Structural Study of Vesicular Stomatitis Virus G Protein in the Virion Envelope

By SHEILA E. TAUBE*† AND PHYLLIS C. BRAUN‡

Department of Microbiology, School of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06032, U.S.A.

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SUMMARY

Some structural properties of the vesicular stomatitis virus (VSV) G protein were examined in virions and isolated envelope fragments. We have shown that in the virion a portion of the G protein extends through the lipid envelope and that this part of the molecule can be cleaved by chymotrypsin. Envelope fragments isolated from VSV without the use of detergents maintained the structural characteristics of the G protein found in intact virions. In addition, we provide evidence that at least some of the isolated envelope fragments have both sides of the bilayer exposed to added reagents, suggesting that this preparation would be useful for in vitro reassociation experiments.

INTRODUCTION

Vesicular stomatitis virus (VSV) assembles at the host cell plasma membrane and is released from the cell by budding. The lipid bilayer envelope of the virus is derived from the plasma membrane and contains only one of the five virus-coded proteins, G, the glycoprotein (Wagner, 1975; Schloemer & Wagner, 1975). The M protein is sandwiched between the envelope and the nucleocapsid and has the properties of a peripheral membrane protein (Emerson & Wagner, 1972; Morrison & Lodish, 1975; Carroll & Wagner, 1979). The G protein is synthesized, processed and inserted into the plasma membrane by the cell in the same manner as cellular glycoproteins (Lingappa et al., 1978; Irving et al., 1979). The concentration of G protein in the host cell plasma membrane increases with time after infection until it becomes the major species late in infection (Lodish & Porter, 1980a). Despite the fact that G is surrounded by many cellular proteins throughout much of the infection, the virions assembled at all times contain predominantly G protein and only a few molecules of cellular proteins (Lodish & Porter, 1980b). A localized aggregation of G protein with exclusion of cellular proteins must occur. However, virus mutants have been described which are defective in the synthesis and/or insertion of G protein; these mutants form enveloped particles (although with lower efficiency) containing normal, competent nucleocapsid cores and any of a variety of glycoproteins which may be present in the host cell plasma membrane (Schnitzer et al., 1979; Lodish & Weiss, 1979). Phenotypic mixing occurs in cells doubly infected with VSV and other unrelated enveloped viruses (McSharry et al., 1971; Zavada, 1976; Lodish & Weiss, 1979).

Most of the mechanisms suggested to explain the selective incorporation of virus glycoproteins during assembly of VSV implicate some kind of interaction between the glycoproteins and either the M protein or the nucleocapsid (McSharry et al., 1975; Shimizu & Nakao, 1975; Knipe et al., 1977a, b; Witte & Baltimore, 1977; Lodish & Porter, 1980a).

* Present address: Genetic Biology Program, National Science Foundation, Washington, D.C. 20550, U.S.A.
‡ Present address: Department of Biology, Fairfield University, Fairfield, Connecticut 06430, U.S.A.
The in vivo systems do not readily lend themselves to detailed exploration of these molecular interactions. We have suggested that alternative in vitro approaches must be developed (Taube & Rothfield, 1978). As a first step toward this goal, we reported the isolation of VSV envelope fragments which retained the biochemical composition of the original virions. We have continued the characterization of these fragments to demonstrate that they also retain the structural characteristics of the envelope of the intact virions. In addition, we present the first direct evidence that the VSV G protein spans the lipid bilayer in the virion; earlier structural studies using fixed virions provided strong support for such a model (Brown et al., 1974) while data derived from in vitro translation systems only showed that G spanned the membrane of endoplasmic reticulum vesicles (Katz et al., 1977; Toneguzzo & Ghosh, 1978).

METHODS

**Cells.** BHK-21 cells were obtained from the American Type Culture Collection (ATCC). Monolayer cultures were maintained in complete medium (MEM-C), which consisted of Eagle's minimal essential medium (MEM) supplemented with 7% calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Mouse L cells (clone 929; obtained from ATCC) were adapted to grow in suspension and were maintained as spinner cultures in complete MEM suspension medium supplemented with 2 mM-glutamine.

