Isolation of the Glycoprotein of Vesicular Stomatitis Virus and its Binding to Cell Surfaces

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SUMMARY

The glycoprotein (G) of vesicular stomatitis virus (VSV) was radiolabelled, extracted and purified so that its potential interaction with host cell surfaces could be studied. When BHK-21 cells were incubated with the radiolabelled virus glycoprotein, the virus component rapidly attached to the cell surface. The attachment was shown to be temperature-dependent and saturated at approx. 3 x 10^5 molecules/cell. The omission of Mg^{2+} or Ca^{2+} from the incubation medium had little effect on the glycoprotein binding. Treating the isolated G protein and intact virions with neuraminidase did not significantly decrease their binding to BHK-21 cells. Pre-incubating cells with trypsin did not decrease the attachment of VSV virions nor the binding of purified G protein. Treating cells with phospholipase A or phospholipase C suggested that the binding of the glycoprotein and the intact virion might have been dissimilar. Unlabelled glycoprotein competitively inhibited binding of the labelled molecules although the presence of intact virions did not inhibit attachment of the G protein. Likewise, saturating amounts of the glycoprotein did not decrease binding of VSV to BHK-21 cells. These observations suggested that either the isolated glycoprotein bound to cell surface components that were distinct from the virion receptor or that the manner of the purified glycoprotein attachment differed from the G protein still associated with the intact virion. Chemical crosslinking and diagonal two-dimensional gel electrophoresis were used to identify and to compare the cell surface components responsible for glycoprotein and virion attachment.

INTRODUCTION

The initial interaction between viruses and the host cell surface has been the subject of many studies. Most investigators, however, have focused on the physical and chemical properties of the virus components that are responsible for virion attachment (Bose & Sagik, 1970; Lonberg-Holm & Korant, 1972; Philipson & Lindberg, 1974). Until recently most studies involving the host cell surface receptor complexes associated with virus attachment have been limited to indirect examination by enzymic analyses and many have been restricted to erythrocyte surfaces. For example, sialic acid of a surface glycoprotein of erythrocytes was shown to mediate influenza virus attachment (Kathan et al. 1961; Hoyle, 1968) and glycoproteins that contain sialic acid, present in serum and respiratory fluid, were shown to prevent virion attachment to susceptible cells (Howe & Lee, 1970). This host cell surface glycoprotein contains most of the N-acetyleneuraminic acid of the erythrocyte membrane (Cook et al. 1961; Dannon et al. 1965) and interestingly, also contains the determinants of the M and N blood group substances (Kathan et al. 1961; Howe et al. 1963).

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A cell surface glycoprotein, devoid of sialic acid, has been isolated and characterized as being responsible for the binding of adenovirus, type 7, to susceptible cells (Neurath et al. 1970). More recently, a glycoprotein (gp71) of murine leukaemia virus has been used as a molecular probe to identify the host cell component responsible for virion attachment. The binding properties and requirements of the gp71 molecule were shown to be similar to the intact virion (DeLarco & Todaro, 1976; Fowler et al. 1977). Further studies of the cell surface component have suggested that the murine leukaemia virus binds to a lipoprotein (Kalyanaraman et al. 1978).

Vesicular stomatitis virus (VSV), a rhabdovirus that possesses an unusually broad host range which includes established cell lines of arthropod, avian and mammalian species, has a glycoprotein (G) that is responsible for virion attachment (Kelley et al. 1972; Bishop et al. 1975). Antiserum prepared against the glycoprotein neutralizes virus infectivity while antibodies directed against other VSV structural proteins do not inhibit virus infection (Kelley et al. 1972; Dietzschold et al. 1974). Schloemer & Wagner (1975) found that the cellular receptor for VSV resisted treatment with neuraminidase and trypsin. Little else is known about the chemical nature of the cellular receptor for VSV or the nature of the virus interaction with the host cell surface. The characterization of the chemical and physical properties of the initial VSV–host cell interaction, however, may be fundamental to our understanding of why this virus has such a broad host range. These studies were initiated to determine if the isolated G protein of VSV could bind to cell surfaces and if the nature of the G protein attachment was related to the interaction of the intact VSV virion with the cell.

**METHODS**

*Cell culture.* Baby hamster kidney cells (BHK-21), obtained from International Scientific Industries, Cary, Ill., U.S.A. were maintained as monolayer cultures by routine cell culture methods (Rabinowitz et al. 1976). The BHK-21 cells were grown in Earle’s salts supplemented with 7% virus-screened foetal calf serum, 1% tryptose phosphate, 2 mm-glutamine, 1% non-essential amino acids, 1% minimal essential medium, vitamins and penicillin (1000 units/l), streptomycin (1 mg/l), fungizone (2·5 mg/l) and gentamicin (20 mg/l) (Grand Island Biological Co., Grand Island, N.Y., U.S.A.). BHK-21 cells were grown to confluence in 32 oz, sterile, glass bottles by incubation at 37 °C in a 5% CO₂-95% air atmosphere in a water-jacketed incubator.

