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Morphogenesis of Vesicular Stomatitis Virus: Electron Microscope Observations with Freeze-Fracture Techniques

DENNIS T. BROWN* AND BIRGIT RIEDEL

Institut für Genetik der Universität zu Köln, 5 Cologne 41, West Germany

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The morphogenesis of vesicular stomatitis virus was examined using freeze-fracture techniques, and the results obtained were compared with those from previously published experiments carried out with influenza viruses and togaviruses. The process of conversion of the host cell plasma membrane into the vesicular stomatitis virus envelope was accompanied by a loss of the intramembranal particles abundant in cell membranes. Frequently a dense accumulation of intramembranal particles could be seen at the base of the developing virion, suggesting that these structures might play some role in the generation of viral envelope. In addition to the viral structures that were seen to develop in the classical fashion, with their long axis perpendicular to the cell surface, structures were also found that suggested the initiation of a process similar to budding, with the long axis of the viral capsid parallel to the plasma membrane. In this situation, as in the "perpendicular" process, intramembranal particles were excluded from the viral structure, and an accumulation of these particles could be seen adjacent to the developing viral membrane.

Vesicular stomatitis virus (VSV) is an enveloped RNA-containing virus which acquires its membranous envelope by the process of "budding" through the plasma membrane of the host cell after that membrane has been modified by the insertion of virus-specified glycoprotein (5, 8, 26). VSV is a structurally complex virion which has the shape of either a bullet, with one end tapered and the other square, or a bacillus, with both ends tapered, when viewed in the electron microscope (4, 14, 19, 25). The core of the virion is helical in structure, has the overall shape of a bullet, and is located in the center of the cylindrial virion (4, 25). The viral membrane is believed to be comprised of two virus-specified proteins, an internally situated membrane protein (M) and an externally located glycoprotein (G) (4, 25). The organization of these proteins in the membrane is not clear. The G protein is clearly exposed on the surface of the virion and serves as the organelle of attachment to susceptible host cells (1, 12, 18, 19, 25). The M protein is believed to be located on the inner surface of the viral membrane; however, it is unclear to what extent (if any) this protein extends into the envelope bilayer itself (25). It is also not clear whether the G protein extends across the membrane bilayer to interact with the M protein. It has been shown recently that the G protein does have a hydrophobic fragment of about 5,000 molecular weight buried in the envelope membrane (13, 20). Little more is known regarding the processes that organize the protein components of the virion during the budding process. Models have been suggested (based on electron microscope observations of thin sections of VSV-infected cells) that show the viral components to be assembled in a stepwise fashion as budding progresses. The maturation process is morphologically depicted as beginning at the tapered end of the virion and progressing in such a manner that the virion buds with its long axis perpendicular to the surface of the plasma membrane (29). This model allows the core to be progressively shaped into a tight spiral as its protein interacts with the modified membrane. The G protein is exposed or reorganized into the spike on the exterior of the developing virion as the process continues (25, 26). No suggestion has been offered as to how the completed virion is released from the cell surface.

We have investigated the process of VSV maturation using the procedure of freeze-fracturing. This procedure has a number of advantages over classical thin sectioning for studying the morphogenesis of membrane-bound viruses. The freeze-fracturing process eliminates artifacts of dehydration and preserves three-dimensional structure. The fracturing process exposes to view the interior of the developing viral membrane, making it possible to observe topological alterations occurring within the membrane during envelopment (17, 24). The

freeze-fracturing technique has been successfully used by Bächi et al. (1) and by Brown et al. (3) in the study of influenza and togavirus morphogenesis. In both cases a distinct change in the morphology of the interior of the cell membrane was demonstrated as it was converted from host plasma membrane to viral envelope. This change was reflected in the loss of the intramembranal particles from the plasma membrane as it became virus envelope. The intramembranal particles have been shown to be membrane structural proteins which extend into or across the nonpolar region of the membrane bilayer (22, 24). Thus, the topological change and the structure-free appearance of the interior of the envelopes of influenza virus and the togavirus Sindbis can be interpreted to support the hypothesis that the envelope structural proteins of these virions do not extend into or across the nonpolar region of the envelope bilayer (3, 7, 10, 21). A freeze-fracture study of VSV structure and development was therefore undertaken to determine if this model rhabdovirus would be similar to the previously studied influenza and togavirus systems.

