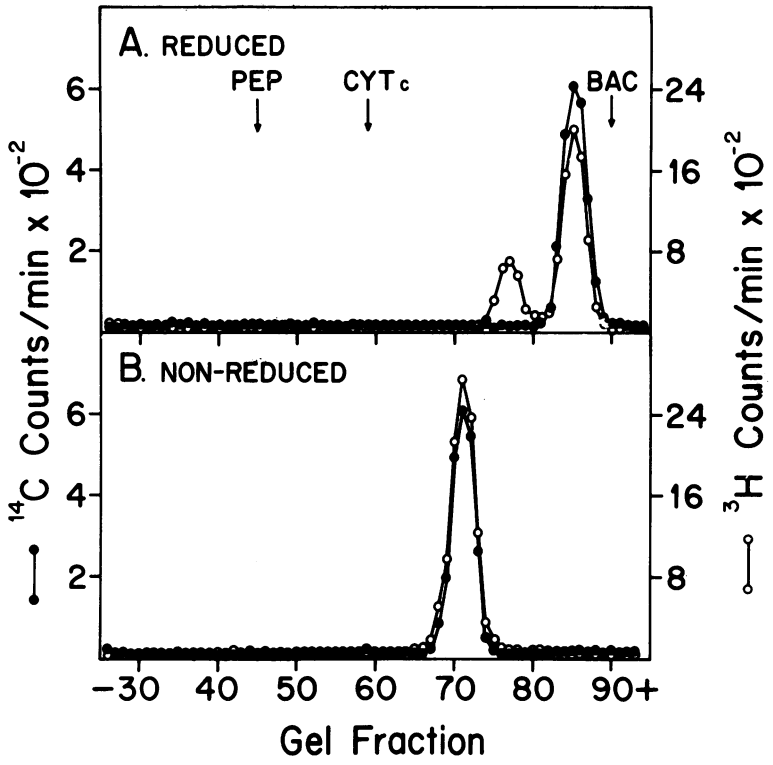

FIG. 3. *Electropherograms of (A) reduced and (B) unreduced thermolysin-resistant, virion-associated fragment following CNBr cleavage. VS virions that had been labeled with [^{14}C]methionine, [^{3}H]valine, [^{3}H]leucine, and [^{3}H]glycine were digested with thermolysin. The virion-associated glycoprotein fragment was then extracted with chloroform/methanol, washed with water, dried, and cleaved with CNBr. Aliquots of the cleaved peptide fragments were (A) reduced in 1% 2-ME or (B) left unreduced before electrophoresis on SDS-12.5% polyacrylamide gels for 18 h at 2.5 mA/gel. Pepsin, cytochrome c, and bacitracin were co-electrophoresed, and positions of these protein markers were determined by staining with Coomassie blue, as described by Swank and Munkres (23). Symbols: ●, [^{14}C]methionine; ○, [^{3}H]protein; PEP, pepsin; CYT c, cytochrome c; BAC, bacitracin.*

attack. An alternative explanation might be that this virion-associated fragment corresponds to regions of hydrophobic cores of exteriorized G protein which are shielded in some manner from proteolytic digestion. To distinguish between these two possibilities, we took advantage of the fact that Triton X-100 binds to integral proteins, whereas hydrophilic proteins bind very little, if any, of this detergent (6). Presumably Triton X-100 is bound by hydrophobic interactions to those same lipophilic regions of proteins which interact with lipids in native membranes.

We investigated the possibility that Triton X-100 which forms mixed micelles with integral proteins may protect the lipophilic peptide segment of the VS viral glycoprotein from proteolytic degradation. Therefore, chloroform/methanol-extracted, delipidated whole glycoprotein, which was also free of Triton X-100, was dissolved in buffer in the presence or absence of 1% Triton X-100. Thermolysin was added to each sample, and the reaction was allowed to proceed for 90 min at 37 C.

Figure 4 shows electropherograms of the VS viral glycoprotein before (A) and after (B) thermolysin digestion. The G protein, which was exposed to thermolysin in the absence of Triton X-100, was completely degraded into small peptides. However, the presence of Triton X-100 in the digestion mixture resulted in the appearance of a peptide which migrates slightly behind the tracking dye, in addition to the smaller peptides seen in the G protein preparation digested in buffer alone. These data imply that Triton X-100 forms a micelle that protects a segment of the glycoprotein from thermolytic attack.