*Virus preparation and purification.* The Indiana serotype of VSV was added to monolayers of BHK-21 cells at 0·1 p.f.u./cell and allowed to adsorb for 30 min at 25 °C. Culture medium was added and whenever appropriate, 5 μCi/ml of 3H-fucose and/or 5 μCi/ml of 35S-methionine (New England Nuclear Corp., Boston, Mass., U.S.A.) were added. The infected cells were incubated at 37 °C for 18 to 24 h. Mature virions released into the medium were isolated by first removing cellular debris by slow speed centrifugation (5000 g for 10 min). VSV virions were then pelleted from the resulting supernatant fluid by ultracentrifugation at 32000 g for 3 h at 4 °C. The virus pellet was suspended in TEN buffer (50 mm-tris-HCl, 100 mm-NaCl, 5 mm-EDTA, pH 7·4), briefly sonicated (Sonifier Cell Disruptor, model W140D, at a setting of 3 for 5 to 10 s) and re-pelleted at 136000 g for 1·5 h at 4 °C. The final VSV pellet was resuspended in either TEN buffer or Hanks’ balanced salt solution (HBSS; Hanks & Wallace, 1949), briefly sonicated and stored at −90 °C.

*Plaque and plaque reduction assays.* Tenfold serial dilutions of purified VSV were prepared in HBSS and 0·1 ml was plated on to monolayers of BHK-21 cells cultured in six-well plates (35 × 10 mm, Linbro Co., New Haven, Conn., U.S.A.). After virus had been allowed to adsorb to cells for 30 min at 25 °C, the monolayers were overlaid with growth media supplemented with 0·37% agar (Difco Laboratories, Detroit, Mich., U.S.A.) as a
solidifying agent. All samples were plaqued in duplicate and plates were incubated at 37 °C for 24 to 48 h. After incubation, the agar medium was removed and the plates stained with crystal violet (Hughes et al. 1979).

Isolation and purification of G protein. The G protein was isolated by a modification of a technique used by Hale et al. (1978). Nonidet P40 (NP40, Bethesda Research Laboratories, Rockville, Md., U.S.A.) was added to purified virions to a final concentration of 1% (v/v) for 20 min at 25 °C with occasional stirring. The non-solubilized nucleocapsids were then separated from the solubilized envelope G and M proteins by pelleting the nucleocapsids through 20% sucrose at 136000 g for 90 min at 4 °C. The virus envelope proteins, which remained above the 20% sucrose, were collected for analysis by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and further purification. The M and G proteins were then separated on a Sephadex G-75 column (56 x 1.1 cm) by using phosphate-buffered saline (PBS: 0.4 M-NaCl, 2.7 mM-KCl, 6.7 mM-Na2HPO4, 1.4 mM-KH2PO4, pH 7.4) to elute the two proteins. The G protein eluted in the void volume, while the M protein eluted in later fractions. The fractions containing only the G protein were pooled and used to measure binding of the VSV glycoprotein to BHK-21 cells.

SDS–PAGE. Virus proteins of VSV and various protein samples were analysed by SDS–PAGE according to the method described by Laemmli (1970). The sample proteins were electrophoresed (2.5 mA/gel) through a 3% polyacrylamide stacking gel (2 cm) and a 9% polyacrylamide separating gel (10 cm). With radioactive samples, the gels were frozen following electrophoresis and sliced into 1 mm slices. The slices were mixed overnight in an NCS:scintillation fluid mixture (Hughes et al. 1979) and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (model 3320). Those gels containing unlabelled protein were stained following electrophoresis with Coomassie brilliant blue and were scanned at 500 nm by using a Gilford spectrophotometer (model 250) with a linear transport module. Areas under virus protein peaks were integrated by using a Numonics electronic graphic calculator. In all instances of gel electrophoresis, samples of bovine serum albumin (mol. wt. 68000), ovalbumin (mol. wt. 45000) and chymotrypsinogen (mol. wt. 25000) were co-electrophoresed as mol. wt. standards to determine the mol. wt. and identity of the virus proteins.