MATERIALS AND METHODS

Cells, virus, and media. VSV (Indiana stereotype) was a gift of Elmer Pfefferkorn (Dartmouth Medical School, Hanover, N.H.). The virus was grown after plaque purification in BHK-21 cells, which were received from Peter Faulkner (Queens University, Kingston, Ont., Canada). Cell and virus growth were carried out at 37°C in Eagle minimal essential medium (6) containing 10% tryptose phosphate broth and 10% fetal calf serum. Virus titration was carried out with a 1% agarose overlay containing the medium described above.

Preparation of specimens for electron microscopy. Monolayers of cells were infected at about 95% confluency with 20 PFU of VSV per cell from the first passage after plaque purification. The infected cells were incubated for 10 h at 37°C and subsequently washed with phosphate-buffered saline (4°C) and fixed in monolayers with 2% glutaraldehyde in cold phosphate-buffered saline for 2 h. The fixed cells were scraped from the monolayers and embedded for ultrathin sectioning as described previously (11) or prepared for freeze-fracturing. Freeze-fracturing was carried out exactly as described by Brown et al. (3), except that both fixed and unfixed infected cells were used. The results obtained were the same regardless of whether fixation was used. Freeze-fracturing was carried out in a Balzers BAF-300 freeze-etching device, and replicas were photographed in a Siemens 101 electron microscope.

The triple-layered unit membrane seen in thin sections is split into two components by the cleaving process (17, 23, 24). Electron micrographs of freeze-fractured membranes can thus present to view one of two possible interior membrane surfaces. The convention of Branton et al. (2) is used to describe the various components of the plasma membrane bilayer. The term "protoplasmic" (abbreviated P) refers to that part of the membrane bilayer that is in contact with the cytoplasmic contents. "Exoplasmic" (abbreviated E) refers to the layer that is in contact with the extracellular fluid. Each of these bilayer leaflets has an inwardly facing (toward the cytoplasm) and an outwardly facing (toward the extracellular fluid) surface. Of these four possible surfaces, only two are revealed by the freeze-fracturing process used in this study: the outwardly facing surface of the protoplasmic part of the membrane bilayer (protoplasmic fracture face, PF) and the inwardly facing surface of the exoplasmic part of the bilayer (exoplasmic fracture face, EF). Both of these interior membrane surfaces possess the 6- to 10-nm intramembranal particles believed to represent membrane proteins which penetrate into or across the fracture plane, the nonpolar region of the bilayer (17, 22–24). These particles can be used as an aid in identifying the exposed fractured faces, because the protoplasmic fracture face (PF) possesses the structures in greater abundance than the exoplasmic fracture face (EF) (3, 17, 23, 24).

Viral envelopes fracture in a fashion similar to that described for plasma membranes. The fracturing process reveals an external fracture face (EF) similar to the exoplasmic fracture face of the plasma membrane (EF) and a fracture face of the envelope bilayer part adjacent to the core. Because the viral interior contains no protoplasm, we feel the symbol PF is inappropriate for describing this surface and have adopted the designation "C" for that part of the envelope bilayer located closest to the core or capsid. Thus, "CF" represents the fracture face in the viral envelope that is analogous to the PF surface exposed by fracturing the plasma membrane.

RESULTS

Ultrathin sectioning of VSV-infected cells revealed the virions developing from the plasma membrane as described previously (25, 29), and a single electron micrograph is provided here as a frame of reference for the reader in examining the following images of freeze-fracture replicas (Fig. 1).

