RECONSTITUTED G PROTEIN-LIPID VESICLES FROM
VESICULAR STOMATITIS VIRUS AND THEIR INHIBITION
OF VSV INFECTION

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ABSTRACT
The single glycoprotein (G) of vesicular stomatitis virus (VSV) was isolated in
nearly quantitative yield by extraction of the purified virions with 0.05 M octyl-
β-D-glucoside (OG) in 0.01 M sodium phosphate, pH 8.0. The extract contained
essentially all of the viral phospholipids and glycolipids, and was free of other
viral proteins. Dialysis to remove OG resulted in the formation of G protein–viral
lipid vesicles having a lipid–G protein ratio similar to that of the intact virions.
The vesicles were 250–1,000 Å in diameter, with a “fuzzy” external layer also
similar to that of intact virions. The vesicles were predominantly unilamellar and
sealed, with both phosphatidyl ethanolamine and gangliosides symmetrically
distributed in the bilayer. G protein was asymmetrically oriented, with about 80%
accessible to exogenous protease. Addition of soybean phospholipid to the viral
extract before dialysis resulted in vesicles that incorporated viral proteins and
lipids quantitatively, but that were markedly decreased in buoyant density. The G
protein–lipid vesicles were effective in eliciting specific anti-G antibodies that
neutralized viral infectivity. Competitive radioimmunoassay showed that both
reconstituted vesicles and a soluble form of G protein (Gs) were indistinguishable
from purified VSV in their antibody binding properties. Addition of G protein–
lipid vesicles of BHK-21 cells before, or simultaneously with, infection by VSV
inhibited viral infectivity, as measured by two independent techniques (viral RNA
production in the presence of actinomycin D and a neutral red assay of cell
viability). The total inhibitory activity of G protein in the vesicular form was,
however, less than 5% of that found for intact virus particles that had been
inactivated by ultraviolet light irradiation. Gs was inactive as an inhibitor as
determined by the RNA production assay.
Vesicular stomatitis virus (VSV) is unique among the well-characterized, enveloped viruses in possessing a single glycoprotein (G) on its surface. G protein is the only protein of the VSV virion that is accessible to exogenous proteases (2, 17) or other nonpenetrating probes (5). Proteolytic removal of G protein from the VSV viral surface decreases infectivity to about 1/100,000 of its original value (2). G protein apparently interacts with the viral bilayer through a small peptide that contains a predominance of hydrophobic amino acids and that remains associated with the particle after proteolytic treatment (20, 26).

In this paper we describe the specific and quantitative extraction of G protein and viral lipid from purified virions using the nonionic detergent octyl-β-D-glucoside (OG). OG has a high critical micelle concentration (0.025 M in water, reference 27), which makes it readily dialyzable and, thus, uniquely suitable for lipid-protein reconstitution procedures. Dialysis to remove OG yields preparations of reconstituted lipid-protein vesicles that demonstrate the functional and immunologic activity of G protein.

OG has previously been used to prepare reconstituted vesicles of lipid and viral glycoprotein from Semliki Forest virus, another enveloped virus (11). Petri and Wagner (22) have recently described the preparation of G protein−egg lecithin protein-lipid vesicles by dialysis of OG.

MATERIALS AND METHODS

Virus

VSV, Indiana serotype, was grown in BHK-21F cells on confluent monolayers and purified as previously described (6). Viral protein was labeled by addition of [35S]methionine to Eagle's minimal essential medium (MEM) that contained 10% of the usual amount of methionine. Viral lipid was labeled by addition of [14C]palmitic acid to the growth medium during the entire period of cell growth and to the medium after viral infection. 35P was added after viral infection to label viral phospholipid in one experiment (Fig. 5).

Octyl-β-D-glucoside

Because these studies were initiated before OG became commercially available, a high-yield synthesis based on the previously published synthesis was developed (21). (Details are available on request.) Presently, commercial OG (Calbiochem-Behring Corp., American Hoechst Corp., St. Louis, Mo.) is being used.

Extraction and Reconstitution of G Protein

A pellet of purified VSV (~7.5 mg. of viral protein) was mixed with 3 ml of 0.05 M OG in 0.01 M phosphate buffer, pH 8.0, at room temperature, repeatedly vortexed and stirred for 40 min, and centrifuged at 40,000 rpm for 50 min at 5°C in a Beckman SW41 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The supernate contained 3−400 µg/ml of nearly pure G protein (Fig. 2) as determined by the Hartree-modified Lowry assay (9) using crystalline bovine serum albumin as a standard. Reconstitution occurred upon dialysis for 36 h at room temperature against three changes of Dulbecco's phosphate-buffered saline (PBS) (11).