Antiserum to G protein. Isolated G protein, approx. 0.5 to 1 mg, was added to complete Freund’s adjuvant and injected subcutaneously into New Zealand white rabbits. A booster injection containing incomplete Freund’s adjuvant was given every 2 weeks after the initial injection. Serum was collected every 2 weeks and tested for the ability of the antiserum to neutralize virus infection by plaque reduction assay. Sera with high neutralizing titres were pooled and IgG immunoglobulins were purified as previously described (Fahey & Terry, 1978; Heide & Schwick, 1978). Briefly, the serum was precipitated repeatedly with sodium sulphate, the precipitate was dissolved in 0.03 M-tris-borate buffer (pH 8.0) and then fractionated on a DEAE-cellulose column (80 x 2.5 cm) previously equilibrated with the tris-borate buffer. The IgG (which eluted in the void volume) was then concentrated in an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass., U.S.A.) by using a type PM-10 ultrafiltration membrane. To reduce non-specific reactions, purified antiserum (0.1 mg) was absorbed overnight at 4 °C with 1 x 10^7 BHK-21 cells before use. Neutralization titres of antiserum were determined by a plaque reduction assay. Fivefold dilutions of antiserum were reacted with an equal vol. of VSV (1 x 10^8 p.f.u.) for 30 min at 37 °C and then plaqued on BHK-21 monolayers as described above.

Binding of G protein to BHK-21 cells. 3H-fucose- and 35S-methionine-labelled G protein, at the designated concentrations, was mixed with 2 vol. of binding solution (28 mM-NaCl, 9 mM-glucose, 7.6 mM-KCl, 1.4 mM-MgSO4, H2O, 3.4 mM-KH2PO4, pH 7.4) and one-third vol. of 12.6 mM-CaCl2. The BHK-21 cells were prepared for the binding studies by scraping
the cell monolayers with a rubber policeman, pelleting the cells by slow speed centrifuga-
tion and then suspending them in HBSS. The labelled G protein was added to $7.5 \times 10^6$
to $1 \times 10^7$ cells for the binding assay. Following incubation, the cells were pelleted and the
supernatant discarded. Cells were washed twice with HBSS and the final pellet was solubil-
ized with 1 M-NaOH. The solubilized cell solution was dissolved in 3.5 ml of Biofluor
scintillation fluid (New England Nuclear), 2 drops of 1 M-acetic acid were added to each
vial and the radioactive incorporation determined in a Packard scintillation spectrometer.
The number of G protein molecules binding/cell was calculated from the specific radio-
activity of the G protein after a zero time point as background was subtracted. The specific
radioactivity of the G protein preparation isolated from labelled VSV virions ranged
from $4.3 \times 10^5$ to $8.7 \times 10^5$ cnt/min/mg protein. Protein concentrations were determined by
the procedure of Lowry et al. (1951) by using bovine serum albumin as a protein standard.

**Enzymic treatment of BHK-21 cells.** Ten ml of a trypsin solution (1:250) or 1 unit of
phospholipase C (Sigma Chemical Co., St Louis, Mo., U.S.A.) were incubated with
$7.5 \times 10^6$ BHK-21 cells for 30 min at 37°C. Five units of phospholipase A (Sigma) were
added to $7.5 \times 10^6$ BHK-21 cells for 30 min at 25°C. After enzymic treatment, cells were
pelleted and washed three times with HBSS before using in a binding assay. Phospholipase
C was suspended in 0.5 mM-phosphate buffer, pH 7.4, and phospholipase A was suspen-
ded in 0.22 M-NaCl, 20 mM-CaCl$_2$ and 1 mM-EDTA, pH 7.5. Treated cells were used imme-
diately to avoid re-expression of cell surface components removed by enzyme hydrolysis.

**Neuraminidase treatment of virus and G protein.** Approx. 5 units of *Vibrio cholera*
neuraminidase (CalBiochem-Behring Corp., La Jolla, Calif., U.S.A.) was incubated with
$1 \times 10^7$ p.f.u. of $^{35}$S-methionine-labelled VSV for 90 min at 37°C. Treated virions were
pelleted by ultracentrifugation, suspended in HBSS and infectivity was analysed by plaque
assay on BHK-21 cell monolayers. Approx. 8 pg of labelled G protein were treated with
0.5 units of neuraminidase linked to agarose beads (Sigma) at 37°C for 3 h and the enzyme
beads were then removed by centrifugation. Attachment of enzyme-treated G protein and
virions was evaluated and compared with that of G protein or virions treated in the same
manner but without neuraminidase.