Figure 2 shows an area of a VSV-infected cell surface after freeze-fracturing. The fracture plane has passed through the exoplasmic part of the membrane bilayer and has exposed to view the PF of the membrane bilayer before breaking through the protoplasmic part exposing the cell cytoplasm. On the exposed surface can be seen large numbers of the intramembranal particles (described above) as well as a number of partially enveloped virions. At the "ice level," where the exoplasmic part of the bilayer has been cut by the fracturing process, both parts of the membrane bilayer follow with-

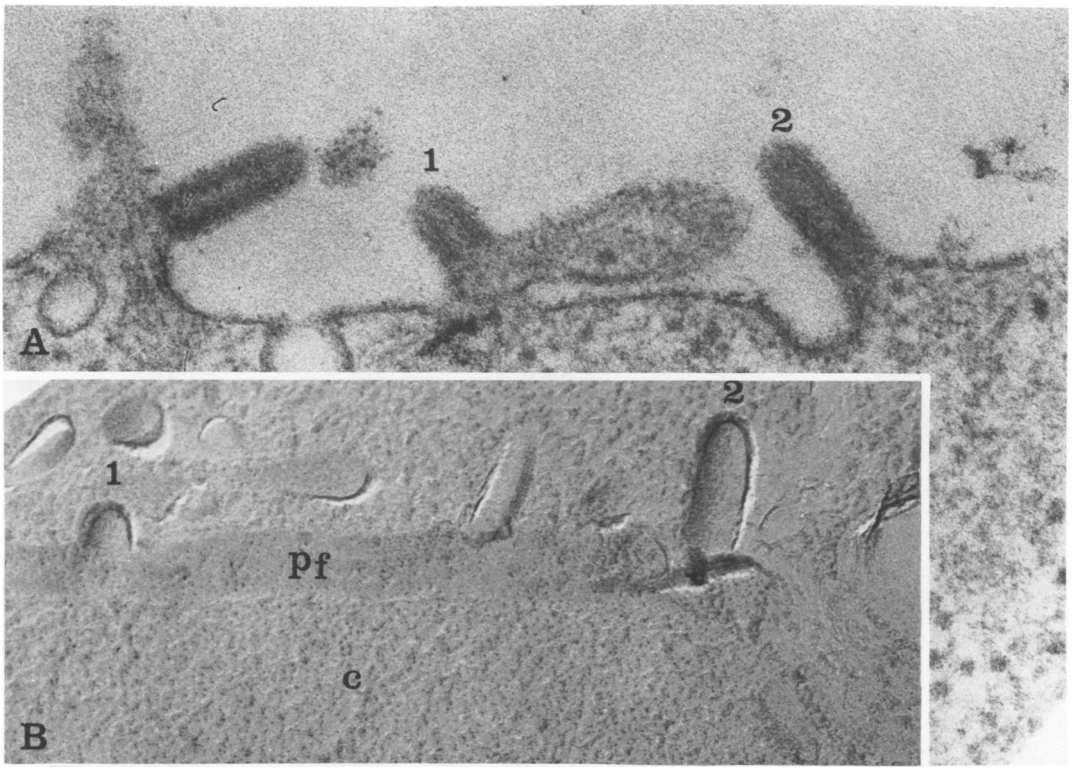

FIG. 1. *Budding of VSV as seen by ultrathin sectioning and freeze-fracturing. (A) Developing virions thin sectioned along their long axis: (1) a virion at an early stage in budding; (2) a more completely developed particle. ×105,000. (B) Developing virions similar to those shown in (A) but observed in a replica of a freeze-fractured cell; (1) and (2) as described for (A). PF, Protoplasmic fracture face. ×105,000.*

out interruption the contour of the developing virion. The membrane that is forming the viral envelope is, therefore, continuous with the plasma membrane from which it is derived. The portion of the membrane clearly incorporated into the viral bud is free of the intramembranal particles that can be readily detected in the membrane at the base of the developing viral envelope. In some instances there is an unusually large accumulation of the particles at the base of the developing virion, where the transition from host membrane to viral envelope takes place. In some instances a halo of material could be seen at the outer surface of the developing viral structures (Fig. 1, 2). This halo of material may represent the appearance of the viral spikes.