**Virus.** Our original stock of VSV Indiana (3B-Glasgow) was obtained from Dr R. Wagner. The selection of a heat-resistant variant and the production of stock virus have been described previously (Taube & Rothfield, 1978). Virus purification and assay were performed according to our standard procedures (Taube & Rothfield, 1978). Virus protein was assayed by the Hartree modification of the Lowry method (Hartree, 1972).

**VSV envelope isolation.** The procedure used was a modification of the one described previously (Taube & Rothfield, 1978). Purified virus, isotopically labelled either with amino acids, lipid precursors or both, was pelleted and resuspended in cold glass-distilled water. Aliquots (15 ml) of the resuspended virus were sonically treated with the standard probe on a Branson Sonifier (model W185) for eight 15 s bursts at 70 to 80 W separated by 45 to 60 s. After sonic treatment, the suspension was concentrated in an Amicon ultrafiltration apparatus (Diaflo PM 10 filter). The concentrated suspension was applied to 2H2O-sucrose gradients, pH 7.7. The material sedimenting to the density region of 1.18 g/ml was collected, diluted 1:2 with tris–saline buffer pH 6, and layered on to a 0.2 ml cushion of 100% glycerol in polyallomer tubes. After 5 h centrifugation at 45 000 rev/min in a Spinco SW50.1 rotor, 1.2 ml samples were collected from the bottom of each tube. The pooled samples were sonically treated and applied to 2H2O-sucrose gradients, pH 6. The isolated envelope fragments were collected in the density region of about 1.75 g/ml. These preparations contained mainly G protein, but always contained some N protein and frequently a detectable amount of M protein.

**Polyacrylamide gel analysis.** All samples were prepared for polyacrylamide gel electrophoresis (PAGE) by boiling for 5 to 10 min immediately following the addition of SDS-containing sample buffer. This procedure appeared to be sufficient to inhibit further proteolysis, since control experiments using phenylmethylsulphonyl fluoride prior to addition of SDS yielded identical results. Our modification of Neville's (1971) discontinuous system has been described previously (Taube & Rothfield, 1978). Two other systems were employed to obtain better resolution of the low mol. wt. peptides. In the first case, solutions containing 10% acrylamide (acrylamide:bisacrylamide, 37.5:1) and 17% acrylamide in 0.38 M-tris–HCl buffer pH 8.8 plus 0.1% SDS were used to prepare linear gradient slab gels. The stacking gel contained 3% acrylamide in 0.13 M-tris–HCl pH 6.8 plus 1% SDS. The electrophoresis buffer was prepared according to Laemmli (1970). Electrophoresis was terminated when the indicator dye was about 1 cm from the bottom of the gel. In the second
high resolution system, recrystallized urea was added to a 17% acrylamide solution (acrylamide:bisacrylamide ratio and running gel buffer as above) to a final concentration of 8 m. Urea was also added to the 3% stacking gel. Electrophoresis buffer was as above.

Gels used for analysis of the tail peptide and its proteolytic cleavage products were processed for liquid scintillation counting immediately. Following removal from the electrophoresis apparatus, the gel was chilled, and the appropriate channel excised and fractionated using a Mickle gel slicer. The slices were incubated in 0.5 ml 1-25% SDS overnight at 37 °C before addition of a scintillation cocktail containing toluene-Liquifluor-BioSolve (334:16:50).

Amidination reactions. Purified VSV was pelleted and resuspended in pyrophosphate-saline buffer (4 mM-sodium pyrophosphate, 0.1 M-NaCl, pH 8). A 200 nmol amount of 14C-labelled isethionyl acetimidate (in 20 μl H2O) was neutralized with 0.1 M-NaOH (1.9 μl) and added to a volume of virus calculated to contain 1 nmol G protein (60 μg G). The volume of the reaction mixture was adjusted to 100 μl with the saline-pyrophosphate buffer and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by the addition of 0.9 ml ammonium acetate-saline buffer (4 mM-ammonium acetate, 0.1 M-NaCl, pH 8). The diluted reaction mixture was incubated for an additional 30 min at 37 °C to be sure all free isethionyl acetimidate either decomposed or reacted with the added ammonium ions.