Similarities of the virion-associated and Triton-associated thermolysin-resistant glycoprotein segments. Although Triton X-100 protects a G protein segment from complete

degradation by thermolysin, further evidence is required to support the contention that this peptide is derived from the same region of the glycoprotein as the virion membrane-associated, thermolysin-resistant peptide. Since the virion-associated glycoprotein segment is devoid of carbohydrates and is soluble in chloroform/methanol, we sought to determine if the glycoprotein fragment which is obtained by thermolysin digestion of the Triton-glycoprotein complex possesses these two properties.

Therefore, delipidated glycoprotein isolated from VS virions that had been labeled with [^{3}H]glucosamine and ^{14}C-labeled amino acids was incubated with thermolysin in the presence of Triton X-100. Chloroform/methanol was added to the reaction mixture, and the organic layer was partitioned. The organic phase was washed several times with water, evaporated, and subjected to polyacrylamide gel electrophoresis. The only peptide which was partitioned into the chloroform/methanol phase was the one protected from thermolysin digestion by Triton X-100; all other peptides were water soluble. Furthermore, no [^{3}H]glucosamine radioactivity was detected in this peptide. Similar treatment of G proteins isolated from VS virions which contained other carbohydrate labels yielded

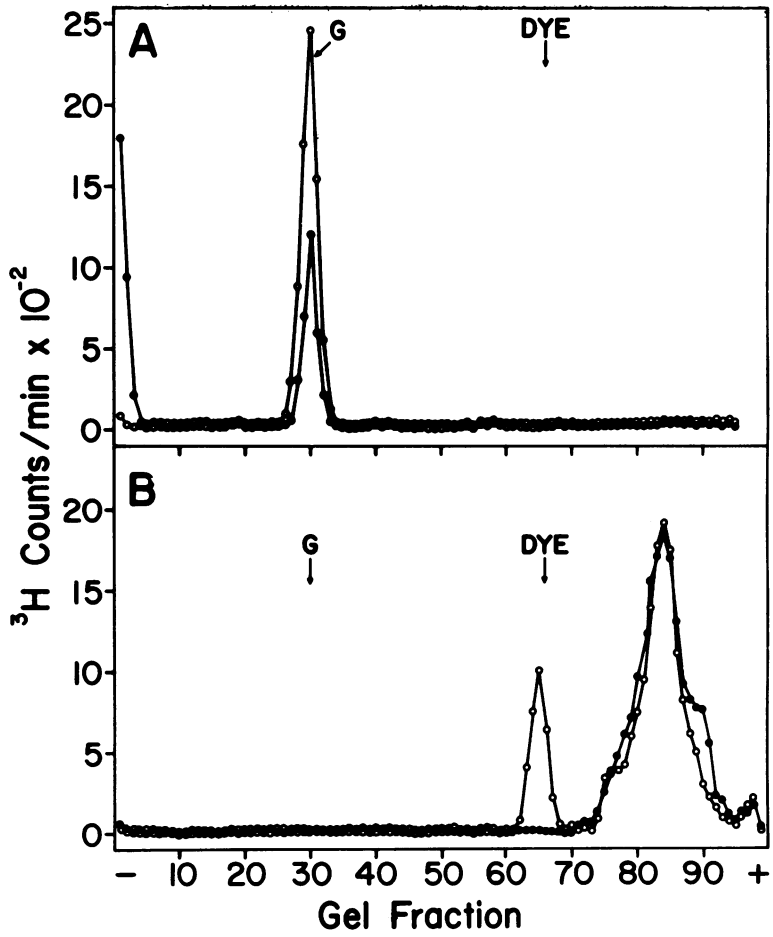

FIG. 4. *Polyacrylamide gel electrophoresis of lipid-free VS viral glycoprotein resuspended in the presence of* (○) *or absence* (●) *of Triton X-100 before* (*A*) *and after* (*B*) *digestion with thermolysin. Glycoprotein labeled with* [^{3}*H*]*amino acids was extracted from VS virions with 2% Triton X-100 in Tris-hydrochloride, pH 7.0; the Triton was removed by successive extraction with acetone and chloroform/methanol. The delipidated G protein was lyophilized to dryness and resuspended in Tris-hydrochloride (pH 7.0) containing 0.1 M NaCl and 5 mM* $CaCl_2$ *either with or without 1% Triton X-100. Each sample was then incubated at 37 C for 90 min with thermolysin at an enzyme:viral protein ratio of 1. Proteins of each digest were solubilized with 1% SDS, 1% Triton X-100, 1% 2-ME, and 8 M urea and were subjected to electrophoresis on 7.5% polyacrylamide gels for 9 h at 5 mA/gel.*

identical results (data not shown).

