Assembly of Enveloped Viruses in Madin-Darby Canine Kidney Cells: Polarized Budding from Single Attached Cells and from Clusters of Cells in Suspension

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ABSTRACT In confluent monolayers of the dog kidney epithelial cell line Madin-Darby canine kidney (MDCK) assembly of RNA enveloped viruses reflects the functional polarization of the cells. Thus, influenza, Sendai, and Simian virus 5 bud from the apical (free) surface, while vesicular stomatitis virions (VSV) are assembled at basolateral plasma membrane domains (Rodriguez-Boulan, E., and D. D. Sabatini, 1978, *Proc. Natl. Acad. Sci. U.S.A.*, 75:5071–5075). MDCK cells derived from confluent monolayers by dissociation with trypsin-EDTA and maintained as single cells in spinner medium for 12–20 h before infection, lose their characteristic structural polarity. Furthermore, when these cells were infected with influenza or VSV, virions assembled in a nonpolarized fashion over most of the cell surface. However, when dissociated MDCK cells infected in suspension were sparsely plated on collagen gels to prevent intercellular contact and the formation of junctions, the characteristic polarity of viral budding observed in confluent monolayers was again manifested; i.e., VSV budded preferentially from adherent surfaces and influenza almost exclusively from free surface regions. Similar polarization was observed in cells which became aggregated during incubation in spinner medium: influenza budded from the free surface, while VSV was produced at regions of cell-cell contact. It therefore appears that in isolated epithelial cells attachment to a substrate or to another cell is sufficient to trigger the expression of plasma membrane polarity which is manifested in the asymmetric budding of viruses.

The function of epithelia as selective permeability barriers between different extracellular compartments requires mechanisms for regulating the passage of diffusible substances along a paracellular transport route. This regulation is effected by tight junctions (zonulae occludentes), which are beltlike regions of intercellular contact located near the apical surface of the cells, where adjacent plasma membranes are engaged in multiple linear fusions (c.f. references 5–7). Tight junctions are also thought to be essential for maintaining the functional polarization of epithelial cells by preventing the intermixing of plasma membrane components, such as surface enzymatic and transport activities, which must be segregated exclusively to apical or basolateral domains.

Evidence for a role of tight junctions in preserving the characteristic composition of the two membrane domains has been obtained from studies demonstrating a redistribution of cell surface markers after cells are dissociated from native epithelia (22, 28) or cultured monolayers (11) and from determinations of the extent to which fluorescent probes inserted in the plasma membrane are transferred from one surface of an epithelial layer to the other (3). It is difficult, however, to evaluate from experiments involving cell dissociation the relative contribution of the junctions to the polarized distribution of plasma membrane markers, since in most instances normal cell-cell and cell-substrate interactions are disrupted simultaneously with the junctions.

Cultured epithelial cell lines, such as the Madin-Darby canine kidney (MDCK) (14), provide useful model systems for studying the development of epithelial polarity and the role of tight junctions in determining and maintaining the polarized distribution of cell surface components (1, 2, 10, 12, 20, 23). We have shown that when polarized monolayers of epithelial cells are infected with enveloped RNA viruses, the distribution of budding virions on the plasma membrane reflects the polar-