**Crosslinking G protein and VSV to cell surfaces.** Saturating amounts of labelled G protein
(50 µg) or intact virions (m.o.i. 100) were adsorbed to $6 \times 10^7$ BHK-21 cells for 30 min.
Cells were washed and 6 mg of the crosslinker dimethyl-3,3'-dithiobispropionimidate
dihydrochloride (DTBP, Pierce Chemical Co., Rockford, Ill., U.S.A.), was added to cells
for 30 min at 4°C according to the procedure outlined by Takemoto et al. (1978). After
incubation with the crosslinker, cell membranes were isolated by treating crosslinked
cells with 1% NP40 for 15 min on ice, removing nuclei by centrifugation and then diluting
the supernatant containing the solubilized cell membranes twice with 0.14 M-NaCl, 3 mM-
KCl and 20 mM-phosphate buffer (pH 7.6). A sufficient quantity of purified IgG (0.2 mg)
directed against the G protein and pre-absorbed with BHK-21 cells was incubated with
the cell membrane fraction for 30 min at 37°C and then placed at 4°C overnight. The
resulting precipitate was pelleted by centrifugation, suspended in sample buffer without
mercaptoethanol (Kelly & Luttges, 1975) and labelled by the chloramine T iodination
method (Moore et al. 1974). As a control, cells not exposed to either G protein or VSV
virions were crosslinked with DTBP, cell membrane fractions were collected and reacted
with antiserum and iodinated in a similar manner.

Crosslinked species precipitated by the IgG were resolved by diagonal two-dimensional
electrophoresis. Approx. $4 \times 10^6$ cnt/min of each sample were electrophoresed (10 mA/gel)
through a 3% acrylamide stacking gel and 9% acrylamide separating gel in the absence of
2-mercaptoethanol. After electrophoresis, sample tracks were cut out, placed horizontally
above another prepared polyacrylamide gel and immobilized on the top of the slab gel
**RESULTS**

*Isolation and purification of the G protein*

Initial studies were carried out to determine the protein and glycoprotein composition of the purified VSV virions and the efficiency of NP40 in separating the virion envelope and nucleocapsid components. SDS-PAGE analysis of complete VSV virions clearly separated the major structural proteins; virus previously labelled with $^3$H-fucose, which
Table I. Fractionation of the VSV proteins

<table>
<thead>
<tr>
<th>Virus protein</th>
<th>% Protein in intact virions*</th>
<th>Nucleocapsid pellet†</th>
<th>Soluble fraction†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>3.2±0.5</td>
<td>100.0±0.0</td>
<td>0</td>
</tr>
<tr>
<td>N</td>
<td>33.7±1.8</td>
<td>96.4±1.2</td>
<td>3.6±1.2</td>
</tr>
<tr>
<td>G</td>
<td>28.5±1.5</td>
<td>25.3±2.8</td>
<td>74.4±2.9</td>
</tr>
<tr>
<td>M</td>
<td>34.6±1.3</td>
<td>43.8±5.3</td>
<td>56.2±5.2</td>
</tr>
</tbody>
</table>

* The VSV proteins were separated by SDS–PAGE, stained with Coomassie brilliant blue and the gels were scanned at 550 nm as in Fig. 1. The area under each protein peak was integrated to determine what % each VSV protein was of the total virion protein content.

† The VSV virions were dissociated by NP40 treatment, followed by centrifugation into a nucleocapsid pellet and soluble fraction as described in the text. The % of each protein present in each fraction was determined from SDS–PAGE analysis of the fractions. All values represent the mean of five to eight independent experiments ± S.E.M.

labels only the virus glycoprotein (G), provided a convenient marker (Fig. 1). The protein composition of the virion was 3.2% L protein, 28.5% G protein, 33.7% N protein and 34.1% M protein (Table I). The NS protein, which normally constitutes only 1 to 2% of the virus protein (Bishop & Smith, 1977), could not be accurately quantified because of the small amount and because it often migrated close to the N protein.

NP40 extraction provided an efficient method of separating the nucleocapsid and envelope proteins of VSV. The nucleocapsid pellet contained 100 and 96% of the L protein and N protein, respectively. Approx. 75% of the G protein was solubilized by treatment with NP40 and SDS–PAGE analysis of this soluble fraction showed that only a trace of the major nucleocapsid protein (N) was present (Table I). The G and M proteins of the virion envelope were separated by gel filtration on a Sephadex G-75 column as described in Methods. SDS–PAGE analysis of the G protein fraction, which eluted in the void volume, demonstrated that the preparation was composed of more than 97% G protein and contained less than 3% M and N (Fig. 2). The purified G protein appeared to be intact in that its migration by SDS–PAGE was identical to that of the glycoprotein of intact virions. This purification procedure allowed a recovery of 55% of the initial G protein of the VSV.

Binding of G protein to BHK-21 cells

In preliminary experiments we found that the purified radiolabelled G protein of VSV bound tightly to BHK-21 cells. To maximize the binding of the G protein to BHK-21 cells, we measured the effects of varying concentrations of G protein, incubation temperature, time of incubation, the composition of the binding medium and cell concentration. When increasing concentrations of G protein were incubated with 1 x 10⁷ BHK-21 cells at 25°C, the amount of G protein bound was essentially maximal at 5 µg per reaction (Fig. 3). Increasing the concentration of G protein to over 15 µg per reaction resulted in little additional binding. These kinetics suggested that there were a definitive number of average binding sites/cell (≈ 2 x 10⁵ to 3 x 10⁶).

