Interactions of Semliki Forest Virus Spike Glycoprotein Rosettes and Vesicles with Cultured Cells

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ABSTRACT Semliki Forest virus (SFV)-derived spike glycoprotein rosettes (soluble octameric complexes), virosomes (lipid vesicles with viral spike glycoproteins), and liposomes (protein-free lipid vesicles) have been used to investigate the interaction of subviral particles with BHK-21 cells. Cell surface binding, internalization, degradation, and low pH-dependent membrane fusion were quantitatively determined. Electron microscopy was used to visualize the interactions.

Virosomes and rosettes, but not liposomes, bound to cells. Binding occurred preferentially to microvilli and was inhibited by added SFV; it increased with decreasing pH but was, in all cases, less efficient than intact virus. At 37°C the cell surface-bound rosettes and virosomes were internalized via coated pits and coated vesicles. After a lag period of 45 min the protein components of the internalized ligands were degraded and appeared, as acid-soluble activity, in the medium. The uptake of rosettes and virosomes was found to be similar to the adsorptive endocytosis of SFV except that their average residence times on the cell surface were longer. The rosettes and the liposomes did not show low pH-induced membrane fusion activity. The virosomes, however, irrespective of the lipid compositions used, displayed hemolytic activity at mildly acidic pH and were able to fuse with the plasma membrane of cells with an efficiency of 0.25 that observed with intact viruses. Cell-cell fusion activity was not observed with any of the subviral components.

The results indicated that subviral components possess some of the entry properties of the intact virus.

Semliki Forest virus (SFV), a togavirus, infects baby hamster kidney (BHK-21) cells by an endocytic route (1, 2). After binding to the cell surface, the virus particles are internalized via coated pits and routed through prelysosomal vacuoles, endosomes, to the secondary lysosomes (1, 2, 3). The penetration of the viral RNA into the cytoplasm is thought to occur when the viruses enter a compartment of pH 6 or lower (1, 3). The low pH triggers fusion between the viral envelope and the vacuolar membrane, resulting in release of the nucleocapsid into the cytosol (1, 3, 4). The cell surface-binding and low pH-dependent membrane fusion activities involve the viral spike glycoproteins (4, 5, 6) whereas the adsorptive internalization is a cellular activity also responsible for fluid uptake and the receptor-mediated endocytosis of physiological ligands (2, 7).

Here we investigate the interaction with cells of particles containing the viral membrane lipids and/or the viral glycoproteins. The study has been undertaken to gain insights into: (a) the endocytic mechanisms by which cultured cells internalize adsorbed particles of various physical form, (b) the ways in which viruses use cellular endocytosis for entry, and (c) the role of viral components in the low pH-dependent fusion reaction. In addition, we have evaluated the potential of subviral components to deliver foreign molecules into the lysosomes and the membranes of living cells. The particles used were lipid-free, octameric, spike glycoprotein rosettes (8, 9), reconstituted vesicles containing the viral spike glycoproteins (virosomes) (10, 11), and protein-free liposomes (4, 12). The rosettes and virosomes were prepared using nonionic detergents, and their structure and composition have been extensively characterized in previous studies (9, 10, 11).

MATERIALS AND METHODS

Cells and Virus: BHK-21 cells were grown on 3.5-cm diameter plastic dishes (Falcon, Labware, Oxnard, CA), 24-well Linbro plates (Flow Laboratories, Hambden, CT) and 11-mm glass coverslips, with Glasgow minimal essential medium (G-MEM, Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). The cells were seeded at a density of $2.5 \times 10^5$ per cm$^2$ and used,
after 40 h, as confluent monolayers (1,2). BHK-21 cell lipids were labeled with [35S]orthophosphate for 24 h as described (10).