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 MARCH 1983 866-874

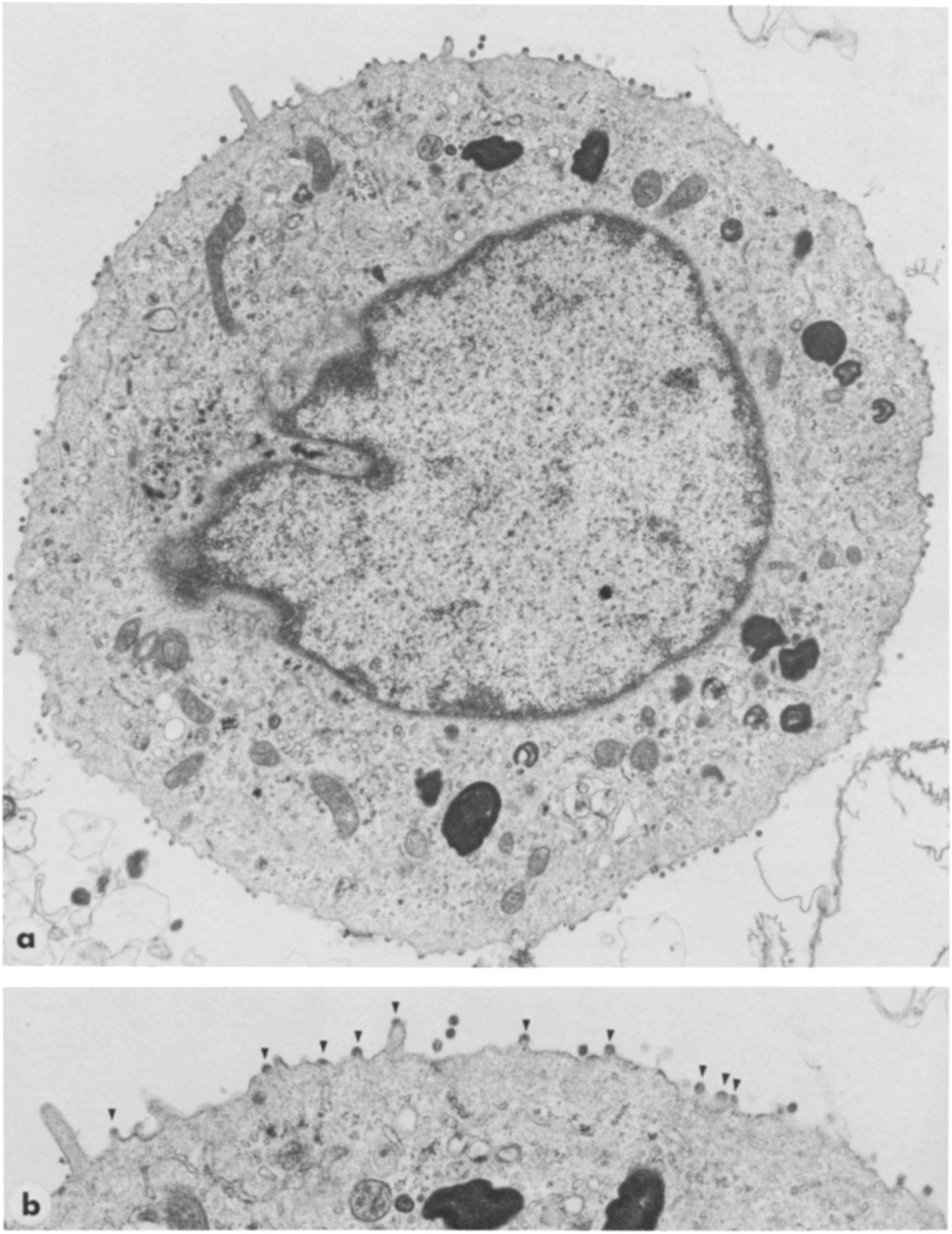


FIGURE 1 Budding of influenza virions from isolated MDCK cells. MDCK cells, dissociated with trypsin-EGTA and maintained in spinner culture overnight were infected with influenza virus at a MOI = 10, incubated in suspension for 8 h, and fixed. (*a*) Note virions budding from most of the surface of a cell in suspension. Higher magnification of the periphery of the cells (arrowheads) point to budding virions. (*a*) × 15,500. (*b*) × 25,000.