The kinetics of G protein binding to BHK-21 cells was shown to be relatively rapid in that 57 and 78% of the binding reaction was completed within 5 min of incubation at 4 and 25°C, respectively (data not shown). The remaining G protein that bound during the subsequent 10 min of incubation appeared to occur at a different and slower rate. Incubation of G protein and BHK-21 cells for periods longer than 15 min at 4 and 25°C did not result in increased binding.
VS V glycoprotein and cell surface binding

40
30
20
10
5
2
1

G protein bound (cell \( \times 10^{-9} \))

1 5 10 15
G protein added (\( \mu g \))

Fig. 3. Binding of radiolabelled G protein to BHK-21 cells. Various amounts of radioactively labelled G protein were added to \( 1 \times 10^7 \) BHK-21 cells for 30 min at 25°C. The cells were then washed and the amount of G protein bound was determined as described in Methods.

Table 2. Effect of cell number on G protein binding*

<table>
<thead>
<tr>
<th>Total BHK-21 cells present ((x 10^{-6}))</th>
<th>Total number of G protein molecules bound ((x 10^{-11}))</th>
<th>G protein molecules bound/cell ((x 10^{-9}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>31.4</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>7.5</td>
<td>26.6</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>5.0</td>
<td>16.2</td>
<td>3.2 ± 0.1</td>
</tr>
</tbody>
</table>

* Different concentrations of BHK-21 cells were incubated with a saturating amount of radiolabelled G protein at 25°C for 30 min. Radioactivity which remained associated with washed BHK-21 cells was evaluated and the number of G protein molecules bound/cell was calculated from the specific activity of the G protein.

Several experiments were carried out to assess the possible role of Mg\(^{2+}\) and Ca\(^{2+}\) in the binding of G protein to BHK-21 cells. However, increasing the Mg\(^{2+}\) or Ca\(^{2+}\) concentration by 2-5-fold had no measurable effect on G protein binding and the absence of these ions in the incubation medium did not decrease glycoprotein binding (data not shown).

Specificity of G protein binding

The kinetics of G protein binding suggested that the interaction of the glycoprotein with the cell surface involved a limited number of sites that may be specific (Fig. 3). If that were true the binding of G protein should have been dependent on cell concentration and an excess of unlabelled G protein should have competed with the radiolabelled glycoprotein. When various concentrations of BHK-21 cells were incubated with a saturating amount of radiolabelled G protein (Table 2), a total of \( 3.1 \times 10^5 \) to \( 3.5 \times 10^5 \) molecules of G protein bound/cell regardless of the number of cells used. Under similar incubation conditions, calculations from 34 independent determinations throughout this study showed that \( 3.3 \times 10^5 \) G protein molecules bound per BHK-21 cell.

Further evidence for a saturable binding of G protein was obtained by mixing known concentrations of unlabelled G protein with a constant amount of radiolabelled G protein. The binding of \( 0.6 \mu g \) of added labelled G protein was inhibited by unlabelled G protein in a competitive fashion in that the measured decrease in labelled G protein bound was consistent with that calculated on the basis of competitive inhibition (Table 3). However, the calculated and observed G protein binding were only consistent when \( 1.4 \mu g \) and \( 2.8 \mu g \) of unlabelled G protein were present. When \( 0.8 \mu g \) of unlabelled G protein was mixed with \( 0.6 \mu g \) of the radiolabelled glycoprotein, 61% of the labelled protein bound,
Table 3. Competitive inhibition of binding of radiolabelled G protein by unlabelled G protein

<table>
<thead>
<tr>
<th>Amount of G protein added (μg)</th>
<th>Number of labelled G protein molecules bound/cell*</th>
<th>Calculated binding† (% of control)</th>
<th>Observed binding‡ (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>$1.25 \times 10^5$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.8</td>
<td>$7.70 \times 10^4$</td>
<td>42</td>
<td>61</td>
</tr>
<tr>
<td>1.4</td>
<td>$3.10 \times 10^4$</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>2.8</td>
<td>$1.65 \times 10^4$</td>
<td>17</td>
<td>13</td>
</tr>
</tbody>
</table>

* Radiolabelled G protein (0.6 μg) was added with various concentrations of unlabelled G protein to 1 × 10⁷ BHK-21 cells for 30 min at 25 °C. The number of radiolabelled G protein molecules bound was determined from duplicate samples as described in Methods.
† The calculated or expected binding was determined from the amount of G protein that should have bound to BHK-21 cells if the binding was saturable and if unlabelled G protein could competitively inhibit labelled G protein binding.
‡ The observed percentages of G protein binding were calculated from the amount bound compared with that bound in the controls with no unlabelled G protein added.