In Fig. 3 and 4 the PF of the infected cell membrane is widely exposed, the axis being perpendicular to the plane of section. Large numbers of budding virions can be seen in both figures. Figure 3 has a number of nearly completed or possibly complete virions at the "ice level." These virions, like the budding forms, have no intramembranal structure. The developing viral envelopes viewed from above in both Fig. 3 and 4 are generally free of the intramembranal particles, as were the developing envelopes viewed from the side shown in Fig. 2. In a survey of 200 budding structures of the type shown in Fig. 3 and 4, only seven virions were found to have particulate structures associated with their surface that were morphologically identical to the host intramembranal particles. Most of the structure seen in the developing viral envelopes could be attributed to fine structure of the platinum-carbon replica itself. In these images, as in Fig. 2, an accumulation of particles at the base of the developing virion can frequently be seen. Occasionally the normal distribution of intramembranal particles, in areas where budding structures were not apparent, was disturbed in such a way as to produce a bead-free area having the size and shape of a VSV particle (Fig. 4) viewed on its long axis. Figure 5 shows an area of the PF of the membrane bilayer which has an extraordinary number of these structures. The

FIG. 2. *Protoplasmic fracture face (pf) of a cell surface producing a large number of virions. The developing particles are viewed from the side. The protoplasmic fracture face of the infected cell has a large number of the intramembranal particles. These particles (small arrows) are absent from the interior of the developing viral envelopes (1, 2, and others unmarked). The large arrow indicates a developing virion that has a tightly packed ring of the intramembranal particles at its base. A virion that was fractured in such a way as to reveal its external fracture face (ef) is also shown. ×103,400.*