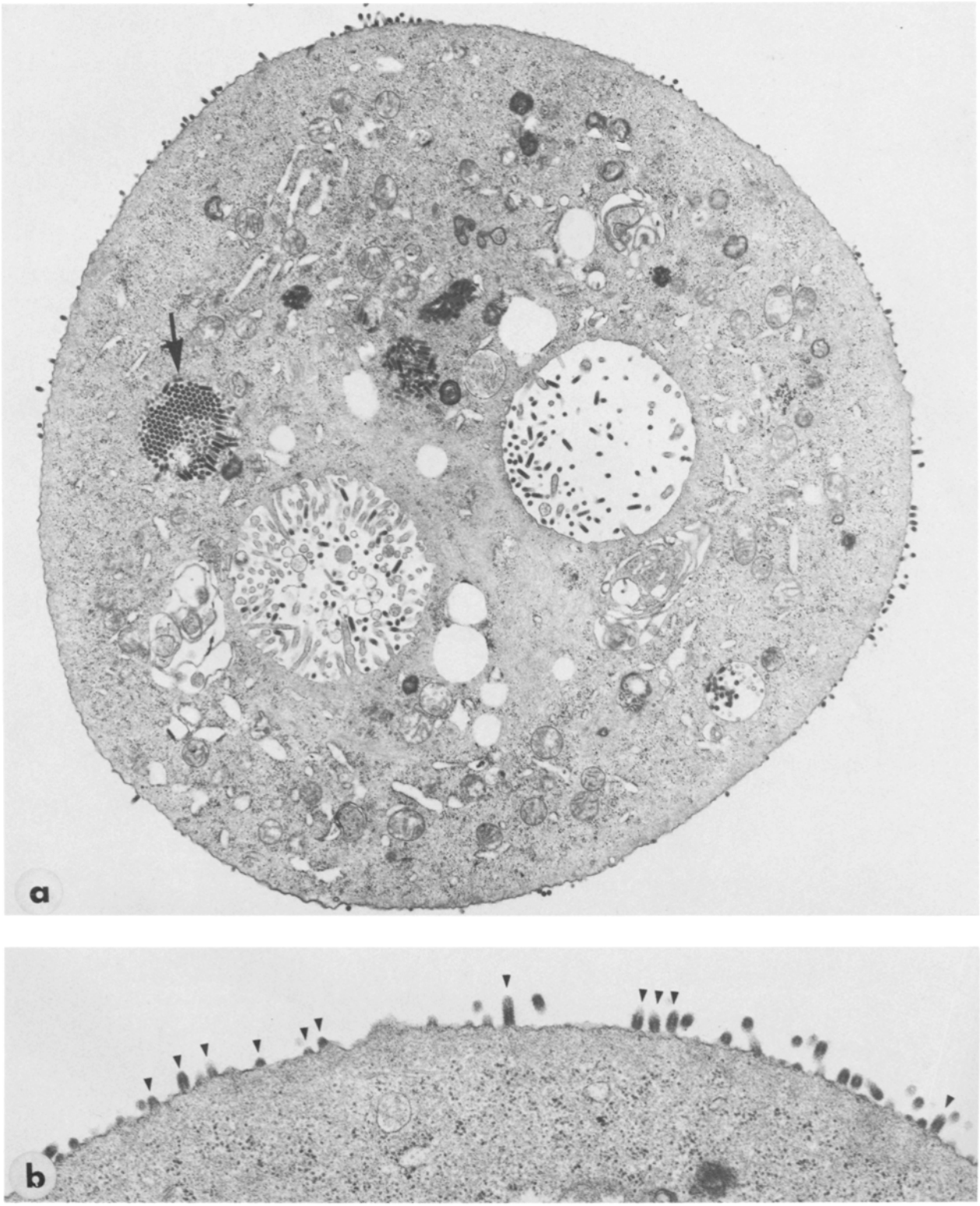


FIGURE 2 Budding of vesicular stomatis virions (VSV) from isolated MDCK cells. Dissociated MDCK cells were infected with VSV at a MOI = 10 and incubated in suspension for 8 h before fixation. (*a*) Budding virions are observed over the entire surface of an isolated cell, but are especially concentrated in two plasma membrane regions. The one on the right-hand side of the cell is represented at a higher magnification in *b*. Virions also bud and accumulate into two large intracellular vacuoles which are surrounded by a filamentous network. Packed virions may form para-crystalline structures (arrow) within smaller vesicles in the cytoplasm. (*a*) × 12,500. (*b*) × 34,000.