Table 4. Effect of enzyme treatment of host cells on attachment of G protein and VSV*

<table>
<thead>
<tr>
<th>Enzymic treatment of BHK-21 cells</th>
<th>Binding component</th>
<th>Untreated</th>
<th>Treated × 100 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>G protein</td>
<td>107</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>VSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase A</td>
<td>G protein</td>
<td>136</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>VSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>G protein</td>
<td>81</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>VSV</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Approx. 7.5 × 10⁶ BHK-21 cells were treated with the various enzymes as described in Methods. Saturating amounts of G protein (8 μg) or 4000 cts/min of radiolabelled VSV were added to the treated cells and binding was determined relative to binding to untreated cells. The values presented are the averages of duplicate measurements.

which was well above the 42% calculated. This indicated that 0.6 μg of G protein was not sufficient to saturate all the cell binding sites and that at least 2 μg of G protein was sufficient to provide saturation kinetics with 1 × 10⁷ cells. This observation was consistent with the saturation curve illustrated in Fig. 3. In addition, this observation eliminated the possibility that the measured number of G protein molecules bound/cell was a result of G protein aggregation.

Comparison of G protein and VSV binding

Since the binding of G protein was saturable and involved approx. 3 × 10⁵ sites/cell, comparisons between the binding of purified G protein and VSV virions could be made with confidence. Since Schloemer & Wagner (1975) reported that the infectivity of VSV could be decreased by the treatment of virions with neuraminidase, we incubated both the virions and the G protein with neuraminidase as described in Methods and measured their subsequent ability to bind to BHK-21 cells. Neuraminidase treatment resulted in a 1.5 to 2 log loss of infectious VSV, as measured by plaque assay, although virion attachment was reduced by only 25% (data not shown). Treatment of the G protein with neuraminidase also had little effect on the ability of the isolated glycoprotein to bind to cell surfaces. Although antibody, directed against the G protein of VSV, clearly neutralizes virus infectivity (Kelley et al. 1972; Dietzschold et al. 1974), it is not known whether the antiserum directly inhibits virion attachment or interferes with later stages of infection.


Table 5. Effect on the binding of G protein and the whole virion of the addition of unlabelled virions or G protein

<table>
<thead>
<tr>
<th>Expt. 1. Competitive inhibition of virus attachment with G protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>G protein added (µg)</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>0 (control)</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>10.0</td>
</tr>
<tr>
<td>15.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expt. 2. Competitive inhibition of G protein attachment with VSV†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus added (m.o.i.)</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>0 (control)</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>100</td>
</tr>
</tbody>
</table>

* 3000 ct/min (5 × 10⁹ p.f.u.) of whole VSV virions, mixed with various amounts of unlabelled G protein, were added to 7.5 × 10⁸ BHK-21 cells and incubated at 25 °C for 30 min. The radioactivity that remained with the washed cells was determined and compared with the amount of VSV bound in the absence of G protein. Values represent the average of four measurements.

† Saturating amounts of radiolabelled G protein (9 µg) were mixed with various amounts of unlabelled VSV before being added to 7.5 × 10⁸ BHK-21 cells. The number of G protein molecules bound was evaluated after 30 min incubation at 25 °C. Values represent the average of four measurements.

We found that incubation of VSV or G protein with anti-G IgG sufficient to reduce virus infectivity by 4 logs did not decrease the binding of either virion or the purified protein to BHK-21 cells (data not shown). Possibly the binding reflected an affinity of cell surface components with the Fc region of the IgG molecules. In any event, these results had little value in discriminating between the attachment properties of the intact VSV virions and the purified G protein.

The ability of BHK-21 cells to bind intact VSV virions and isolated G protein was not diminished by pre-incubation of the cells with trypsin (Table 4). However, phospholipase A treatment increased their ability to bind G protein while the attachment of intact VSV virions was somewhat reduced. The enhanced binding of G protein by pre-exposure of the cells to phospholipase A was most likely a reflection of an increased exposure of cell surface receptors to the glycoprotein. Treatment of BHK-21 cells with phospholipase C slightly reduced G protein binding although the enzyme had little effect on the attachment of intact VSV (Table 4). Although this was the first evidence that the nature of the binding of the isolated G protein and the VSV virions was different, the difference was small and a more direct assessment was necessary.

Competitive binding by the virions and the G protein

Since the binding of radiolabelled G protein was inhibited by unlabelled G protein (Table 3), a more direct approach was available to compare the attachment of the glycoprotein and the VSV virions. The presence of a saturating quantity of G protein did not interfere with the binding of radiolabelled VSV virions to BHK-21 cells (Table 5). In addition, the presence of a saturating quantity of intact virions did not reduce the amount of radiolabelled G protein that was bound (Table 5). These observations clearly indicated that the purified glycoprotein did not bind in a fashion that was identical to the intact virion. In support of this conclusion, we also could not find evidence that the G protein, bound to BHK-21 cells before the addition of VSV, was displaced when virions were added.