A prototype strain of SFV was propagated in BHK-21 cells grown in 850 cm2 roller bottles (Corning Glass Works, Corning, NY) (13). SFV, labeled with [35S]methionine, was grown as described (13) and had specific activities of ~1 x 10^6 particles per cpm. The radiolabeled components were obtained from the Radiobiological Research Center, Amersham, UK. Viral proteins in 0.1% SDS, was determined by the Lowry procedure (14) using bovine serum albumin (BSA) as a standard. A viral protein content of 57% (15) and molecular weight of 48 x 10^6 was used for calculations (B. Jacrot, personal communication). Quantities of ligand are given as micrograms of protein.

Preparation of Subvirial Components: Spike glycoprotein rosettes were prepared by sucrose gradient centrifugation after Triton X-100 solubilization of whole virus (8). For reconstituted vesicles, the SFV spikes were solubilized in β-octylglucoside (Calbiochem-Behring Corp., La Jolla, CA) and reconstituted with lipids by dialysis against 50 mM Tris pH 7.4 containing 0.1 M NaCl (TN buffer) (10). Reconstitutions were made into four different lipid mixtures: (a) egg lecithin, (b) BHK-21 cell lipid extracts (16), (c) SFV-lipid extracts (10), (d) a mixture of phosphatidylcholine, phosphatidyl ethanolamine, sphingomyelin, and cholesterol, 1:1:1:1.5 by weight (9) (purified lipids were obtained from Sigma Chemical Co., St. Louis, MO). Each milligram of spike protein, 1.5 mg of unlabeled lipid and a trace amount of [35S]-labeled BHK-21 cell lipid was used (10). After 48 h dialysis the reconstituted vesicles were fractionated on step gradients consisting of 0.5 ml of 60% sucrose, 3 ml of 30% sucrose, and 40% of the SFV bound to the cells. This was equivalent to 4 x 10^6 rosettes and 3.7 x 10^6 glycoprotein spikes in the form of virosomes (~2 x 10^9 virosomes) bound per cell.

The values are in agreement with our previous estimate of ~10^8 SFV binding sites per cell (2, 22). Binding of rosettes and virosomes was inhibited in the presence of increasing amounts of SFV (Fig. 2). Virus binding is limited by receptor number and not by accessibility (1, 2, 22). The possibility of direct interaction between ligand and virus in solution, which could explain the decreased binding, was excluded, as the ligands show no interaction when analyzed on sucrose velocity and isopycnic density gradients and by sedimentation in the presence of sucrose. The result thus indicated that viruses and subviral particles bind to the same sites on the BHK-21 cell surface.

When virostone preparations containing [35S]methionine-labeled glycoproteins and [3P]-labeled phospholipid were used, twice as much 35S-labeled associated with the cells as 3P-label, suggesting preferential binding of particles with a high protein-to-lipid ratio. To obtain a homogeneous population of virosomes, the reconstituted preparations were fractionated by isopycnic sucrose gradient centrifugation and the intermediate density fraction containing about two thirds of total 35S- and 32P-activities was collected (see Materials and Methods). The average buoyant density of the virosomes in this fraction was 1.08 g/cm^3 and the protein-to-lipid ratio 1:1.5 by weight.

RESULTS

Binding to Cell Monolayers

The binding of rosettes, virosomes, protein-free liposomes, and intact SFV to BHK-21 cells was determined. The temperature was 0°-4°C to eliminate endocytosis (2). The viral proteins were labeled with [35S]methionine, and the virosomes and the liposomes contained a trace amount of [3P]-labeled lipid to facilitate quantitation. Of the four ligands, all except the protein-free ligosomes were able to bind to the cells (Fig. 1). The rosettes and virosomes bound less efficiently than intact SFV and the binding was more explicitly dependent on a mildly acidic pH (Fig. 1). During 1 h at pH 6.4 (the pH used in most subsequent experiments) 9% of the rosettes, 27% of the virosomes, and 40% of the SFV bound to the cells. This was equivalent to 4 x 10^6 rosettes and 3.7 x 10^6 glycoprotein spikes in the form of virosomes (~2 x 10^9 virosomes) bound per cell.