However, the two thermolysin-resistant glycoprotein fragments are not identical in all aspects. Co-electrophoresis on 12.5% polyacrylamide gels of the virion-associated glycoprotein fragment (amino acid ^{14}C label) mixed with the segment obtained from thermolysin digestion of the Triton-glycoprotein complex ([^{3}H]protein label) showed that the virion-associated fragment migrates slightly slower than the glycoprotein segment obtained by thermolysin digestion of the Triton-G protein complex (Fig. 5). This datum indicates that the lipophilic segment obtained from the delipidated Triton-associated glycoprotein has a molecular weight of ~4,500, which is slightly smaller than that of the segment obtained after thermolysin proteolysis of glycoprotein in intact virions.

CNBr cleavage of the glycoprotein segment remaining after thermolysin digestion of glycoprotein-Triton micelles. Since the glycoprotein segment remaining after thermolysin digestion of the isolated G protein in the presence of Triton X-100 appears to be somewhat smaller than the corresponding fragment recovered from thermolysin-treated virions, it was of interest to determine the molecular weights of the peptides obtained from CNBr cleavage of the Triton-associated, thermolysin-refractory glycoprotein segment. Therefore, delipidated glycoprotein isolated from virions that had been labeled with [^{14}C]methionine and [^{3}H]amino acids was digested with thermolysin in the presence of Triton X-100. The thermolysin-resistant peptide was recovered by extraction with chloroform/methanol, dried, cleaved by CNBr, and then electrophoresed on SDS-12.5% polyacrylamide gels before and after reduction with 2-ME.

Table 3 summarizes the molecular weights of the reduced and nonreduced CNBr peptides of the VS viral glycoprotein fragment generated by thermolysin digestion of the Triton-glycoprotein complex. Molecular weight values of peptides obtained by identical CNBr cleavage of the virion-associated, thermolysin-resistant glycoprotein fragment (Fig. 3) are also included in Table 3 for comparison. Under nonreducing conditions, the CNBr peptides of the Triton-associated, thermolysin-resistant fragment mi-

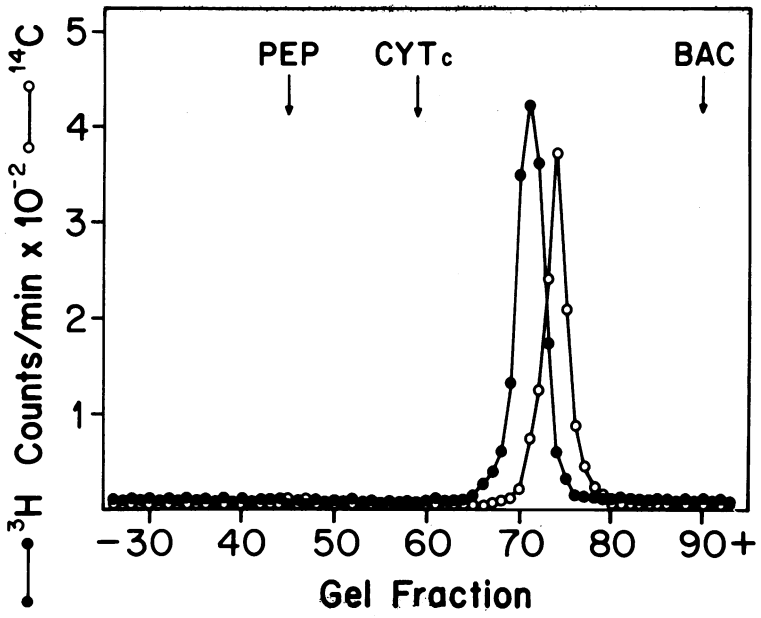

FIG. 5. *Co-electrophoresis of peptide fragments remaining after thermolysin digestion of whole VS virions (●) or of extracted glycoprotein suspended in Triton X-100 (○). The virion-associated glycoprotein labeled with [^{3}H]amino acids was digested with thermolysin, and the undigested peptide fragment was extracted with chloroform/methanol. Intact glycoprotein labeled with ^{14}C-labeled amino acids was extracted from virions with Triton X-100, delipidated with acetone and chloroform/methanol, and digested with thermolysin in the presence of 1% Triton X-100, and the chloroform-soluble peptide was isolated. These two peptide samples were mixed and electrophoresed on an SDS-12.5% polyacrylamide gel for 18 h at 2.5 mA/gel. Positions of co-electrophoresed protein markers were determined by Coomassie blue staining and gel scanning before the gels were sliced for radioactivity determinations by scintillation spectrometry. Symbols: PEP, pepsin; CYT c, cytochrome c; BAC, bacitracin.*

