DIRECT CELL-TO-CELL TRANSMISSION OF VESICULAR STOMATITIS VIRUS

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SUMMARY
Vesicular stomatitis virus (VSV) infection of kidney-derived, LLC-PK1 epithelial cells resulted in the budding of new viral particles into the basolateral space of the cultures. In lateral regions where cells were in close apposition, the majority of assembling viral particles in the process of budding from the producing cell had their apex already engaged in clathrin-coated pits of the neighbouring cell surface. These observations suggest that the viral envelope–plasma membrane interaction triggers the focal formation of clathrin-coated pits; they also show how VSV infection could spread throughout a tissue with only minimal exposure to a host's extracellular environment.

INTRODUCTION
Cellular infection by enveloped viruses starts by the binding of viral particles to the plasma membrane, followed, in most cases, by receptor-mediated endocytosis through clathrin-coated pits and vesicles (Marsh, 1984). After intracellular replication of the viral genome, and synthesis and transport of viral proteins, new viral particles are released by budding from the plasma membrane (Rodriguez-Boulan, 1983; Simons & Fuller, 1985); these newly formed viruses can in turn infect additional cells. We report here that vesicular stomatitis virus (VSV) particles budding from the basolateral surface of epithelial cells in monolayer culture are engaged into clathrin-coated invaginations of neighbouring cells before their complete release from the producing cells. This association between budding viruses and coated membrane segments, which appears to be the consequence of a non-random process, suggests a possible mechanism for the direct cell-to-cell transmission of enveloped viruses.

MATERIALS AND METHODS
Pig kidney-derived LLC-PK1 cells (Hull et al. 1976) (D+Sc clone; Wohlwend et al. 1986) were grown in Eagle's minimum essential medium (MEM) (Gibco Laboratories, Grand Island, NY) supplemented with 110 units ml⁻¹ penicillin (Pfizer, Zurich), 110 μg ml⁻¹ streptomycin (Streptothelin, Grunenthal, Bern), 1.2 mg ml⁻¹ NaHCO₃ and 5% foetal bovine serum (FBS) (Gibco Laboratories). Stock cultures were seeded at 0.3 × 10⁴ to 1 × 10⁴ cells cm⁻² in 100 mm tissue culture Petri dishes (Falcon Plastics, Division of Bioquest, Oxnard, CA), and incubated at 37°C, 3.5% CO₂ in air. For passage, cells were washed once with phosphate-buffered saline.

Key words: LLC-PK1, coated pits, clathrin.
(PBS), and dispersed with 0.5% trypsin (Difco Laboratories, Detroit, MI) containing 3 mM-(ethylenedinitrilo)tetraacetic acid.

For infection, cultures were established in 35 mm tissue culture Petri dishes and used 2–3 days later, when reaching confluency. The cultures were washed twice in PBS containing 2 mg ml⁻¹ bovine serum albumin (BSA), and infected for 1 h at 31°C with VSV (MVOD and Summers Indiana Strain, kindly provided by Dr L. Roux) at a dose of 10⁸ plaque-forming units per Petri dish, in PBS+2 mg ml⁻¹ BSA. After washing with PBS+2 mg ml⁻¹ BSA, the cultures were incubated at 37°C for 8 h in MEM supplemented with 0.1% FBS and 1 mg ml⁻¹ BSA. At the end of the incubation, the cells were washed in PBS+2 mg ml⁻¹ BSA and fixed for 30 min with 2.5% glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4. After washing with cacodylate buffer, the cultures were postfixed in 1% osmium tetroxide, stained en bloc with 2.5% uranyl acetate in 50% ethanol, dehydrated in a graded series of ethanol and embedded in situ in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM300 electron microscope.

For the immunocytochemical localization of clathrin, infected cultures were fixed with 1% cacodylate-buffered glutaraldehyde and processed for Lowicryl K4M low-temperature embedding (Armbruster et al. 1982). Thin sections picked on nickel grids were floated overnight at 4°C on drops of affinity-purified anti-clathrin immunoglobulin G (IgG) (lot 3-124, kindly provided by Dr D. Louvard) (Louvard et al. 1983). After washing, sections were incubated with protein A–gold (Roth et al. 1978) for 1 h at room temperature, washed again, and stained with uranyl acetate and lead citrate.

**RESULTS**

Monolayer cultures of pig kidney-derived LLC-PK1 epithelial cells were exposed to VSV for 1 h. After extensive washing, the cultures were further incubated for 8 h, fixed, and processed for electron microscopy. As expected from previous reports on VSV infection of polarized epithelial cells (Rodriguez-Boulan & Sabatini, 1978; Lombardi et al. 1985), viral release from infected cells was almost exclusively directed towards the basolateral space. Free viral particles were readily observed between the basal plasma membrane and the tissue culture dish, and in dilated regions of the lateral space. However, in those lateral regions where cells were in close apposition, a large number of viral particles in the process of budding (i.e. whose limiting membrane was in continuity with the plasma membrane of the infected cell) had their apex already engaged in invaginated regions of the surface of the neighbouring cell (Figs 1, 2); the cytoplasmic side of the plasma membrane limiting these pit-shaped invaginations was decorated by a coat, morphologically indistinguishable from that seen on coated pits and vesicles involved in receptor-mediated endocytosis (Goldstein et al. 1979) (Figs 1, 2). More than 60% of viral particles budding in narrow lateral extracellular spaces were associated with such invaginations (Table 1). Budding particles that did not appear in intimate contact with the neighbouring cell in the plane of the section were often associated with a coated plasma membrane segment in a consecutive serial section. Thus, our quantification represents a lower limit, and we conclude that the phenomenon we describe concerns a large majority, if not all, of viruses budding in closely apposed lateral regions within the cell monolayer. Viral particles were also observed in endosomes, multivesicular bodies and lysosomes located close to the lateral plasma
membrane (Fig. 1, insert); thus, the interaction between budding viral particles and coated membrane segments may result in endocytosis of the virus, and it could therefore contribute to the spread of the infection.