Separation of G Protein from Viral Lipid

The OG extract of VSV was layered on a linear 20−40% sucrose gradient prepared in 50 mM OG in 0.01 M sodium phosphate, pH 8.0 (11), and centrifuged for 16 h at 40,000 rpm at 5°C in a Beckman SW41 rotor (2.2 x 10^11 g-min). Protein-containing fractions were identified as described by Hartree (9), and lipid-containing fractions by the use of cholesterol oxidase (19).

Preparation of Soluble Monomeric G

A soluble monomeric form of G (Gs) is spontaneously released by VSV-infected cells (15). Gs was obtained from the serum-free culture medium of infected cells that had been concentrated on an Amicon P-10 ultrafilter (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and clarified by ultracentrifugation at 50,000 rpm for 1 h at 5°C in a Sorvall SW50 rotor (DuPont Instruments-Sorvall, DuPont Co., Newton, Conn.) (4 x 10^11 g-min). An SDS gel of the dialyzed [35S]methionine-labeled protein (Fig. 2) as determined by autoradiography. This component could be specifically precipitated with anti-G antiserum and staphylococcal protein A (Staph A) (not shown). Because of the immunochromatographic similarity of Gs and G (see below), concentrations of Gs in these samples were determined by radioimmunoassay.

Anti-G Antibody Preparation and Immunochromatographic Characterization

Antibodies were initially produced in rabbits from the G protein−lipid vesicles in PBS (50 µg/ml) with an equal volume of complete Freund's adjuvant (Calbiochem-Behring Corp.) by repeated subcutaneous injections (25 µg/injection). Serum collected 25 d after the last injection gave one precipitin line on Ouchterlony plates with either isolated G protein or intact VSV but not against VSV from which G protein had been removed by treatment with protease. A 1:1,000 dilution of this serum reduced a
VSV plaque titer of 1.5 × 10^4 plaque-forming units (PFU) to 1.5 × 10^6 PFU after a 30-min incubation at 31°C.

After an 18-ho lapse, the rabbits were boosted with 100 μg of G protein mixed 1:1 with Mlyanta (Stuart Pharmaceuticals, Div. of ICI United States Inc., Wilmington, Del.; aqueous magnesium aluminum hydroxide) followed in 2 wk by 30 μg of the same mixture. 4 The rabbits were then boosted weekly with 30 μg of G protein mixed with Mlyanta as described above. All injections were intramuscular, 0.5 ml/rabbit, half into each hind flank.

Antibody prepared using the latter procedure was used for all immunoassays reported in this and the following paper (18).

The antibody binding capacity of the serum produced was determined by precipitation of [3H]methionine-labeled G protein--antibody complex with Staph A protein (13). In the specific procedure that follows, 1 ml of antiserum caused precipitation of 50% of 4 μg of labeled G protein. 50 μl of a 1:75 dilution of antiserum was incubated with 50 μl of antigen solution (containing 0.5-5.0 μg of G or Gs) and 200 μl of 1% ovalbumin in all Ca- and Mg-free PBS containing 1 mM EDTA and 0.5% Trition X-100 for 60 min on ice. 50 μl of a solution of 10% Staph A was added, and the samples were incubated for an additional 30 min on ice, centrifuged for 10 min at 2,500 rpm (International centrifuge, Damon Corp., I. E. C. Div., Needham Heights, Mass.), and the precipitate was washed twice with 2 ml of the buffer containing 0.05% Triton X-100 for 60 min on ice. The Staph A pellets were suspended in 1 ml of 1% SDS and counted. The inhibition of precipitation of an [3H]methionine-labeled G standard by various solutions was similarly measured by radioimmunoassay, with the exception that 50 μl of the inhibitor (containing 0.02-30.0 μg G or Gs) was preincubated for 60 min at 0°C with the antibody and the ovalbumin before addition of the labeled G solution.

Electron Microscopy

Vesicles were fixed by dialysis against PBS containing 1% glutaraldehyde at concentrations of 200 μg protein/ml or less and dialyzed further against PBS to remove excess glutaraldehyde. The vesicles were pelleted and resuspended in 0.145 M sodium acetate. 50 μl of the vesicles was mixed with 50 μl of 2% ammonium molybdate. 10 μl of the resulting mixture was adsorbed for 1.5 min on Butler-coated grids (Monsanto Co., St. Louis, Mo.), and the excess fluid was removed. Microscopy was performed on a JEOL 100 C electron microscope at 80 kV.