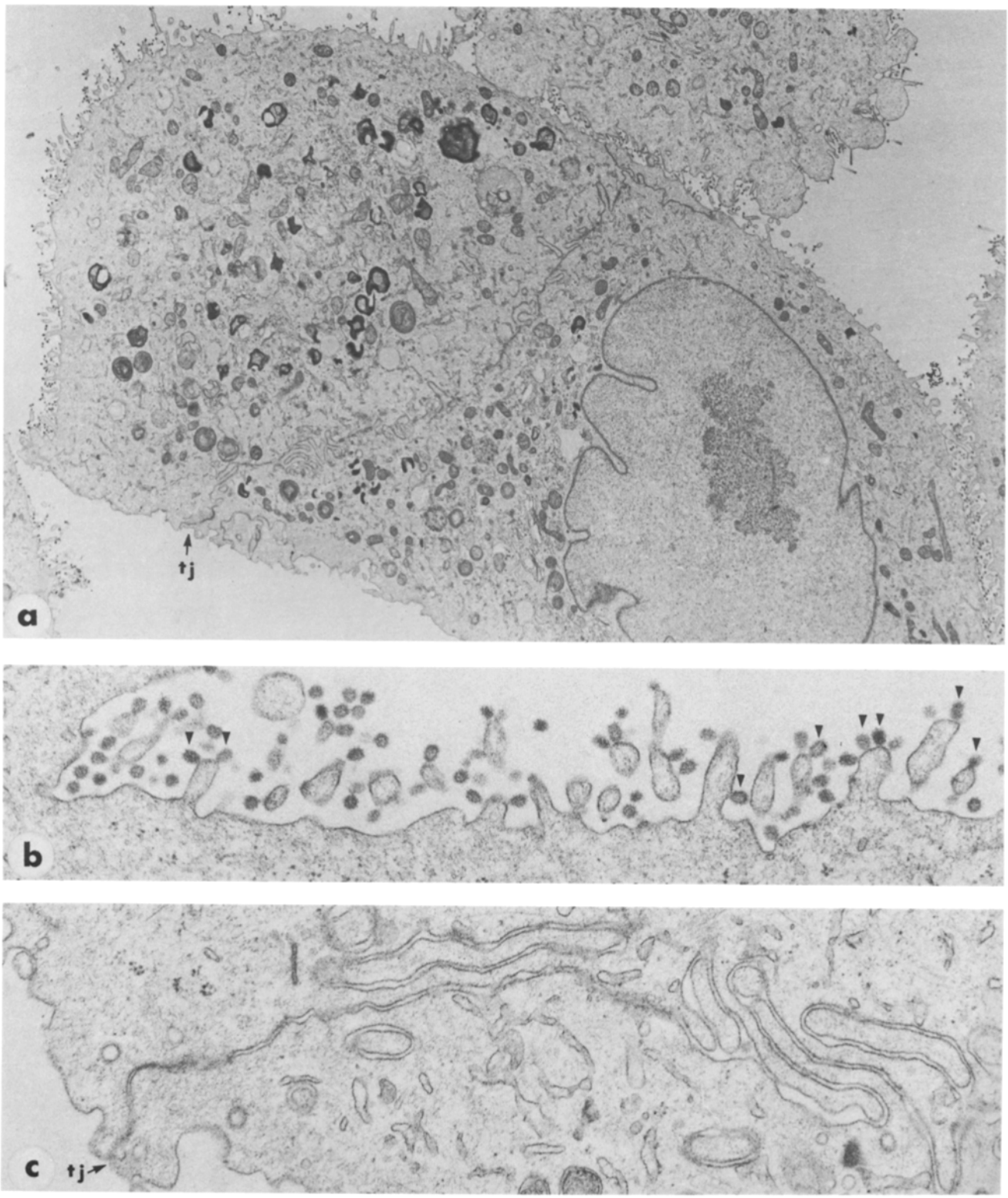


FIGURE 3 Budding of influenza virus from aggregated MDCK cells. Some MDCK cells maintained in suspension form aggregates of two or more cells. (*a*) Influenza virions budding from the free surface of a cluster of two cells. Plasma membrane domains facing intercellular space are delimited by tight junctions (*tj*). Other single cells showing budding influenza virions from their surface are partially represented in this field. These nonpolarized cells have co-sedimented with the clusters to which they are loosely attached. × 8,000. (*b* and *c*) Portions of free and adherent domains, respectively, of cells in the same aggregate. Arrowheads (*b*) point to budding virions. × 37,000.

ized nature of the cell (25). Thus, influenza, sendai, and simian virus 5 particles are assembled exclusively on apical domains, while vesicular stomatitis virus (VSV) particles are produced from basolateral surface regions. In this work, we have used virally infected MDCK cells to study the role of tight junctions in generating epithelial polarity. We found that the polarized state, which is reflected in the asymmetric budding of viruses, can be generated in isolated cells which are attached to a substrate and lack intercellular junctions, as well as in cells which form aggregates in suspension and develop junctions but are not attached to a solid support.

MATERIALS AND METHODS

MDCK cells were grown as previously described (24, 25). Confluent monolayers were dissociated by treatment with 0.25% trypsin, 10 mM EGTA. Cells resuspended in minimal essential medium (S-MEM) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) with 10% dialyzed fetal calf serum (FCS) were maintained overnight as suspension cultures (10^6 cells/ml) under constant magnetic agitation in siliconized spinner flasks (Bellco Glass, Inc., Vineland, NJ). Typically, 50–75% of the cells remained viable the following day, as determined by dye exclusion.

After 15–18 h of spinner culture cell suspensions were filtered through a nylon mesh to remove aggregates and aliquots were inoculated (1 h, 37°C) with VSV (Indiana serotype, obtained from Dr. J. Vilcek, New York University School of Medicine) or influenza (WSN strain, obtained from Dr. P. Palese, Mt. Sinai School of Medicine) at a multiplicity of infection (MOI) of 5–10 plaque forming units/cell (PFU/cell). Virus stocks were prepared and infectivity titers determined as described before (24). 1 h after infection, the cells were sedimented by centrifugation in a table-top centrifuge and resuspended in the same volume of medium to remove most of the free virus. Half of each sample was then maintained in suspension culture and the other half plated on collagen-coated glass cover slips (24) at a density of about 10,000 cells/cm^2. Uninfected control cells were processed similarly. The density of plating, which is considerably lower than the density at confluency (1.5×10^6 cells/cm^2), was chosen to minimize cell-cell interactions necessary for the formation of intercellular junctions. At different times, control or infected cells in suspension were sedimented and fixed (4°C, overnight) as a pellet with 2% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4. Cells plated on collagen were fixed for 5 min, scraped with a razor blade, sedimented in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY) and processed thereafter as a pellet. Samples were prepared for electron microscopy as described elsewhere (24, 25) and sections were examined on Phillips EM 301 or JEOL 100CX electron microscopes.