TABLE 3. *Comparative molecular weights of the peptides generated by CNBr cleavage of unreduced and reduced thermolysin-resistant tail fragments of VS virion-associated glycoprotein and of VS viral glycoprotein-Triton micelles*[a]

Treatment	Mol wt of thermolysin-resistant peptides obtained from:	
	Virion membrane	Triton micelle
None	5,200	4,500
CNBr – 2-ME (unreduced)	5,100	4,500
CNBr + 2-ME (reduced)	2,100	2,100
[^{14}C]homoserine peptide		
COOH-terminal peptide	3,300	2,400

[a] Thermolysin-resistant glycoprotein fragments were extracted with chloroform/methanol either from VS virions which had been incubated with thermolysin or from isolated glycoprotein complexed with 1% Triton X-100 before thermolysin digestion. Each of the glycoprotein tail fragments which had been labeled with [^{14}C]methionine and [^{3}H]amino acids was subjected to CNBr cleavage, and the resulting peptides were electrophoresed on parallel SDS-12.5% polyacrylamide gels before or after reduction with 1% 2-ME. Pepsin, cytochrome *c*, and bacitracin were co-electrophoresed, and positions of these protein markers as determined by staining with Coomassie blue (23) were used to estimate the molecular weights of the glycoprotein tail peptides.

grated as one peptide with a molecular weight of ~4,500, suggesting the presence of a disulfide bridge. If the CNBr peptides are reduced prior to electrophoresis, one [^{14}C]homoserine-containing peptide was detected, the molecular weight of which was ~2,100. Although the peaks of ^{3}H radioactivity were not well separated, the molecular weight of the carboxyl terminal peptide was estimated to be ~2,400.

These data suggest that the same N-terminal homoserine-containing CNBr peptide is protected from thermolysin digestion of VS virion-associated glycoprotein and of isolated glycoprotein forming a micelle with Triton X-100.

Is the N-terminus of the glycoprotein buried in the VS virion membrane? Amino end group analysis was used to determine the amount of heterogeneity present in the isolated, delipidated glycoprotein, in the glycoprotein fragments of thermolysin-treated virions which were solubilized by exposure to chloroform/methanol, and in the chloroform-soluble peptide remaining after thermolysin digestion of the Triton-G protein complex. In all cases, only two dansylated derivatives were detected using either thin-layer chromatography or high-voltage paper electrophoresis (pH 4.4) to separate the dansylated amino acids. The major spot in all three samples corresponded to alanine, whereas the minor spot was identified as ϵ-lysine. These data confirm the homogeneity of the isolated glycoprotein preparation and of the two lipophilic, thermolysin-resistant glycoprotein fragments.

The fact that alanine is the N-terminal amino acid of the VS virion intact glycoprotein as well as of the lipophilic glycoprotein segments recovered from thermolysin-treated virions and from the Triton-glycoprotein complex indicates, but does not prove, that the thermolysin-resistant glycoprotein fragment represents the N-terminal portion of the glycoprotein. Since the thermolysin-resistant, virion-associated glycoprotein fragment has at least nine alanine residues among ~52 amino acids (Table 2), there would be a 20% chance of having an N-terminal alanine after thermolysin digestion of a C-terminal membrane-embedded glycoprotein. To obtain further evidence that the N-terminus of the G protein is inserted in or through the viral membrane, we took advantage of the fact that Trition X-100 shields the glycoprotein fragment from digestion by thermolysin (see Fig. 3). We reasoned that if the N-terminal amino acid of the glycoprotein is the same as that of the thermolysin-resistant lipophilic peptide, then if the G protein is dansylated prior to thermolysin digestion in the presence of Triton X-100, the dansylated amino acid should be recovered in the thermolysin-refractory glycoprotein fragment.