Materials. Reconstituted protein hydrolysate—algal profile—3H-amino acids, 14C-amino acids and isethionyl [14C]acetimidate (59.4 mCi/mmol) were purchased from Amersham. Deuterium oxide (99.8% pure) was obtained from Sigma, and BioSolve solubilizer formula BBS-3 was purchased from Beckman.

RESULTS

G protein spans the virion envelope

It has been assumed, although not directly demonstrated, that the segment of the VSV G protein which is embedded in the virion envelope extends through the bilayer and is exposed on the inner surface. During translation in an in vitro synthesizing system in the presence of microsomal vesicles, a peptide from the C-terminus of the G protein can be removed by proteases (Katz et al., 1977; Toneguzzo & Ghosh, 1978).

We reasoned that if there is a chymotryptic cleavage site in the peptide extending through the microsomal membrane, perhaps this site is also available in the virion and in the isolated envelope fragments. We chose to examine the virion first. When intact VSV is treated with chymotrypsin, PAGE (using appropriate conditions) reveals little protein in the region of G and a new peptide of mol. wt. about 5000 (Mudd, 1974; Schloemer & Wagner, 1975). To be sure that the peptide of mol. wt. 5000 reflected the complete digestion of the exposed portion of the G protein, we incubated intact virions (labelled with 3H-amino acids) with chymotrypsin for increasing periods of time, pelleted the treated virus, assayed the radioactivity released into the supernatant fluid, and electrophoresed the pelleted virus on a 10 to 17% gradient SDS-polyacrylamide gel (see Methods). Fig. 1 (insert) shows that digestion was complete by about 20 min. At the 10 and 20 min time-points, the gels showed a single band migrating more slowly than the dye front (data not shown). After 40 min digestion there was still only a single band in this region of the gel (Fig. 1, G-tail), indicating that there was no further cleavage of the envelope-associated tail of the G protein. Residual G protein seen in Fig. 1 is the result of virion aggregation; complete elimination of such aggregation requires conditions which result in some virion disruption or leakiness.

Osmotic shock and vigorous sonication of VSV cause loss of infectivity and partial disruption of the virions (Taube & Rothfield, 1978). Such treatment results in uncoiling of the nucleocapsid but the envelope remains intact. The inside of the virion envelope should be
Fig. 1. PAGE analysis of intact VSV incubated for increasing time periods with chymotrypsin. Aliquots of \(^3\)H-amino acid-labelled virus were incubated for 0, 10, 20 or 40 min with chymotrypsin at 37 °C, diluted with cold buffer and pelleted. The supernatant fluids were assayed for radioactivity released, and the pellets were applied to a 10 to 17% gradient SDS-polyacrylamide slab gel (see Methods). Channels of the gel were excised and fractionated and the fractions assayed for radioactivity. The insert shows the radioactivity released into the supernatant. •, Untreated control; ○, 40 min incubation with chymotrypsin.

accessible to added protease at least in the sections where the nucleocapsid has uncoiled and no longer apparently adheres to the envelope. If this assumption is correct, and if G extends through the bilayer, it should be possible to decrease the size of the membrane-associated tail of G by cleavage from the inside. The following experiment was designed to test this hypothesis. Chymotrypsin was added to intact unlabelled VSV to give a final enzyme:virion protein ratio of 1:4, and the virus was incubated for 30 min at 37 °C. A 200-fold molar excess (over G protein) of \(^{14}\)C-labelled isethionyl acetimidate was added and incubation at 37 °C continued for an additional 30 min. The reaction was then stopped by dilution with ammonium acetate buffer. The sample was divided in half and placed on ice. One half was subjected to vigorous sonic treatment. Fresh chymotrypsin was added to each half of the sample and both were incubated at 37 °C for 30 min. Following this incubation, the samples were diluted with cold buffer and the virus pelleted. The pellets were solubilized with gel sample buffer and electrophoresed through a 17% SDS-polyacrylamide gel to which urea was added (see Methods). Since isethionyl acetimidate at pH 8 is polar and cannot penetrate the lipid bilayer, the free amino terminus of the G-tail peptide created by chymotrypsin treatment of the virions would be the only non-lipid group expected to be amidinated. If, as proposed above, sonic treatment allowed chymotrypsin to reach areas of the inside of the envelope, a fraction of the \(^{14}\)C-labelled peptide molecules should be cleaved in the sonicated sample and a new labelled moiety should be seen in a lower mol. wt. region on the gel. Fig. 2 presents the results of this experiment. After sonic treatment of the isethionyl \(^{14}\)C-acetimidate-labelled virus, further treatment with chymotrypsin results in a new peak at fractions 68 to 70 (Fig. 2 b). There is a small peak in this region in Fig. 2 (a) (no sonic treatment) and in Fig. 2 (c) (control virus, labelled in protein and phospholipids, treated only with chymotrypsin) which reflects the fact that all virus stocks have a small percentage of partially disrupted particles. The distribution of label in the G-tail and the smaller peptide
regions was determined by integrating the area under the peaks of the tracings. This analysis indicated that label in the G-tail peak had decreased by 5 to 10% in the sonically treated sample, about the amount expected based on the extent of virion disruption. The increase seen in the smaller G-tail region was somewhat greater than expected, but this may be accounted for by small losses in the region between M and the G-tail. A small amount of M protein also is consistently labelled by isethionyl [14C]acetimidate, in agreement with the observations of others when using external labels such as pyridoxal phosphate or lactoperoxidase-catalysed iodination (Eger et al., 1975; Moore et al., 1974).