Chemical crosslinking of the G protein to BHK-21 cells

To compare the cell surface components that primarily bound the isolated G protein and the G protein in the intact virion, we reacted the bound molecules with DTBP, a bifunctional crosslinker containing an imido-ester group at each end that reacts with
Fig. 4. Chemical crosslinking of the G glycoprotein or whole VSV virions to BHK-21 cells. Isolated G glycoprotein or VSV was incubated with BHK-21 cells for 30 min at 25 °C. After chemical crosslinking with DTBP, precipitation of the solubilized G protein–cell surface complexes with anti-G IgG and radiolabelling the molecules with $^{125}$I, the radiolabelled crosslinked species were resolved by two-dimensional gel electrophoresis as described in Methods. The gels were then fixed and stained for protein, dried and exposed to Kodak XRP1 film. (a) BHK-21 cells incubated with neither G protein or VSV. (b) BHK-21 cells incubated with the purified G protein. (c) BHK-21 cells incubated with VSV virions. The figures present diagrammatic composites of several exposures of the radiolabelled protein species. The species which migrated as expected from their mol. wt. migrated only to the diagonal (---) since the proteins should be electrophoresed equally in the first and second dimension gels. All of the proteins below the diagonal line represent protein species which were covalently linked by disulphide bonds in the first dimension that were broken in the second gel: protein species found below the diagonal line in cells incubated without G protein or VSV (⊙), in cells incubated with isolated G protein (●) or in cells incubated with VSV (○). The species which migrate similarly to some crosslinked species as detected in (a) were also labelled (⊙) in (b) and (c) as they were not associated with either G protein or virion binding. The arrows in (b) and (c) show the position of the G protein.

primary amines. Between the reactive imido-ester groups lies a disulphide that is cleaved by mercaptoethanol. The G protein or intact VSV virion was first bound to BHK-21 cells, reacted with DTBP and the membranes solubilized with NP40 as described in Methods. The crosslinked G protein–surface membrane complexes were precipitated with anti-G IgG and the proteins were iodinated with $^{125}$I. When radiolabelled G protein was used to measure total recovery by this method, over 65% of the initial G protein was recovered by precipitation with the antibody. The iodinated components were then resolved by two-dimensional diagonal electrophoresis to allow the identification of cell surface moieties that were associated with the free and virion-bound G protein.

In the absence of both G protein and the DTBP crosslinker, only two cell surface components were found to be linked by disulphide bonds (Fig. 4a, arrows). Based on their positions in the slab gel and their migration in the first dimension (the crosslinked form), these cell surface components appeared to exist naturally as homodimers and homotetramers. In the presence of the DTBP crosslinker, but in the absence of either isolated G protein or VSV virions, four additional host cell membrane components were found to migrate at positions out of the diagonal (Fig. 4a). When G protein was bound to the cells before crosslinking with DTBP, 10 additional host cell proteins were found to be crosslinked (Fig. 4b). Of those, only components 1 (mol. wt. 79,000) and 2 (mol. wt. 26,000) appeared to be crosslinked with the G protein. According to their migration in the first dimension as crosslinked species, the G protein could have been complexed to both cell components. For example, cell surface component 1, crosslinked with the G protein, could migrate at mol. wt. 137,000 rather than the accumulative mol. wt. 148,000 because
the G protein itself contains an internal disulphide bond and will migrate faster in the absence of mercaptoethanol (Mudd & Swanson, 1978). In a similar fashion, a crosslinked complex of three cell components 2 and a G protein would migrate at approx. mol. wt. 137000 in the first dimension.

Although not covalently crosslinked to the G protein by DTBP, cell components 3, 4, 5 and 6 were crosslinked as a result of the addition of the glycoprotein (Fig. 4b). Most likely those cell components were originally complexed with the G protein and precipitated by the anti-G IgG, although amine groups of the G protein and these cell components were not situated properly for DTBP to form a crosslink. This would escape direct analysis since the presence of SDS would dissociate the G protein from the chemically crosslinked cell components. These four crosslinked species were not visualized when intact virions were reacted with the cell surface (Fig. 4c).

In addition to the above mentioned four cell components that were crosslinked when G proteins, but not VSV virions, were added, other differences were clearly established that explained the inability of the isolated G proteins and the virions to compete in surface binding. For instance, only cellular component 2 (mol. wt. 26000) and not component 1 was crosslinked to the G protein when intact virions were bound to the cell surface (Fig. 4c). In addition, cell components 7 and 8, as well as 9 and 10, were crosslinked when intact virions were used. None of these four cell proteins was crosslinked when isolated G protein was bound (Fig. 4b).