RESULTS

When MDCK cells dissociated from confluent monolayers by trypsin-EGTA were maintained overnight as suspension cultures in spinner medium, some cells remained isolated while others formed aggregates. The isolated cells were usually rounded and, although occasional microvilli were present at their surface, no morphologically distinct domains could be identified in the plasma membrane (Figs. 1 and 2). On the other hand, cells which formed clusters were joined by complete tight junctions located near their free surface (Figs. 3 and 4). Two plasma membrane domains could therefore be recognized in these cells, a free one with abundant microvilli and an interior one which frequently showed membrane interdigitations (Fig. 3). The orientation of MDCK cells within the clusters is the same as that described for thyroid (4, 21) and mammary (16) cells, which, in suspension, form spherical vesicles with apical plasma membrane domains delimited by junctions facing the medium.

In cells that were infected and maintained in suspension, viral assembly reflected the different state of polarization of the plasma membrane. Cells that remained single after infection produced VSV or influenza virions from the entire cell surface without any apparent polarity (Figs. 1 and 2). The distribution of budding virions, however, was not uniform and assembly seemed to take place preferentially within patches of membrane. In the case of cells infected with VSV, abundant budding into microvilli-rich large intracellular vacuoles was frequently observed (Fig. 2). Occasionally, VSV virions were also found tightly packed within intracellular vacuoles, sometimes forming paracrystalline arrays (Fig. 2). In contrast, polarity of viral budding was evident in cells which had formed aggregates (Figs. 3 and 4). While influenza virions budded from the free surface of the clusters, VSV particles preferentially emerged from regions of the plasma membrane facing the intercellular spaces, where they accumulated.

When sparsely plated on a collagen coated surface, MDCK cells infected in suspension became attached and spread as normal cells, acquiring a characteristic dome-shaped profile (Fig. 5). After 5–6 h, most normal or infected cells were still isolated (Fig. 5 *d*) or only partially in contact with neighboring cells, through regions where incomplete tight junctions could be identified by electron microscopy (Fig. 5 *a*). A striking characteristic of the attached cells, whether single or in contact with other cells, was their structural polarization. Typical microvilli were present on the free surface, and in the basal region of the cell adhesion plaques were apparent in areas where the plasma membrane was closely apposed to the substrate. Cytoplasmic filaments were usually found near the basal region, frequently forming peripheral bundles which delimited the area of attachment (Fig. 5 *a–c*). Even though tight junctions were either absent or incomplete in the sparsely plated cells, the distinct character of the free and adherent plasma membrane regions and their functional equivalence to the luminal and basolateral domains characteristic of cells within confluent monolayers were manifested after viral infection. The polarity of viral budding was clearly expressed in the attached cells (Fig. 5), although not as stringently as in confluent monolayers; influenza virions predominantly emerged from the free surfaces (Fig. 5 *a–c*) and VSV from regions adjacent to the collagen support (Fig. 5 *d–g*). Only occasionally were VSV virions observed budding from free surfaces and even less frequently patches of budding influenza virions were seen at the adherent face.

DISCUSSION

We observed that, after dissociation from confluent monolayers with trypsin-EGTA, polarized MDCK cells maintained in suspension lose their structural and functional polarization. No morphologically distinct plasma membrane domains could be detected in such cells and, when infected, they produced VSV and influenza virions indiscriminately from the entire cell surface. Recently, Roth and Compans (26) have also mentioned that the polarity of viral budding is abolished when, after trypsinization, MDCK cells doubly infected with influenza and VSV are maintained in spinner culture for 7 h. These observations are in agreement with previous reports demonstrating that the polarized distribution of plasma membrane markers is lost when cells are dissociated from native epithelia. Thus, apical markers of toad bladder epithelial cells redistributed uniformly in the plasma membrane following cell dispersal in Ca^{++}-free medium with EDTA (22). Similarly, the brush border enzymes leucine aminopeptidase and alkaline phosphatase were found to be homogeneously distributed on the surface of isolated intestinal cells (28).