2,4,6-Trinitrobenzene sulfonic acid (TNBS) Reaction

Accessibility of phosphatidyl ethanolamine (PE) in [3H]palmitate-labeled vesicles was determined by reaction with the nonpenetrant reagent TNBS using a minor modification of the procedure of Fong et al. (8). To 0.2 ml of G protein--viral lipid vesicles in PBS was added 0.2 ml of TNBS (1 mg/ml) in PBS solution (30 mg/ml in water) was added, and the reaction was carried out at 30°C. The reaction was terminated by the addition of 0.02 ml of 0.1 N NaOH and 0.2 ml of PBS, and extraction was carried out with chloroform-methanol-1 N HCl as described above. Chromatography and autoradiography were performed as described above, except that the chromatogram was developed at 30°C. The gangliosides appeared as a triple peak (Rf = 0.4) and the asialogangliosides as a peak with Rf = 0.6. The extent of reaction was determined from the integrated intensities of these peaks as determined from a densitometer trace of the autoradiogram.

Proteolytic Digestion

Vesicles containing G protein labeled with [3H]methionine (~100 μg/ml) were treated with chymotrypsin (1 mg/ml, 61 U/mg, Worthington Biochemical Corp., Freehold, N.J.) for 30-120 min in PBS at 37°C. Disappearance of G protein was monitored on SDS polyacrylamide gels (14). Formation of TCA-soluble radioactivity due to proteolysis was determined as previously described (25).

Inhibition of Viral Infectivity

Two different assays were used to measure the inhibition of infection of VSV. For both assays, confluent monolayers of BHK-21 cells in 35-mm Petri dishes (Corning Glass Works, Science Products Div., Corning, N.Y.) (~10^4 cells) were preincubated with 0.5 ml of a solution of the substance to be tested in MEM containing 100 mg of bovine serum albumin and 10.67 g of N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid per liter (MEMBB), followed by the addition of stock VSV to the level of 2-10 PFU/cell. A calibration curve, varying PFU/cell in the absence of any inhibitor, was determined for each experiment. Initial assays were based on the cytopathic effect of viral infection as determined by measurement of the decreased ability of infected cells to take up neutral red dye (1). The second assay measured [3H]thymidine incorporation into viral RNA.

Neutral Red, Cell Viability Assay

Cells were washed twice with PBS and once with MEMBB (pH 7.4) at 39°C and incubated at 37°C for 90 min with the substance to be tested for inhibition. The cells were then chilled to 5°C for 5 min. VSV was added to make a 40-ul volume, and the inhibitor and test virus were incubated for 30 min at 3°C. The solution was removed, and the cells were incubated for 10 h at 37°C in 1 ml of MEM containing 5% fetal calf serum. Sterile aqueous neutral red solution (0.4 ml, OD<sub>560</sub> = 2.0) was added, and each plate was incubated for an additional 4 h at 37°C. The
monolayers were washed with 1 ml of PBS, and the remaining dye was extracted from the cells with 1 ml of a 1:1 solution of absolute ethanol and Sorenson citrate buffer, pH 4.2. The extract was spun in a clinical centrifuge to remove cell debris and the OD was measured in a Cary 110 double-beam spectrophotometer (Cary Instruments, Monrovia, Calif.). Each experimental point was determined from quintuplicate samples.

**Viral RNA Assay**

The cells were incubated with the substance to be tested in a 1:1 mixture of PBS and MEM (pH 7.0). The VSV test virus in MEM that contained 20% fetal calf serum and 20 ng/ml of actinomycin D was added in an equal volume to the cell cultures, and the plates were incubated for an additional 30-60 min at 37°C. After a wash with 2 ml of PBS, each plate received 1 ml of MEM containing 10% fetal calf serum, 10 μg/ml actinomycin D, and 3-5 μCi/ml [3H]uridine (5 Ci/mmol, Amersham Corp., Arlington Heights, Ill.). After incubation for 4-6 h at 37°C, the plates were washed 2 times with PBS, and the cells were solubilized by incubation with 1 ml of 0.1% SDS for 10 min at room temperature. The RNA in 0.5 ml of each sample of the SDS solution was precipitated with 1 ml of cold 8% TCA. After a 10-min incubation on ice, the samples were filtered on Whatman GF/C glass-fiber filter papers (Whatman, Inc., Clifton, N.J.), washed four times with 2 ml of cold 5% TCA, and incubated in 0.5 ml of 0.5 N NaOH overnight at room temperature in scintillation vials. The samples were neutralized in 0.5 N HCl and counted with 10 ml of Liquiscint (National Diagnostics Inc., Parsippany, N.J.). Each experimental point was determined from triplicate samples.