These data strongly support the idea that the G protein spans the envelope bilayer in the virion. The design of the experiment precludes the alternative explanation that sonication alters the envelope structure to allow further cleavage from the outside; such cleavage would result in loss of the 14C label.

These experiments were repeated numerous times with very consistent results. An attempt was made to decrease the labelling of phospholipids by pretreating the virions with unlabelled ethylacetimidate; phospholipid label was not significantly decreased and there was evidence that the pretreatment had made the virions leaky. Attempts were also made to further
Fig. 3. Electron micrographs of purified VSV envelope fragments. Preparations were applied to Formvar-coated grids and stained with 2% ammonium molybdate pH 6.5. Solid arrows indicate flattened sheets; open arrows indicate vesicular structures.

Fig. 4. PAGE analysis of envelope fragments before and after incubation with chymotrypsin. Envelope fragments were prepared from VSV labelled with 14C-amino acids. The isolated envelope was incubated with chymotrypsin at an enzyme:virion protein ratio of 1:5 (30 min at 37 °C). (a) Untreated; (b) chymotrypsin-treated.

separate the smaller G-tail from the phospholipid peak; the gel system used for the data shown (i.e. 17% acrylamide plus 8 M-urea) gave the best results.

Morphology of isolated envelope fragments

In our original study (Taube & Rothfield, 1978), we only briefly considered the structure of the isolated envelope fragments; we concentrated mainly on the isolation procedure and the biochemical integrity of the fragments. To further examine the overall morphology of the isolated envelope fragments, several preparations were negatively stained and examined in the electron microscope. Fig. 3 shows representative samples of the electron micrographs. There appear to be flattened sheets (solid arrows) and vesicular structures (open arrows). We expect the flattened sheets to be open structures. We cannot determine from these negatively stained preparations whether the vesicular structures are sealed vesicles or whether they are open allowing components in the medium access to both sides of the envelope. This question is addressed in the following experiments.

G protein structure is maintained in the isolated envelope

Isolated envelope fragments were treated with chymotrypsin at an enzyme:virion protein ratio of 1:5 for 30 min at 37 °C. The treated envelope was pelleted by centrifugation, solubilized in SDS, and subjected to PAGE. The gels were prepared for fluorography using
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Fig. 5. PAGE analysis of isolated envelope fragments incubated with chymotrypsin. Envelope fragments prepared from VSV labelled with ³H-amino acids were incubated with buffer or chymotrypsin (enzyme: virion protein ratio of 1:5) for 30 min at 37 °C, pelleted, solubilized and electrophoresed on a 10 to 17% gradient SDS-polyacrylamide slab gel. Channels were excised, fractionated and the fractions were assayed for radioactivity. (a) Intact virions incubated with chymotrypsin; (b) envelope fragments incubated with buffer (without chymotrypsin); (c) envelope fragments incubated with buffer (without chymotrypsin).

the technique of Bonner & Laskey (1974). Fig. 4 shows that essentially all the G protein was removed by the chymotrypsin treatment. All the fragments in the preparation must have the envelope face equivalent to the virion exterior accessible to the added protease. If a significant portion of the vesicles was sealed exterior face in, the G protein could not have been removed by the chymotrypsin. We therefore conclude from these results that the vesicular structures we see in the electron micrographs (Fig. 3) are either sealed with the exterior side out or unsealed.