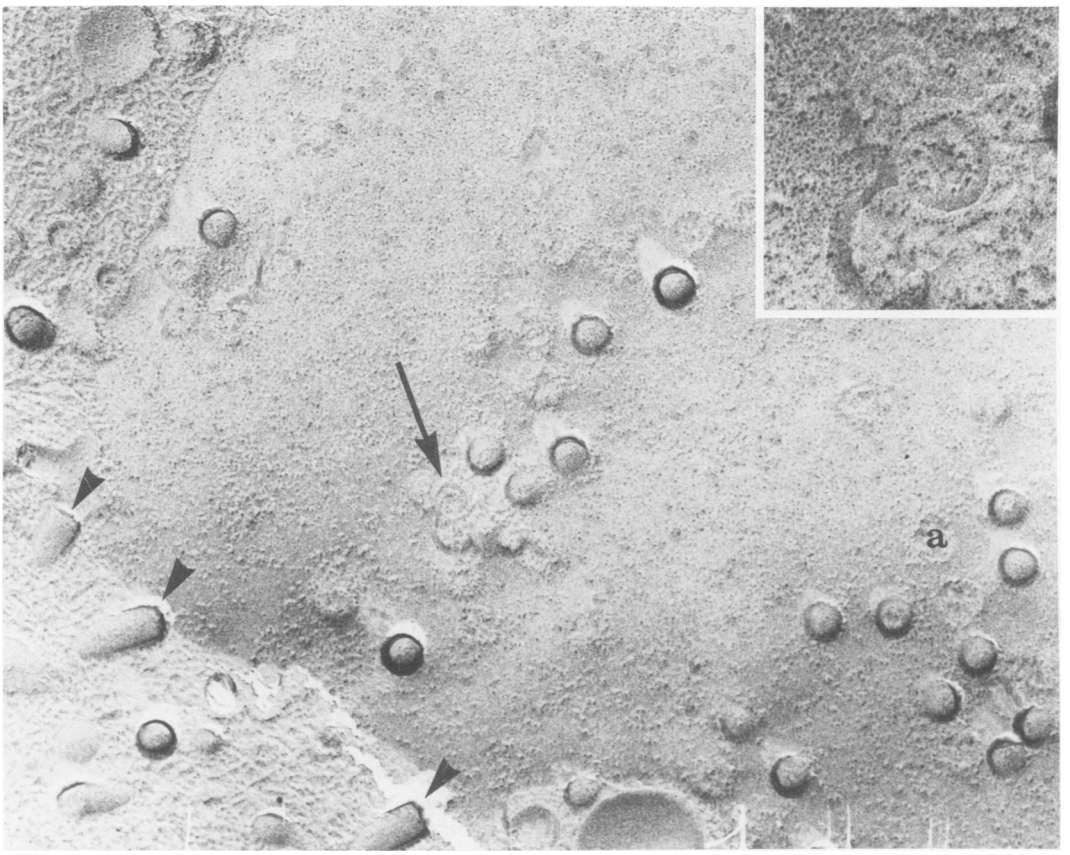

FIG. 3. *Area of the protoplasmic fracture face of a VSV-infected cell. The view is from a position perpendicular to the cell surface. A number of budding structures are viewed on their short axis. The intramembranal particles are absent from the developing buds, although they are abundant in other areas of this surface. At the edge of the exposed cell surface (where the outer leaflet of the bilayer is cross-fractured and the surrounding medium is exposed) are seen a number of completed virions which have remained on the cell surface (arrows). A number of budding virions have been cut from the cell surface by the fracturing process (a). Occasionally this cross-fracture leaves the broken membrane with a more orderly appearance (arrow and insert). ×57,000; insert, ×168,000.*

structures have the size and shape of VSV virions and, like the partially developed virions shown in Fig. 2 through 4, lack the intramembranal particles that can readily be demonstrated adjacent to the virus forms. These structures occurred rarely compared with the budding structures shown in Fig. 2 through 4 (1 in 80 to 100 budding forms) but do suggest that a process of budding can at least be initiated in which the viral core structure interacts with the modified cell plasma membrane in such a way as to deform it into a shape with the dimensions of a virion viewed perpendicular to its long axis. Accumulations of the intramembranal particles can occasionally be detected at the square end of those particles having a bullet shape (Fig. 5B), whereas other such structures appeared to be surrounded by the beads (Fig. 5C).

Because the freeze-fracture technique is so ideally suited to study of the contours of membranes, it was hoped that this study might provide information on how the virion is released from the cell surface after attaining its characteristic shape. Although a large number of budding forms were examined in this study, no indication of an infolding of the membrane at the base of the virion was detected. The only structures seen that might be related to some kind of release mechanism were of the type shown in Fig. 3. The ring-shaped structure shown in this figure does not have the rough-edged appearance of a virion that has been broken from the cell surface by the fracturing