The length and number of coated regions along the apical and lateral plasma membrane domains were quantified (Table 2). Coated pits were increased, in particular in the lateral regions of the cells, in VSV-infected as compared to uninfected cultures; this suggested that the presence of viral particles might be
responsible for local assembly of the coat. Finally, the presence of clathrin in the plasma membrane-associated coat was demonstrated by immunocytochemistry: an anti-clathrin antibody, revealed by the protein A–gold method, specifically labelled the cytoplasmic side of the membrane segments surrounding infecting viral particles (Fig. 2).

Fig. 2. VSV budding in lateral regions of LLC-PK1 cells in monolayer culture: a gallery. E, Immunocytochemical localization of clathrin using the protein A–gold method. Gold particles selectively label the cytoplasmic coat of the coated invagination. A, ×29 200; B, ×39 150; C, ×58 300; D, ×79 200; E, ×64 000.
Table 1. Budding VSV particles engaged in coated pits

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<tr>
<th>Lateral membrane analysed* (µm)</th>
<th>Budding VSV† engaged in coated pit</th>
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<td>1117</td>
<td>278</td>
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* Only lateral regions with an intercellular space not exceeding the length of a VSV particle were included in the analysis.
† Budding viral particles sectioned along their longitudinal axis and showing a clear continuity with the producing-cell surface in the plane of the section were included in the analysis.

**DISCUSSION**

We have shown here that budding VSV particles become associated with discrete clathrin-coated membrane domains of neighbouring cells before their release from the producing cell, and that this interaction can lead to endocytosis of the virus. The high degree of coincidence between budding viruses and clathrin-coated pits cannot be the consequence of random, unrelated events; indeed, coated segments represented only some 0.5-5% of total plasma membrane surface area (see also Anderson et al. 1976; Orci et al. 1978; Goldstein et al. 1979), whereas at least two-thirds of viruses budding in closely apposed regions of the lateral domain of LLC-PK1 cells were associated with such coated segments. Thus, viral budding and coated pits must be somehow related. The presence of a coated membrane segment on a neighbouring cell could restrict the prospective site of viral budding to a region facing this coated segment; alternatively, viral proteins expressed on the surface of the infected cell could be recognized by binding sites on the ‘recipient’ cell plasma membrane, and this recognition could trigger the focal assembly of the coat. In view of the increased number of coated pits along the lateral cell membranes in infected cultures, and of the well-documented involvement of coated membrane segments in cellular infection by free VSV (Marsh, 1984), we believe that the coated pits described here are a consequence, rather than a cause, of viral budding and attachment. Since viral budding must impose a degree of spatial constraint on localization of this ligand, and thereby probably prevents its migration from a site of

Table 2. Effect of VSV infection on coated pits of LLC-PK1 cells

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<tr>
<th>% Membrane length occupied by coated pits</th>
<th>Number of coated pits/mm of membrane</th>
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<tr>
<td>Apical</td>
<td>Lateral</td>
</tr>
<tr>
<td>Non-infected cells</td>
<td>0.49 ± 0.12</td>
</tr>
<tr>
<td>VSV-infected cells</td>
<td>0.98 ± 0.12</td>
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The length of the apical and lateral plasma membranes, and the length and number of coated pits were measured on electron micrographs of sections cut perpendicularly to the substratum, using a graphic tablet connected to an IMSAI microprocessor. At least 1500 µm of membrane was evaluated for each condition. Results are expressed as the mean ± standard error of three determinations carried out on different regions of the monolayers.
first attachment into pre-existing coated pits, we conclude that coated pits form in response to ligand–receptor interaction, at the site of this interaction.

A recent paper has described the engagement of budding retroviruses into coated pits of adjacent AtT20 cells (Tooze, 1985). In this case, however, many viral buds appeared to remain connected to the surface of the ‘parental’ cell, and endocytosis did not go to completion. No such ‘blocked’ structures were observed in our studies; differences in the cell and, or, virus types, possibly related to the phenomenon of viral interference (Weiss, 1982), may account for the differing results. Nevertheless, the association of budding viruses with coated pits in this system also supports our contention that the formation of coated pits is triggered by a ligand–receptor type interaction.

Whatever the precise mechanism of the phenomenon reported here may be, our observation suggests how a viral infection can spread throughout a cultured epithelium without VSV particles being released free into the extracellular space. This hitherto unrecognized mode of propagation of VSV, and perhaps of similar enveloped viruses also, throughout a tissue may significantly limit exposure of the virus to the host’s extracellular environment in vivo. Thus, the successful containment of such viral infections must rely on the immune attack of infected cells and on mechanisms involving direct cellular resistance to infection, for instance those elicited by the interferons.

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REFERENCES


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