Treatment with EGTA, which leads to the fragmentation of tight junctions, has also been reported to abolish the difference in the concentration of intramembranous particles detected by freeze-fracture (17) in apical and basolateral plasma membrane

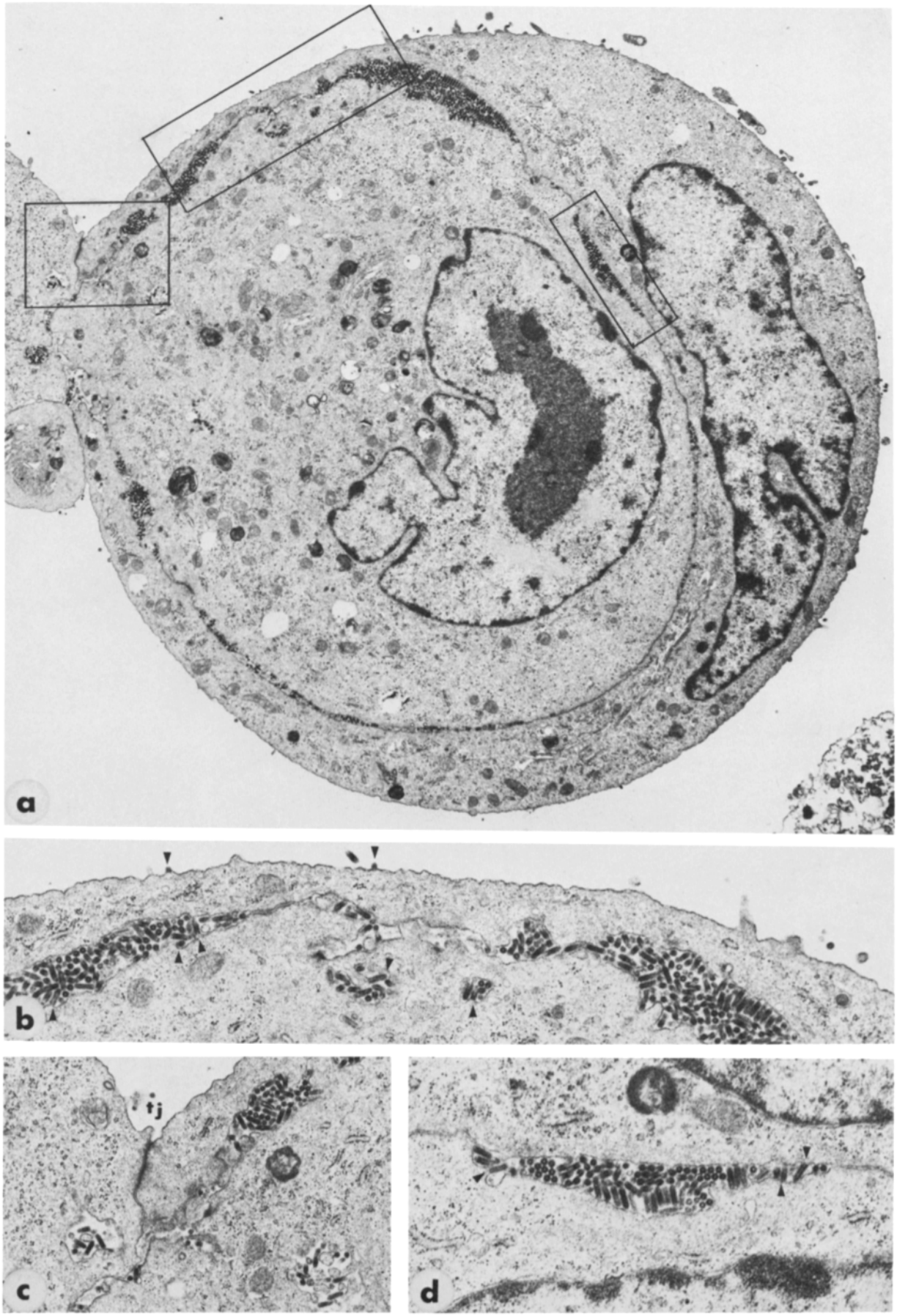
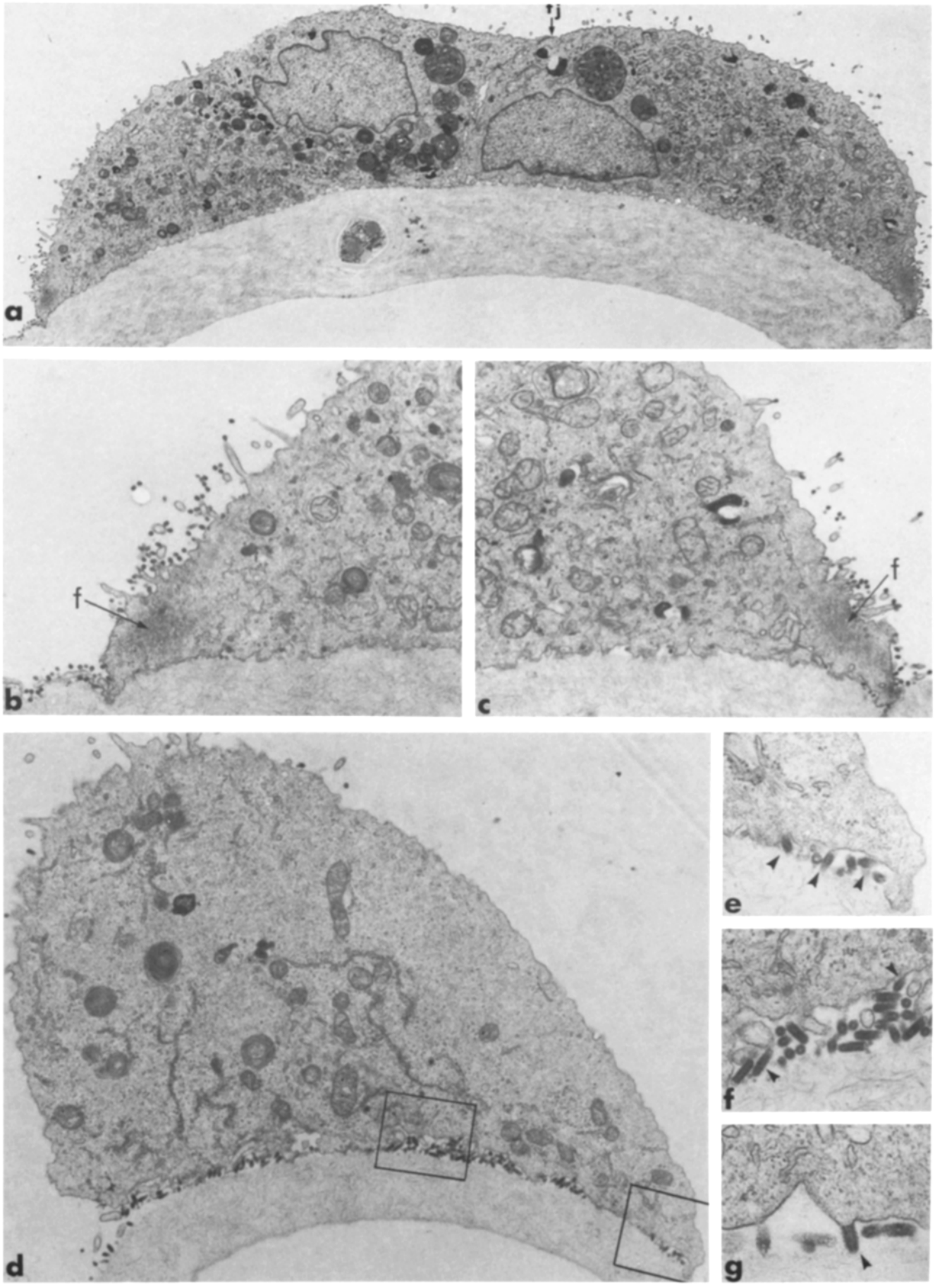