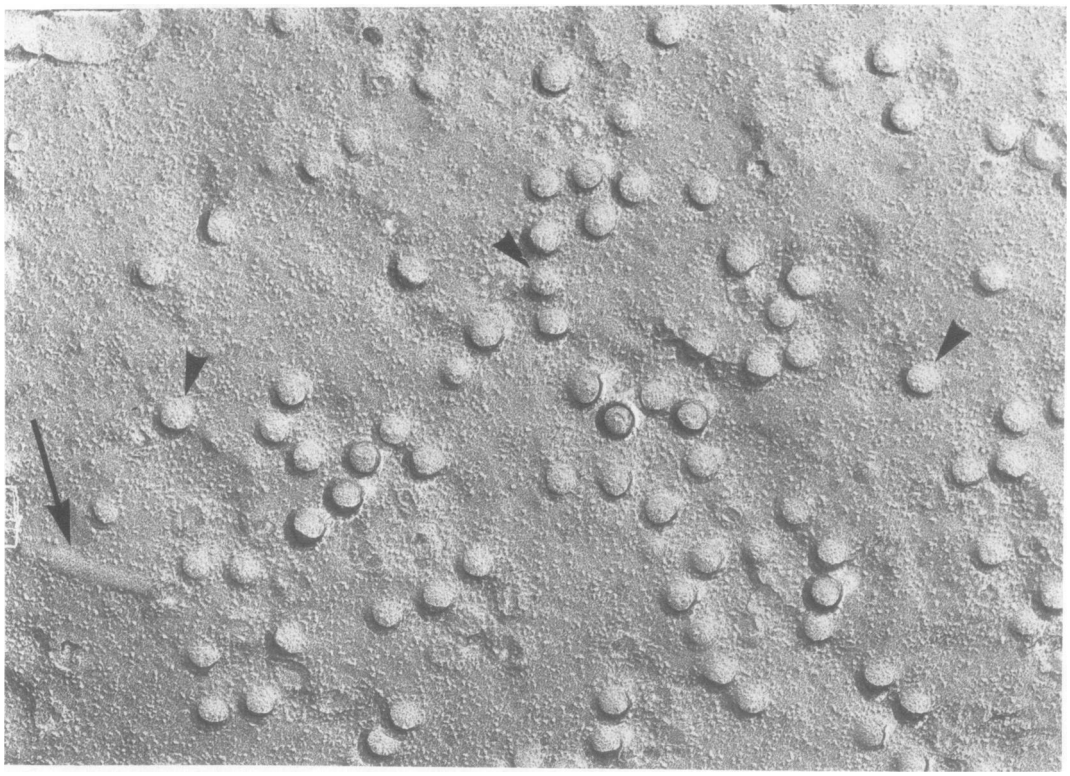

FIG. 4. *Protoplasmic fracture face of a VSV-infected cell. A large number of budding virions are seen, as in Fig. 3. The arrowheads point out a few viral structures that appear to have intramembranal particles. The arrow points out an area of the inner fracture face where the interior membrane beads are not present. This area has dimensions similar to those of a VSV virion viewed on its long axis. ×63,000.*

process. Such structures may represent the process of structural reorganization occurring at the base of a virion as it is released from the host cell plasma membrane.

Membrane fracture faces complementary to those presented here (exoplasmic fracture faces) were also found and were examined in this study (Fig. 6). The exoplasmic fracture face of the developing and mature viral envelopes were found to be free of intramembranal particles or other detectable structure. It should be pointed out, however, that because the analogous surface of the host plasma membrane has itself very few associated intramembranal particles, the morphological change described above could not be detected.

DISCUSSION

This freeze-fracture study of VSV-infected cells revealed similarities and differences in the development of this rhabdovirion compared with previously studied influenza and togaviruses. VSV, like both influenza and Sindbis viruses, was found to be free of intramembranal particles when in a nearly mature state of development. Like influenza, but unlike Sindbis virus, VSV budding structures were found to be generally free of intramembranal particles as soon as the buds could be morphologically distinguished (1). Sindbis virus was found to possess intramembranal particles in its envelope early in development, but these structures were completely lost before the viral core was half enveloped (3). VSV differed from both Sindbis and influenza viruses in that an increase in the density of the intramembranal particles could be frequently found at the base of the developing VSV envelope. This area is the region where the transition from host membrane to virus envelope takes place and may imply some special topological event accompanying the generation of the viral membrane. The viral N and M proteins must have some mechanism for identifying the presence of G protein on the outer surface of the cell plasma membrane in order for formation of the envelope to take place and to be able to incorporate the glycoprotein into the mature virion in a relatively uniform ratio to the internally located N and M proteins. The absence of intra-