Microscopy: Negative staining for electron microscopy of rosettes and virosomes was carried out on Formvar-coated copper grids; samples were stained with 2% phosphotungstic acid pH 7.2 for 5-15 s (10). Thin-section transmission electron microscopy was done as described (2). The rosettes were visualized by an immunoferritin technique using affinity purified rabbit anti-SFV antibodies and goat anti-rabbit IgG conjugated to ferritin (21).

FIGURE 1 Binding of SFV, virosomes, rosettes, and liposomes to BHK-21 cells. Radiolabeled SFV (0.01 µg/well; O), virosomes (0.5/µg/ well, ●), rosettes (10 µg/well; △), and liposomes (25 µg/well; ▼) were allowed to bind to cells, in Linbro wells (10^4 cells/well), for 60 min at 0°C in 0.25 ml of serum-free binding medium (BM) adjusted to the indicated pH with HCl. The efficient binding of SFV at neutral pH has previously been observed at high, as well as low, multiplicity of viruses over cells (22).
and SFV. After 1 h, unbound ligand was washed away and the amount of bound ligand determined as described. Values are expressed as percentages of binding measured in the absence of added virus: the multiplicity of virus per cell is given on the abscissa.

Transmission electron microscopy after negative staining revealed a population of intermediate-size vesicles (50–100 nm in diameter) with characteristic 7-nm spikes protruding from their surface (10). Small vesicles, protein aggregates and large vesicles with few spikes seen in the original mixture were not present. Digestion with thermolysin (10) followed by SDS-PAGE indicated that 90% of the spike glycoprotein in the sample was accessible to the bulk solution. When the fractionated material was added to cells, binding of both labels was identical. This virosome fraction was used in all subsequent experiments.

The binding of rosettes and virosomes to the cell surface in the cold was also studied by indirect immunofluorescence (not shown) and by transmission electron microscopy of thin sections (Fig. 3). It was found that, like SFV, the virosomes (Fig. 3a) and rosettes (not shown) bound preferentially (>60%) to the microvilli. No preferential binding was observed to coated pits, which occupy 2–3% of the BHK-21 cell surface. The virosomes on the cell surface appeared as single shelled vesicles frequently somewhat flattened, with no visible spike structures (Fig. 3a).

Adsorptive Endocytosis

The rate of internalization of rosettes, virosomes, and SFV was studied using proteolytic digestion to monitor the disappearance of bound ligand from the cell surface (2). With [35S]methionine-labeled rosettes a trace of [3H]uridine SFV was added to the same plates as an internal standard. With virosomes labeled with 35S and 32P, [35S]SFV standards were run on parallel plates. The ligands were bound to cells at 0°C, for 1 h, whereafter the cells were warmed to 37°C in an incubator. At various times individual dishes were removed, cooled to 0°C, and the medium and cells assayed for radioactivity (2). The cell-associated radioactivity was determined on duplicate plates with, or without, prior Proteinase K treatment at 0°C.

The rosettes and the virosomes were found to be internalized, but the rate was slower than that of control SFV (Fig. 4). The average residence times for the prebound rosettes and virosomes on the cell surface were 90 and 120 min, respectively, compared to 60 min for SFV. The rates of degradation of the ligands were approximately equal to that of the virus, suggesting that the intracellular phase of the endocytic pathway was not different. Fig. 4b shows, moreover, that when virosomes labeled in both lipid and protein were allowed to internalize, both components entered at similar rates. Morphological studies confirmed that the uptake followed the same pathway seen with SFV (1, 2). The virosomes (Fig. 3b and c) and the rosettes (Fig. 3f) could be observed to enter coated pits, and the virosomes were seen in coated vesicles (Fig. 3d and e). We conclude that rosettes and virosomes are internalized by adsorptive endocytosis in a fashion similar to that of intact virus, but at a markedly slower rate.