**DISCUSSION**

When VSV virions were solubilized with NP40, the two envelope proteins, M and G, were found in the soluble fraction. A single separation step using Sephadex G-75 allowed the recovery of 55% of the virion G protein in a fraction that was 97% pure. Although Triton X-100 (Kelley *et al.* 1972) was also used, it was found to be less efficient than NP40. The purified G protein appeared to be intact and to migrate on SDS-PAGE gels identical to the original virion glycoprotein. The G protein bound efficiently to BHK-21 cells, at a rapid rate and in a saturable manner, resulting in $3 \times 10^5$ molecules bound/cell (Table 2). This was similar to previous observations involving isolated proteins of murine leukaemia virus (DeLarco & Todaro, 1976) and adenovirus (Philipson *et al.* 1968) but in contrast to the unsaturable kinetics measured with myxoviruses (Lonberg-Holm & Philipson, 1974). The fact that unlabelled G protein competed with the binding of labelled molecules suggested that there were a definite number of receptor sites on the cell surface and that the attachment kinetics did not reflect an aggregation of the glycoprotein molecules (Table 3). Similarly, Weissman *et al.* (1977) showed that the binding of the murine leukaemia virus was saturable and not a result of aggregation. Although the VSV G protein was bound more rapidly at 25 than at 4 °C, the total number of molecules bound at 15 min was similar. Longer incubation periods did not lead to more binding. Increasing concentrations of either Mg$^{2+}$ or Ca$^{2+}$, or their omission from the incubation medium, had no effect on G protein binding to BHK-21 cells. This is in contrast to the cation sensitivity of binding of the gp71 of murine leukaemia virus to isolated membranes of 3T3 cells (Kalyanaraman *et al.* 1978).

Several experiments were carried out to compare the binding properties of the VSV G protein and the intact virion. Treatment of the VSV glycoprotein with neuraminidase did not reduce its ability to attach to BHK-21 cells. Neuraminidase reduced virion infectivity by 99%, although the ability of the virions to attach was only decreased by 25%. In the light of the report of Cartwright & Brown (1977) who showed that neuraminidase treatment of VSV resulted in a reduction of the virions' ability to inhibit influenza virus haemagglutination but not a reduced infectivity in mice, it is most likely that sialic acid of the G protein...
protein has no role in infectivity. Pre-incubation of the BHK-21 cells with trypsin did not affect the binding of the purified G protein or the intact virions. A similar observation has been reported by Schloemer & Wagner (1975) where trypsinization of BHK-21 cells actually increased the ability of VSV to attach. Treatment of cells with phospholipase A and phospholipase C, suggested that the cell surface components responsible for the binding of the G protein and the virions may have been different (Table 4). The differences were small compared to the observations of Kalyanaraman et al. (1978) and were therefore difficult to interpret. However, the inability of the G protein to compete with the binding of the intact virion also suggested that there were independent attachment sites for the glycoprotein and the virion (Table 5). In a similar fashion, the virion was unable to compete against the binding of the G protein. Even pre-adsorption of either the G protein or the VSV virion did not affect the subsequent binding of the other. We found no evidence that the relative affinity of the virion for the cell surface receptor was greater than that of the purified G protein.

A direct examination of the cell surface moieties responsible for the binding of the G protein and the intact virion involved the use of a chemical crosslinker and a purified anti-G IgG preparation. In both cases the presence of the ligand caused the crosslinking of several cell surface components (Fig. 4). Some surface proteins appeared to be associated with the binding of both the purified G protein and the intact virions. More important and consistent with their inability to cross-compete for binding, the majority of cell surface components associated with the attachment of the G protein and the intact virion were not shared. Evidently, the manner in which the G protein is presented, when freed from the virion envelope, is quite different from that of the closely packed G protein molecules at the virion surface. Possibly the clusters of G protein in the virion envelope may have a different selectivity for all surface receptors from the single G protein molecules. Perhaps the insertion of the G protein into liposomes would provide a cell surface probe that would be more similar to the intact virion. In any event, it is clear from our data that isolated glycoproteins known to be responsible for virus attachment, must be used cautiously as molecular probes for the identification of surface receptors associated with virus attachment. This may be particularly important with viruses like VSV that have a very broad host range and, perhaps, can attach to a wide spectrum of host cell surface components. Perhaps this experimental approach is most appropriate for viruses that have a highly restricted host range and therefore interact with a limited number of cell surface components (Neurath et al. 1970; DeLarco & Todaro, 1976; Kalyanaraman et al. 1978).

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REFERENCES


VSV glycoprotein and cell surface binding


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