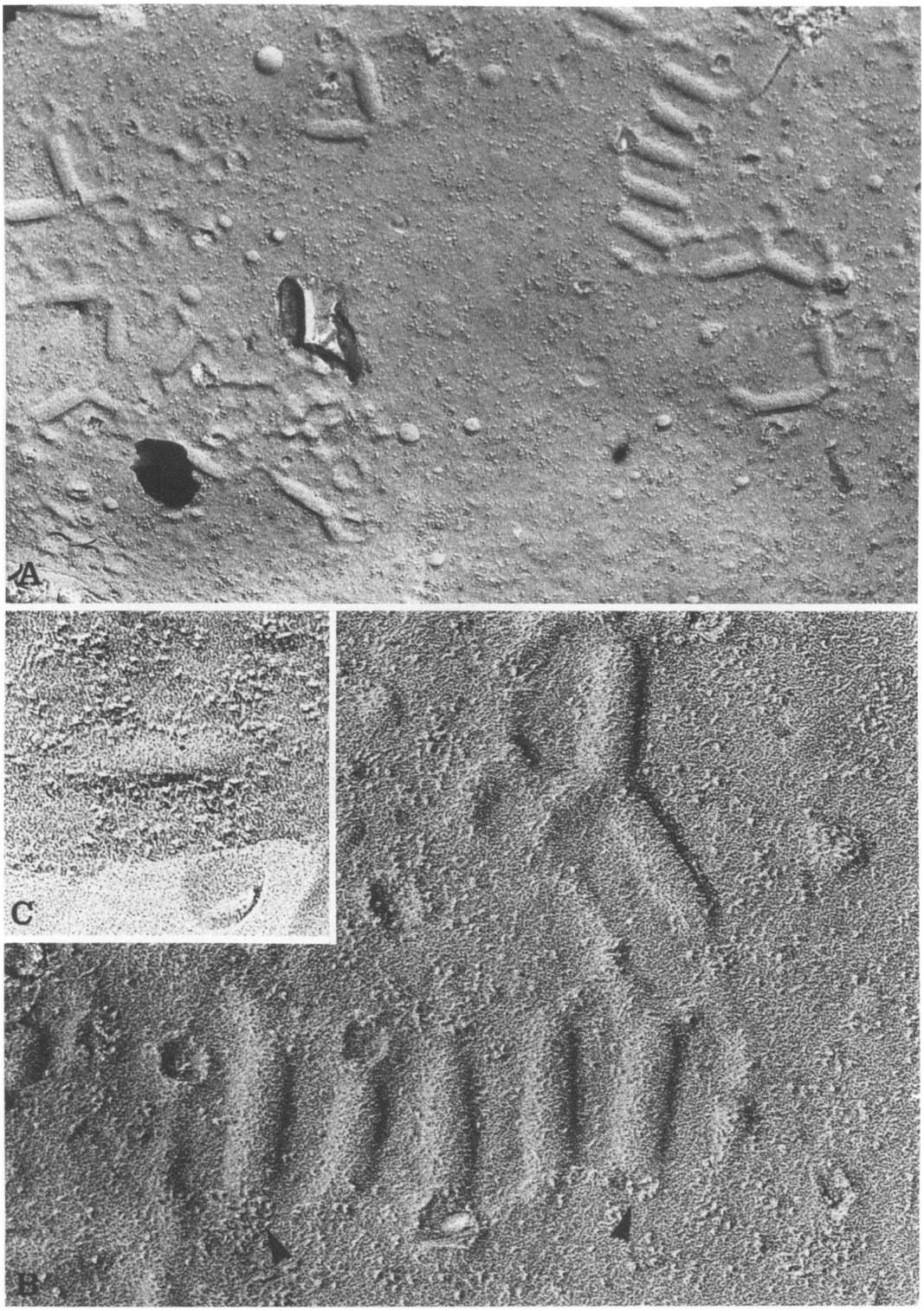

FIG. 5. *Protoplasmic fracture face of a VSV-infected cell having a large number of structures that appear to be virions developing with their long axis parallel to the cell membrane. The areas where the virus structures are seen are free of the interior membrane particles. Some of the structures have a large accumulation of the particles at the square end of the bullet (B, arrows). Other particles were surrounded by the interior membrane particles (C). (A) Low-magnification general view. (B) High magnification of developing virus structures from an area in (A). (C) High magnification of a developing viral envelope from another cell. (A) ×43,000; (B) ×110,000; (C) ×115,000.*