In our previous studies on SFV-uptake in the same cell line, and with the same relatively slow but synchronous warming method, we measured considerably faster rates of internalization than those observed here (2). The reason for the apparent discrepancy was found to be a pH effect (Fig. 5a). At pH 6.8 the rate of uptake was about double that observed at pH 6.4. There was also a difference in the rate of degradation, reflecting the lower rate of endocytosis (Fig. 5b). The overall pinocytic activity was, however, unaffected by the pH as judged by the uptake of two fluid phase markers, [3H]sucrose and horseradish peroxidase (Fig. 5c). The reason for the pH effect on virus uptake is not clear but may reflect an alteration in virus attachment to the cell surface at lower pH.

Membrane Fusion

The fusion activity of SFV can be demonstrated experimentally as virus fusion with liposomes (4), as virus fusion with the plasma membrane of cells (21), as virus-induced cell-cell fusion (18), and as virus-induced hemolysis (19, 20, 23). It has been shown that the spike glycoproteins are involved in the fusion reaction (4) but it is not known whether they alone are sufficient for fusion. The test systems chosen to demonstrate fusion activity in rosettes and virosomes were (a) cell-cell fusion in BHK-21 cell monolayers—a semiquantitative assay for the ability of a ligand to fuse two separate target membranes (18), (b) hemolysis—a qualitative but highly sensitive assay for fusion activity of viruses and virus components (19, 20), and (c) fusion of the spike glycoproteins into the plasma membrane—a reaction which can be quantitated using Proteinase K (21).

**CELL-CELL FUSION:** No detectable fusion at acid or neutral pH occurred when SFV spike glycoproteins were bound to cells in the form of rosettes or virosomes; only the intact viruses had cell-cell fusion activity. The equivalent of five times the amount of SFV needed to fuse a BHK-21 cell monolayer to a single polykaryon (18) was used in each case without effect.

**HEMOLYSIS:** It has been shown that SFV damaged by sonication, or by freezing and thawing, has hemolytic activity at low pH (19, 20, 23) and that rosettes lack this activity (19). We confirmed the latter result (Fig. 6) and, in addition, tested the activity of virosomes reconstituted with phosphatidylcholine or with extracted BHK-21 cell lipids. Fig. 6 shows that, unlike the rosettes, both types of virosomes are hemolytic but only when the pH is reduced to 6 or below.

**FUSION WITH THE PLASMA MEMBRANE:** For a quantitative fusion assay we used the observation that SFV, bound to the plasma membrane, is removed by Proteinase K at 0°C, whereas the virus spike glycoproteins, introduced into the
plasma membrane by fusion, are not (21). SFV, virosomes and rosettes (all 35S-labeled) were bound to cells, and the unbound ligand was removed. The cells were briefly (0.5–1 min) incubated with pH 5.0 or 5.5 media at 37°C and subsequently returned to pH 6.4 media 0°C and treated with Proteinase K. We found that ~50% of the virus 35S activity remained cell-associated in pH 5.5-treated cells (Table I). An average of <1% of the rosettes remained cell-associated, whereas with the virosomes 10–16% of the 35S activity remained cell-associated.

At 37°C, in contrast to 0°C, Proteinase K removes the external portion of the SFV spike glycoproteins (21). When native [35S]methionine-labeled virus and virome preparations were digested with Proteinase K at 37°C, 34 and 20% of the activity, respectively, remained resistant. This protected activity is the capsid protein in the virus and the COOH-terminal pieces of the spike glycoproteins left in the membrane (21, 24).