**RESULTS**

**Extraction of VSV with OG**

The efficiency of extraction of G protein with OG in 0.01 M phosphate was found to depend upon the pH of extraction, with complete solubilization above pH 8.0 (Fig. 1). When samples of VSV solubilized in OG-0.01 M phosphate at pH 8.0 were analyzed in SDS gels after ultracentrifugation, essentially all of the G protein was found in the OG supernate, and none could be detected in the pellet (Fig. 2). A trace amount of M protein was occasionally seen in the OG supernates, but this could be removed by an additional ultracentrifugation (not shown). Viral lipid was also recovered quantitatively in the extract; >90% of the label was found in the extract from VSV labeled with [14C]palmitate. Thin-layer chromatograms showed that each lipid component was present in similar amounts in the virion and in the OG extract (not shown).

**Properties of Reconstituted Vesicles**

Vesicles were seen by negative staining after dialysis of the G protein–viral lipid extract. Most were in the range of 250–1,000 Å, with a fringelike structure suggestive of G protein frequently evi-
reacted with TNBS within 60 min, and this amount did not increase upon further incubation. In contrast, the addition of 0.1% Triton X-100 to the vesicles resulted in nearly complete reaction in the same time period (Fig. 4). These results indicate that the vesicles are predominantly unilamellar and sealed, with PE distributed symmetrically between the two surfaces of the bilayer.

Neuraminidase treatment of the vesicles provided a further indication that the vesicles were sealed. Treatment of G protein–viral lipid vesicles with neuraminidase (0.3 mg/ml) for 30 min at room temperature resulted in hydrolysis of 48–52% of the viral gangliosides. Further digestion
FIGURE 4 Reaction of TNBS with reconstituted vesicles in the presence (●) and absence (○) of 0.1% Triton X-100.

was not observed when the vesicles were incubated for 90 min or when the vesicles were incubated with ten times as much neuraminidase for 90 min. However, in the presence of 0.1% Triton X-100, >90% of the gangliosides were digested by a similar incubation. Thus, gangliosides, like PE, are symmetrically distributed between the two surfaces of the reconstituted vesicles. Using a similar procedure, Stoffel et al. (28) found that all of the gangliosides of intact VSV grown in BHK cells were accessible to digestion and hence externally located. This finding provides an additional indication of the predominantly unilamellar nature of the vesicle preparation.

Proteolytic digestion of the G protein–lipid vesicles suggested that the protein was asymmetrically distributed, with most of it facing outward from the vesicle. After treatment of vesicles in PBS at 37°C with 1 mg/ml of α-chymotrypsin, ~20% of the original G protein was detected on autoradiograms of SDS polyacrylamide gels, indicating that ~80% of the protein was susceptible to proteolytic digestion. This result is in agreement with measurements of the rate of formation of TCA-soluble peptides made under the same proteolytic condition. TCA-soluble material was generated by proteolysis of intact vesicles at a rate that was ~80% of that generated from the vesicles dispersed in 0.1% Triton X-100 (data not shown).

It is of interest that the ~20% of the G protein remaining on the gel after proteolysis retained its original molecular weight. A slightly smaller molecule would have been expected if the molecules were facing inward with their cytoplasmic, COOH-terminal peptides exposed to the outer surface (10, 24). Thus, although the reason for the inaccessibility of this fraction of G protein is not known, it does not arise as a result of the molecules being positioned normally in the bilayer but facing inward.

The reconstituted material sedimented as a single peak in sucrose equilibrium density gradients (Fig. 5 A). The addition of unlabeled soybean phospholipid to the extract before dialysis as a solution in 50 mM OG–0.01 M phosphate, pH 8.0, resulted in the formation of vesicles of lower buoyant density that possessed approximately the same
relative amounts of viral protein and viral phospholipid as were found in the vesicles to which extra lipid had not been added (Fig. 5 B and C). Changes in buoyant density arose from incorporation of increasing amounts of unlabeled soybean phospholipids into the vesicles. It is thus possible to control the lipid content and composition of the reconstituted vesicles.

To demonstrate structural similarities between G in the reconstituted vesicles, G in irradiated virions, and G in the Gs form, their ability to inhibit the precipitation of a labeled G extract by anti-G antibody and Staph A was measured. All 3 forms inhibited antibody precipitation equally (Fig. 6), indicating that there was no major difference in the antigenic structure of the molecules under the conditions of the assay.