FIGURE 4 Budding of VSV into the intercellular space of an aggregate consisting of a cluster of three cells (*a*). Enlargements of boxed regions in *a* are represented in *b*, *c*, and *d*. Many VSV virions (arrowheads) are observed budding from the plasma membrane region facing the intercellular spaces where they accumulate (*b* and *d*). A few virions (arrowheads) can be seen on the free cell surface (*b*). A tight junction has formed between two of the cells (*c*). (*a*) × 13,000. (*b*, *c*, and *d*) × 28,000.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domains of pancreatic acinar cells and MDCK cells in confluent monolayers (1, 11). Recently, it has been reported that (26) the strict polarity of viral maturation is also lost when MDCK monolayers doubly infected with influenza and VSV are treated with EGTA to disrupt tight junctions. All these observations although consistent with a possible role of tight junctions in maintaining the segregation of plasma membrane components to distinct domains, do not exclude the possibility that other cellular elements play a primary role in determining the segregation of plasma membrane components, since the agents employed to separate cells from their neighbors and/or the substratum may affect cell organization in many other ways besides disruption of tight junctions. In fact, it is known that Ca^{++} deprivation and treatment with EGTA can markedly affect cell shape, probably through a reorganization of cytoskeletal elements (15, 18), which it could be speculated may be involved in controlling the distribution of plasma membrane components.