Therefore, delipidated, [^{3}H]protein-labeled glycoprotein was dansylated, a procedure which resulted in the labeling of 85% of the available N-terminal analine residues. The excess dansyl chloride was removed by repeated acetone washings, and the acetone powder was extensively lyophilized and resuspended in buffer containing Triton X-100. Thermolysin was added and the reaction mixture was processed as described earlier. The chloroform-soluble glycoprotein fragment was isolated, and the previously dansylated amino acids were separated by thin-layer chromatography as described in Materials and Methods. Only one spot was detected and it corresponded to alanine. Moreover, after correcting for losses due to elution of the dansylated amino acid from thin-layer sheets, 90% of the previously dansylated alanine residues of the glycoprotein was recovered in the thermolysin-resistant, lipophilic glycoprotein fragment. Since this dansylated alanine residue could have only been derived from the N-terminal alanine of the glycoprotein, these data provide further evi-

dence that the lipophilic glycoprotein peptide segment represents the N-terminus portion of the glycoprotein.

DISCUSSION

As an initial step in determining the manner in which the VS viral glycoprotein is inserted into the virion membrane, we have isolated and characterized a fragment of the glycoprotein which is resistant to proteolysis of intact virions. The peptide segment, which represents about 9% of the membrane glycoprotein, exhibits characteristics which are attributed to protein segments embedded in membranes (20); that is, being lipophilic in nature and devoid of carbohydrates. Furthermore, it appears likely that this thermolysin-resistant peptide is located in the viral membrane where it is protected from proteolysis, because extracted glycoprotein free of lipids and detergent is completely digested by thermolysin. Therefore, it seems likely that this hydrophobic peptide fragment represents that portion of the glycoprotein which is buried in the viral membrane.

A peptide of similar properties can be recovered when isolated glycoprotein is digested with thermolysin in the presence of Triton X-100. Presumably, Triton X-100 protects the peptide from complete degradation by forming mixed micelles with the hydrophobic region(s) of the G protein (6). Although the peptide recovered after thermolysin digestion of the Triton-glycoprotein complex is slightly smaller than that obtained from thermolysin-treated virions, it appears likely that these two peptides are derived from the same region of the glycoprotein. Both peptides contain the same N-terminal amino acid (alanine), are soluble in chloroform/methanol, lack carbohydrates, and have a disulfide bridge contributing to their secondary structure. Furthermore, cyanogen bromide digestion of the two thermolysin-resistant peptides in each case yields homoserine-containing peptides of identical molecular weight.

The C-terminal CNBr peptide of VS viral glycoprotein contains carbohydrate and has an approximate molecular weight of 3,500 (J. M. Kelley and S. U. Emerson, personal communication). A sugar-free portion of a peptide of this size could conceivably traverse the viral membrane since a sequence of only 23 amino acids in length is necessary to span a membrane (M. S. Bretscher, personal communication). However, it does not appear likely that the C-terminus of the VS viral glycoprotein is associated with the membrane since the virion-associated, thermolysin-resistant glycoprotein fragment does not contain carbohydrate but does possess a methionine residue. Our data indicate that the N-terminal peptide of the glycoprotein is associated with the viral membrane since the N-terminal amino acid of the G protein and of the hydrophobic segments is alanine. Moreover, when intact glycoprotein was dansylated and then treated with thermolysin in the presence of Triton X-100, the dansyl label was recovered only in the chloroform-soluble peptide; the dansylated amino acid was identified as alanine in the whole glycoprotein and the lipophilic fragment.

The question as to whether the virion-associated glycoprotein penetrates through the membrane is not yet resolved. Cartwright et al. (2) demonstrated that phospholipase C treatment of VS virions selectively removes the matrix (M) protein without disturbing the spikes (G protein). The electron microscopic observation that glutaraldehyde-fixed virions treated with SDS still retain 60% of their glycoprotein despite the loss of most of the lipid also suggests that the G protein penetrates through the membrane and interacts with the nucleocapsid (1). However, no proof is available to indicate that all of the viral envelope (lipid) has been removed. The hydrophobic segments are of sufficient length (~50 amino acids) to span the virion membrane. However, we have no indication of how close to the viral membrane thermolysin is cleaving the external segment of the glycoprotein.

The experiments reported here enable us to say with some confidence that the VS viral glycoprotein is an amphipathic protein in the same category as other membrane proteins such as cytochrome b_5 (21), the glycoproteins of Semliki Forest virus (24), and the red blood cell membrane glycoprotein (8, 9). All these proteins have external hydrophilic segments and lipophilic tail portion which serves to anchor the macromolecule into the membrane.

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