After fusion of SFV, or virosomes, with BHK-21 cells and Proteinase K treatment at 37°C, 33 and 20% of the fused activities respectively remained cell-associated. The good agreement between the protected protein in the starting prep-

![Figure 4 Internalization of SFV, virosomes, and rosettes. [35S]methionine- and [32P]phospholipid-labeled virosomes (3.5 μg/well), [35S]methionine-labeled SFV or 35S-labeled rosettes (5.0 μg/well) were bound to BHK-21 cells in Linbro wells at 0°C in BM pH 6.4 for 60 min. The unbound ligand was washed away, and the cells were overlaid with fresh medium and placed at 37°C. At the indicated times the cells were assayed for 35S-acid-soluble activity in the medium (A) and cell-associated Proteinase K-resistant (internalized) 35S-activity (O or O). (A) [35S] SFV. (B) Virosomes; internalized [32P] phospholipid (C) and [35S]methionine (O) activity, 35S-acid-soluble activity (A); degraded 32P-activity does not appear in the medium (45). (C) [35S] rosettes.

![Figure 5 Endocytosis and degradation of SFV, and fluid phase endocytosis at pH 6.8 and pH 6.4. [35S]methionine SFV was bound to BHK-21 cells in pH 6.8 (A) or pH 6.4 (B) binding medium (BM) for 1 h at 0°C. Unbound ligand was washed away, and the plates were overlaid with 0°C BM (pH 6.8 or 6.4) and placed at 37°C for the indicated times. Thereafter, cells and media were analyzed for the viral activity as described. (A) The proportion of bound SFV internalized during 90 min at pH 6.8 (A) or pH 6.4 (O). (B) The proportion of bound activity which appears as degraded acid-soluble activity in the medium. (C) Accumulation of [3H]sucrose into cells. Sucrose was made up in BM pH 6.8 (A) or pH 6.4 (O). Similar results were obtained using horseradish peroxidase.

![Figure 3 Binding of virosomes and rosettes to BHK-21 cells. In (a) virosomes (4 μg per 11-mm coverslip) were bound to cells for 1 h at 0°C; the unbound ligand was removed by washing and the cells were fixed without washing. Virosomes are seen mainly, but not exclusively, associated with microvilli. In b, c, d, and e, the cells were warmed to 37°C for 1 min before fixation. The virosomes are seen within coated pits and coated vesicles. In some cases several virosomes occur in the same pit. The arrows in (a) and (b) show virosomes flattened to increase the contact area with the cell surface. In (f) rosettes (4 μg/11-mm coverslips) were bound at 0°C for 1 h; unbound ligand was washed away and the cells were warmed to 37°C for 1 min before fixation. Bound ligand was localized using anti-SFV antibodies and immunoferritin. The rosettes, localized by the ferritin grains, are seen associated with coated pits. Bar, 0.3 μm, × 100,000.
virosomes may have practical consequences. Quantitative de-
terminations indicate that up to $2 \times 10^5$ virus spike proteins
be inserted into a lipid bilayer. A critical de-
pendence on the lipid composition for fusion was not detected.

Characteristic structural features of the intact virus such as the
presence of a nucleocapsid, a high density and geometrically
defined arrangement of spike glycoproteins (46, 47, 48) and the
relative rigidity in the lipid bilayer (49) are not, therefore,
absolute requirements for SFV entry and fusion. We found,
however, that the binding, internalization, and fusion of sub-

| Ligand | $^{35}$S-Activity bound | Proteinase K-resistant $^{35}$S-activity after incubation at pH 5.5* |
|--------|-------------------------|---------------------------------------------------------------|
| SFV    | 18.5 (1.80)             | 6.9 (37)                                                      |
| Rosettes | 27.3 (1.50)           | 14.9 (46)                                                    |
| Virosomes | 5.5 (0.40)           | 0.02 (0.4)                                                   |
|        | 5.2 (0.17)             | 0.12 (2.0)                                                   |
|        | 53.6 (0.45)            | 5.4 (10)                                                     |
|        | 74.2 (0.58)            | 11.6 (16)                                                    |

Ligands were bound to cells at pH 6.4 in BM for 1 h. The pH 5.5 incubation
was done for 60 s at 37°C. The results for two experiments are given.

Virosomes were reconstituted with BHK-21 cell lipids.