The observation that in isolated suspended MDCK cells VSV frequently buds into intracellular vacuoles may indicate that some of the basolateral membrane is removed from the cell surface by endocytosis and is sequestered in an intracellular pool, from where it could be mobilized upon attachment to a substrate. In confluent monolayers, on the other hand, budding into intracellular vesicles is very rarely seen indicating that, when in contact with a substratum, little basolateral membrane is stored intracellularly.

Although the polarized budding of viruses from cells which formed clusters in suspension suggests that establishment of complete tight junctions is sufficient to create the conditions necessary for the differentiation of the two domains, our experiments with sparsely plated cells demonstrate that such conditions can also be created in the absence of tight junctions or in the presence of incomplete junctions. When cells are sparsely plated and allowed to attach to a collagen coated substrate isolated from neighboring cells, free and attached cell surfaces acquired distinct morphological features normally associated with the surfaces of polarized cells. When infected, viral budding occurs these cells in the polarized manner characteristic of infected monolayers. This observation differs from that of Roth and Compans (26), who observed complete loss of polarity in the budding of influenza virions from cells which were replated at low density after dissociation with trypsin-EGTA from doubly infected monolayers. Given that we have shown (8) that the development of polarity after trypsinization requires protein synthesis, we suggest that this discrepancy is explained by the fact that surface components damaged by trypsin, which may be required for polarization, cannot be produced by cells trypsinized after several hours of infection, since host protein synthesis is most probably suppressed.

The observation that attachment to a surface was sufficient to determine polarized viral budding in MDCK cells is in striking contrast to the finding that VSV or influenza bud in a nonpolarized fashion from infected fibroblasts (25). Thus, the development of adhesion plaques by fibroblasts is not sufficient to determine asymmetric budding. It can be presumed that attachment to a solid substrate, which in all cells may cause cytoskeletal changes as well as a redistribution of plasma membrane proteins (9, 19, 27), produces in epithelial cells a more comprehensive response which induces a differentiation of two plasma membrane domains. It is possible that the circumferential region of attachment where bundles of filaments are seen to approach the cell membrane plays in single isolated epithelial cells a role equivalent to that of tight junctions in delimiting the different surface domains of cells in contact with each other. It is interesting to note that fibroblasts do not appear to exhibit prominent circumferential bundles of filaments as those we frequently observed in the isolated attached epithelial cells, and that a circumferential filamentous bundle has been described just below tight junctions in epithelial cells within monolayers (1).

In an epithelial tissue, three main factors may play a role in the establishment and maintenance of surface polarity: cell-substrate interaction, cell-cell contact, and the existence of complete tight junctions. The results of this work indicate that the presence of complete tight junctions is not essential for the expression of epithelial polarity. Our experiments indicate that the interaction with a substrate, or with another cell, may serve as sufficient signal to trigger the segregation of plasma membrane components responsible for polarized viral budding. They do not demonstrate, however, whether the two surface domains reach the degree of molecular segregation observed in confluent monolayers. Experiments involving direct localization of intrinsic plasma membrane proteins by immunocytochemistry on isolated attached cells are needed to answer this point.

We wish to thank Ms. Anne Russo, Ms. Myrna Chung, and Ms. Sonia Martinez for typing the manuscript and Mr. Alan Lieberman, Mr. Brian Zietlow and Ms. Jody Culkin for photography.

This work was supported by grants from National Institutes of Health (AG 01461; GM 20277), and National Science Foundation (PCM 8011053).

Received for publication 23 August 1982, and in revised form 5 November 1982.

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FIGURE 5 Budding of influenza or vesicular stomatitis virions from sparsely plated MDCK cells attached to a collagen layer. Isolated cells kept in spinner culture for 16 h were infected with influenza virus or VSV (MOI = 10), plated sparsely on a preformed collagen gel 1 h later, and fixed 8 h after infection. (*a*) Two attached MDCK cells showing a region of intercellular contact limited by junctions (*tj*). On the opposite sides the cells do not make contact with other neighbors × 4,000. (*b* and *c*) Higher magnification views of the edges of each cell where the free and attached membrane domains converge. × 11,000. Influenza virions bud from the free surface, preferentially near the edges; a few viruses appear to have been trapped under the adherent surface. A circumferential bundle of microfilaments (arrows labeled *f*) is associated with the edge of both cells. (*d*) VSV particles bud from the adherent surface and accumulate between the cell and the collagen layer. A few virions are seen on the free surface × 11,000. (*e* and *f*) Higher magnification views of portions of the basal region of the cell in *d*. × 28,000. (*g*) Portion of the adherent surface of a different cell. Arrowheads point to several budding virions. × 44,000.

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