**Inhibition of Viral Infectivity**

The ability of reconstituted vesicles to inhibit VSV infectivity was measured by both the neutral red, cell viability assay and the viral RNA assay. Standard curves were made for each assay (Fig. 7). To generate these curves, the amount of neutral red released or the $[^3H]$RNA formed in response to infection at varying doses of infectious virus was measured in the absence of any inhibitor (Fig. 7). The 0% inhibition point was defined as the OD$_{540}$ or cpm formed by an appropriate multiplicity of infection (m.o.i.) of VSV in the absence of inhibitor (e.g., 4 PFU/cell; Fig. 7 inserts). Inhibition was determined by infections with the identical m.o.i. in the presence of inhibitor and comparison with the standard curve. Each assay measured a different response of the cells to the VSV infection. In the neutral red assay, analysis was of a late event, essentially cell death. The viral RNA assay measured a much earlier viral event. This difference may be the cause for the difference in the scales of the inhibition plots; in the region of m.o.i. used, the neutral red assay had a logarithmic response to input multiplicity, whereas the RNA assay had a linear response.

Comparison of the inhibitory activity of the reconstituted vesicles to that of ultraviolet light-
irradiated, intact virions indicated that only 2–5% of the original activity was present after reconstitution. Lipid-free G protein rosettes were generally less effective as inhibitors than the reconstituted vesicles, and protein-free viral lipid vesicles and Gs were essentially devoid of inhibitory activity (Fig. 8). Preincubation with cells was not necessary for inhibition of viral RNA synthesis by vesicles or by irradiated virions, but both inhibitors were markedly more effective when they were incubated with cells for 1–2 h before the addition of infectious virus. Enhancement of inhibition of up to 50% was observed for both inhibitors under these conditions.

The concentration of irradiated VSV giving 50% inhibition after 60 min of preincubation, with a test dose of 1.5 PFU/cell, was ~0.6 µg of viral protein/10⁶ cells, which corresponds to ~0.2 µg of G protein (3). This is more than three orders of magnitude less than the concentration of G protein (prepared by a different method) used by McSharry and Choppin (16) to induce partial inhibition of cellular DNA synthesis. Reconstituted vesicles, although 20-fold less effective than irradiated virions in their ability to inhibit VSV infection, still exhibited 50% inhibition at a concentration of G protein that was less than one-tenth of that used by McSharry and Choppin (16). Reconstituted vesicles had no effect on cell viability in the absence of viral infection as measured by the neutral red assay.

**DISCUSSION**

G protein is generally considered to function in the initial events of viral binding to cells and in cellular penetration, although its precise mechanism of action in the many steps involved in viral uncoating is not known. It is known that proteolytic removal of G protein from VS virions causes a dramatic loss of infectivity (2) and that antibodies that prevent infection by VSV ("neutralizing antibodies") are directed against G protein. It seems likely that purified preparations of G protein will provide useful experimental tools in elucidating the precise role of G protein in the early events of viral infection. Because G protein is the only protein extracted from purified VS virions by OG under the conditions employed, G protein can be easily prepared as lipid-free G protein rosettes or as reconstituted vesicles consisting of G protein in combination with viral or exogenous lipids.

The reason for the relatively low inhibitory activity of the G protein-lipid vesicles as compared with that of irradiated virus is not known. One possibility is that a combination of viruslike rigidity and G protein polyvalency may be required for full inhibitory activity. Thus, the vesicles, some of which are similar in size to the virus particles, might lack the required rigidity, whereas the more rigid rosettes might lack the required degree of polyvalency. Gs, lacking both of these characteristics, would thus be completely inactive as an inhibitor.

A second possibility for the low inhibitory activity of the vesicles is that one of the internal VSV proteins (M or N) may be required for full biological activity. However, irradiated virions of ts III31, a thermolabile mutant with a defective M protein, are fully active after thermal inactivation (see Table I in reference 18), suggesting that functional M protein is not required for inhibitory activity.

A third possibility for the low inhibitory activity of the vesicles is that extraction under the conditions employed might cause conformational changes in the individual G protein molecules. Conformational changes have been shown to arise in the G protein of certain ("thermolabile") mutants possessing temperature-sensitive lesions in G protein as a result of heating the intact virions at
the nonpermissive temperature. Conformational changes in the G protein of these virions is evidenced by inactivation and by a tendency to form aggregates (12). Irradiated virions of these mutants inhibit a wild-type infection as effectively as do wild-type inactivated virions (Fig. 8), but this inhibitory activity is completely lost after heating (18). Vesicles prepared from the G protein of these mutants inhibit infection as well as those prepared from wild-type G protein, but, surprisingly, this activity is not lost after heating (data not shown). This finding implies that the mechanism of inhibition by vesicles may differ from that by irradiated virions in that inhibition by vesicles does not depend upon a specific action of native G protein. For this reason, further investigations regarding the role of G protein in penetration and uncoating were carried out using irradiated virions (18) rather than G protein vesicles.

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