* Background radioactivity, proteinase K-resistant activity after “mock fusion”
at pH 6.4, is removed: in all cases, the backgrounds were <10% of bound
activity. Values in parentheses indicate the amount of ligand and percentage
of original bound activity.

Our results demonstrate that the SFV subviral particles mimic many of the key functions involved in virus entry. The minimal requirement for endocytosis, and intracellular trans-
port to the lysosomal compartment, seems to be the attachment
of the viral spike glycoproteins to the cell surface. The low pH-
dependent fusion reaction, however, requires that the spike
glycoproteins be inserted into a lipid bilayer. A critical de-
pendence on the lipid composition for fusion was not detected.

Characteristic structural features of the intact virus such as the
presence of a nucleocapsid, a high density and geometrically
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relative rigidity in the lipid bilayer (49) are not, therefore,
absolute requirements for SFV entry and fusion. We found,
however, that the binding, internalization, and fusion of sub-

In summary, the fusion studies indicated that rosettes do not
have membrane fusion activity, nor do they interact irreversibly
with the plasma membrane when the pH is lowered to values
which cause fusion of intact viruses and virosomes with the
cells. The virosomes, on the other hand, display low pH-
duced hemolytic activity and fuse with the plasma membrane
of cells. The efficiency of fusion is, however, only about 0.25
that observed with equivalent amounts of intact virus. In
addition, the fusion potential of the virosomes was not influ-
enced by the lipid compositions used in these reconstitutions.

**DISCUSSION**

Rosettes and virosomes have been prepared from a variety of
enveloped animal viruses including influenza viruses (25–27),
rhabdoviruses (28, 29), paramyxoviruses (30–34), and togavi-
ruses (9, 10, 35–37). The interest in these particles is based on
their potential use as subunit vaccines (38, 39), as vehicles for
 verschiedenen with the SFV system is exceptionally well suited for
the basis of protease sensitivity, we know that the structures
serve as receptors are proteins. The efficiency of binding,
in terms of the proportion of free ligand bound, was different
for the three ligands and, although binding increased with
decreasing pH for all, SFV showed a significantly higher
binding efficiency at pH 7.0. This may, in part, be explained
by differences in valency; the rosettes have only a few spikes
accessible for binding, whereas the virosomes and the viruses
have 50 to 200 and can form multiple receptor-spike interac-
tions to increase the affinity of binding. Our previous data with
lymphoblastoid cells indicate that the number of binding sites
for rosettes is larger than for viruses but the apparent affinity
is two to three orders of magnitude lower (22). The present
data indicate that the SFV subviral particles have an interme-

tiate binding affinity.

Differences in the endocytic uptake of prebound ligands
were also observed. The virosomes were internalized more
slowly than either the rosettes or intact viruses. The rate of

Thus, future investigation of the fusion mechanism can focus
on the spike glycoprotein. In addition, the fusion activity of
virosomes may have practical consequences. Quantitative de-
terminations indicate that up to $2 \times 10^5$ virus spike proteins
can be inserted into the cell plasmalemma using virosome
fusion and indicate the feasibility of using virosomes to introduce specific membrane proteins and lipids into the plasma membrane of living cells. We have previously shown that vesicles which contain both the SFV spike glycoproteins and other isolated integral membrane proteins can be prepared by simultaneous reconstitution using β-D-octylglucoside dialysis, yielding asymmetric vesicles with the ectodomains of the proteins oriented almost exclusively outward (50). The fusion of such vesicles with the plasma membranes of cells should result in the introduction of protein components in the same orientation as the glycoproteins in the plasma membrane. However, the internal volume of the virosomes, as prepared here, is <1% of the total volume of a reconstitution mixture which may limit the potential usefulness of virosomes for the introduction of content components into cells. In addition, the data presented here show that cells will endocytose virosomes and deliver them to the lysosomes. Exploitation of the low pH within these organelles (51) and the fusion properties of the virosomes may enable delivery of the virosome contents to the cytosol or membrane components to vacuole membranes.

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