We demonstrated above that chymotryptic treatment of intact virions resulted in formation of a single peptide of mol. wt. about 5000, and that subsequent exposure of the inside of the virion envelope to chymotrypsin resulted in cleavage of the mol. wt. 5000 peptide to one of lower mol. wt. If the structure of the G protein is maintained in the isolated envelope fragments, the G-tail peptide should be cleaved by chymotrypsin from both sides of the envelope and the appropriate gel analysis should reveal a peptide smaller than the 5000 mol. wt. peptide. Indeed, chymotryptic treatment of the isolated envelope fragments followed by PAGE analysis revealed two low mol. wt. peptides, one co-migrating with the 5000 mol. wt. tail peptide from intact VSV and a smaller peptide similar to that seen in the sonically treated virions (Fig. 2). These results are shown in Fig. 5. The small size of the peptides involved and the efficiency of labelling with amino acid precursors prevented preparation of samples with
higher radioactivity; the same two low mol. wt. peaks were obtained in four repetitions of the experiment with separate envelope isolates. All samples were counted for at least 20 min to minimize the error of counting.

Attempts to treat virions with chymotrypsin and isethionyl [14C]acetimidate before envelope isolation were unsuccessful because, as expected, enzyme-treated virus has a lower density and the established isolation procedures could not be applied. In addition, preliminary results indicated that fragments obtained were of a different size and may not have maintained the integrity of the envelope. We therefore relied on the reproducibility of the data. It is clear that the radioactivity in the peak areas is above the background levels. The excess counts in the G-tail and smaller G-tail regions are the result of greater efficiency of elution from gel slices of the small peptides compared with whole G. There are no apparent losses from the N or M peaks (see Fig. 5 b, c). These data along with the previous experiments provide strong evidence that the G protein spans the envelope bilayer in the isolated envelope fragments as it does in the virion. Determination of the percentage of G molecules cleaved at sites on both sides of the bilayer is difficult due to the low level of radioactivity. However, we can conclude that at least a portion of the envelope preparation has both surfaces of the bilayer available to reagents added to the medium. This could represent the fragments seen as flattened sheets but also may include unsealed vesicular structures.

DISCUSSION

We previously described the preparation of VSV envelope fragments without the use of detergents. These fragments had the same phospholipid profile as intact virions, contained predominantly G protein with small and variable amounts of N and M proteins and maintained the G:phospholipid ratio found in intact virions (Taube & Rothfield, 1978). Our present studies demonstrate that the structural characteristics of the G protein found in the virion are retained in the isolated fragments.

The demonstration that chymotrypsin can cleave the membrane-embedded tail of the G protein from the inside of the virion envelope (the isethionyl [14C]acetimidate labelling experiment) provides the first direct evidence that the VSV G protein spans the lipid bilayer in the mature virus particle. The implication of this finding is that the configuration of G assumed during translation in the rough endoplasmic reticulum is probably maintained through the further processing and transport of the protein to the plasma membrane. As virus assembles in the infected cell, there are presumably interactions between internal virus components (M and nucleocapsids) and the inside of membrane containing G protein. Models of virus assembly based on interactions between these internal virus components and a portion of the envelope glycoprotein extending into the cytoplasm now become more tenable. Since VSV particles can be formed in the absence of G, we can now ask questions about the nature of the molecular interactions with G that enhance the efficiency of virus particle formation.

Our structural analyses of the isolated envelope fragments demonstrate that they would be useful in in vitro studies of virus assembly, since these fragments appear to be native by all the criteria so far examined. Preliminary experiments in our laboratory have shown that isolated nucleocapsids reassocciate with the envelope fragments; the nature of this association has not been examined in detail.

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