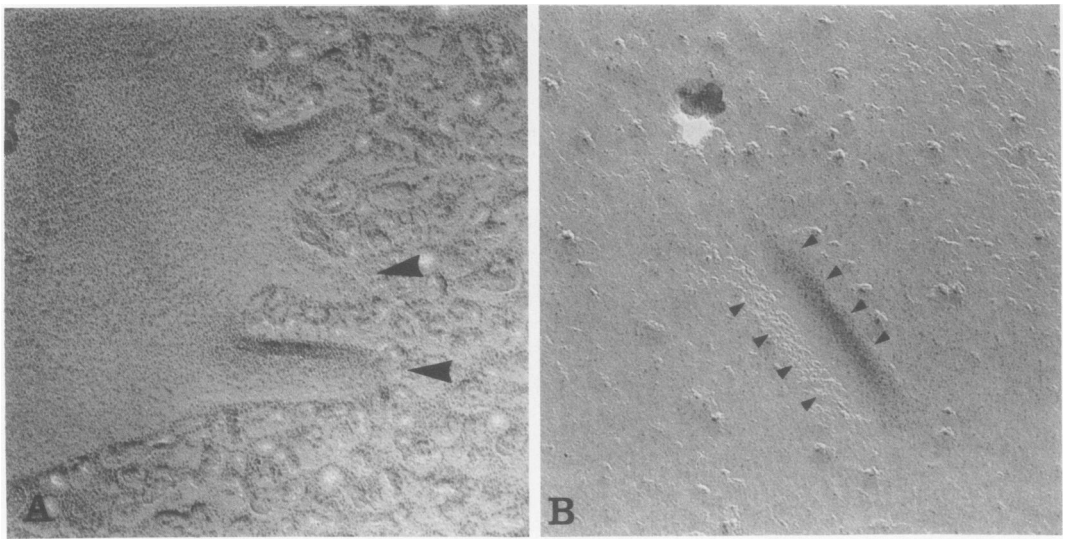

FIG. 6. *Exoplasmic fracture faces of VSV-infected BHK cells. (A) Virions maturing with long axis perpendicular to cell surface (fracture face complementary to those presented in Fig. 1). ×100,000. (B) A depression in the exoplasmic fracture face (bordered by arrow heads) possibly created by a virion budding as shown in Fig. 5. As in Fig. 5, the intramembranal particles (seen only in B) are absent from that part of the membrane interior associated with the virion. ×100,000.*

membranal particles in the cleaved envelope of the mature and partially mature virions supports the notion that the structural proteins of the viral envelope do not penetrate into or cross the nonpolar region of the envelope bilayer (10). It is possible, however, that at some very early point in their being incorporated into the viral envelope, the VSV membrane proteins interact with one another across the nonpolar region of the membrane bilayer. As the proteins become a fixed part of the envelope, this transmembranal interaction may be lost, producing the structurally smooth envelope interior and the appearance of the virus spikes on the surface of the developing envelope. In such a case one might propose that the dense accumulation of intramembranal particles at the base of the developing envelope "C" fracture face represents such a complex of virus envelope proteins (G and M), which have been accumulated by the presence of the nucleocapsid just prior to such a structural transition.

The longitudinal association of the VSV core with the plasma membrane (Fig. 5) is similar to that of structures observed in thin sections by Orenstein et al. (16) and suggest that the form-determining characteristics of the nucleocapsid can function in more than one way. (i) One way is the classical development of the virus structure along its long axis (which is by far the predominating process seen), with the nucleocapsid being progressively organized into a helical structure. (ii) The nucleocapsid is also capable of organizing itself into the form that it will ultimately assume in the virion without being completely in contact with the modified membrane (the other side of such a nucleocapsid would be exposed to the cell cytoplasm), as is the case in the former situation. It is not possible to determine by electron microscopy if such laterally arranged core structures ever bud to a completed virion. The morphological changes occurring within the membrane bilayer accompanying this lateral budding process are, however, identical to those occurring in the predominating "normal" process.

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