Blocked Coated Pits in AtT20 Cells Result from Endocytosis of Budding Retrovirions

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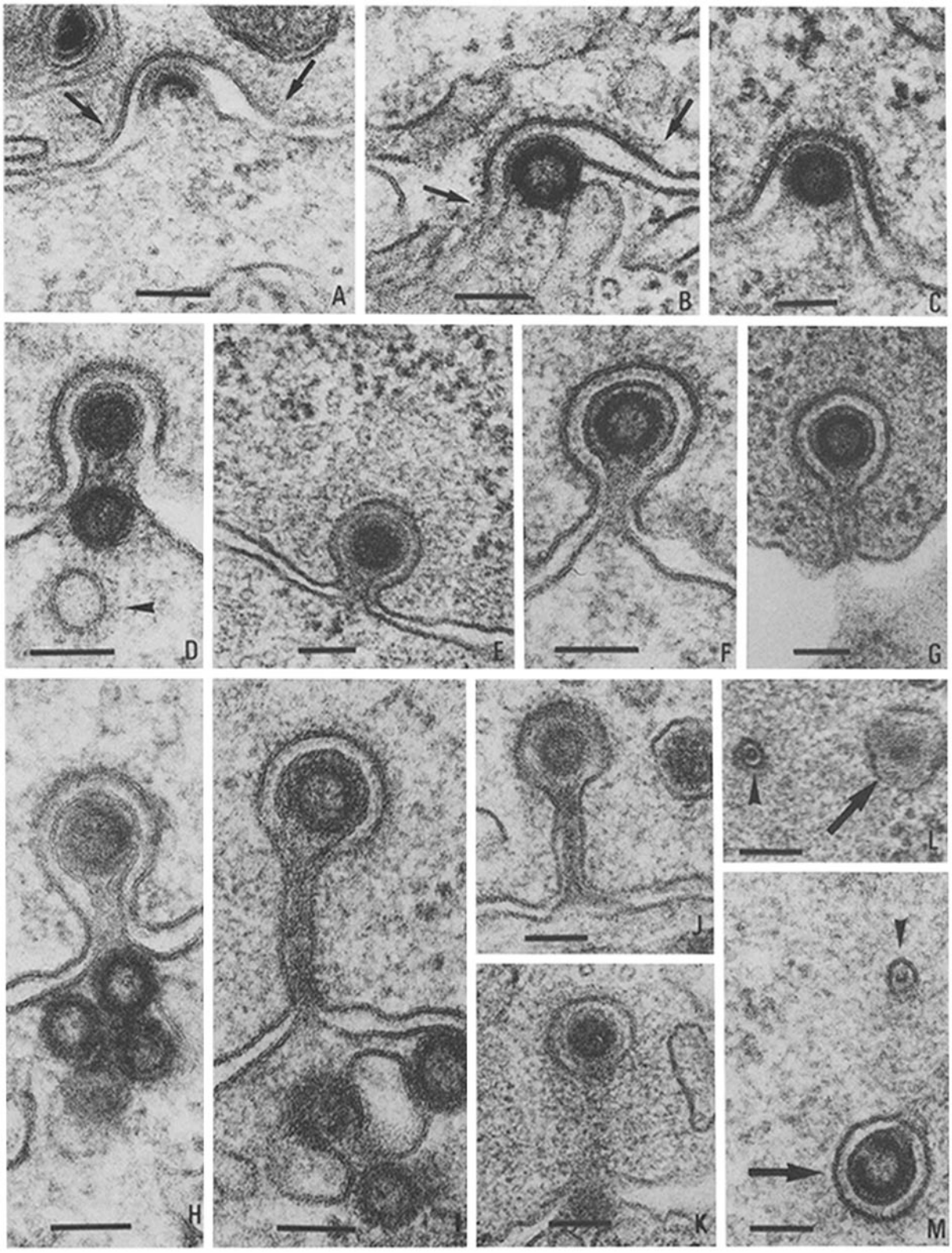
ABSTRACT AtT20 cells support the replication of two endogenous retroviruses, a murine leukemia virus and a mouse mammary tumor virus. On glass or plastic substrates, AtT20 cells grow in clumps. In this situation, retroviruses budding from the plasma membrane of one cell can, on rare occasions, be invested by coated pits in the plasma membranes of contiguous cells. These pits can invaginate to depths of 2,000–4,000 Å within the cytoplasm drawing with them the viral buds which remain connected to their parental cells by tubular stalks, some of which are only 225 ± 15 Å in diameter. These stalks run down the straight necks of the pits from the buds to the parental cell surfaces. Several lines of evidence indicate that these unique structures are blocked such that neither endocytosis nor budding can go to completion, and that they persist for several hours. The properties of these blocked coated pits are relevant to models of both endocytosis and viral budding. First, they indicate that the invagination of a coated pit is not absolutely dependent on its pinching off to form a coated vesicle, but that uncoating appears to be dependent upon the generation of a free vesicle. Secondly, they suggest that the final stages in the maturation of a retroviral core into a mature nucleoid are dependent on the detachment of the bud from its parental cell and that the driving force of budding is the association of viral transmembrane proteins with viral core proteins. An explanation is offered to account for the formation of these structures despite the phenomenon of viral interference.

AtT20 cells are an established line of murine pituitary epithelial tumor cells. They synthesize and secrete ACTH and β endorphin and also support the replication of two endogenous retroviruses, a murine leukemia virus (MuLV)[1] (12) and a mouse mammary tumor virus (MuMTV) (24). These cells have proved valuable for the study of exocytotic transport pathways from the Golgi apparatus to the cell surface. Gumbiner and Kelly (12) have shown that the major envelope glycoprotein of the MuLV is transported by a constitutive pathway to the plasma membrane. On the other hand, the transport of the secretory protein ACTH to the cell surface is primarily along a second regulated pathway, inducible with 8-bromo-cAMP. In contrast to these exocytotic events, endocytosis in AtT20 cells has not been extensively studied.

Receptor-mediated endocytosis is the route of entry into cells of many enveloped viruses of diverse taxonomic groups including toga-, orthomyxo- and rhabdoviruses (for review see reference 14). Glycoprotein components of the viral envelopes have evolved to bind to specific cellular receptors in the plasma membrane. Bound virions are concentrated into coated pits which then pinch off from the plasma membrane to give rise to free coated vesicles. The latter, losing their clathrin coats, move to and fuse with endosomes delivering the virus into the acid endosomal environment. The low pH induces fusion of the viral envelope with the endosomal membrane ejecting the viral nucleoid into the cytoplasm. Though these steps in virus entry have been elucidated primarily using the Semliki Forest, vesicular stomatitis, and influenza viruses, it seems clear that the retroviruses also enter host cells by this pathway (1, 4).

Cells supporting the replication of a retrovirus are usually resistant to superinfection by the same virus, or any other virion having the same envelope glycoproteins as the resident retrovirus. This phenomenon, known as virus interference, results from a reduction in available surface receptors. The receptor molecules in the plasma membrane are saturated by the excess of viral envelope glycoproteins specified by the

[1] Abbreviations used in this paper: DME, Dulbecco's modified Eagle's medium; MuLV, murine leukemia virus; MuMTV, mouse mammary tumor virus.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resident viral genome (for review see reference 29). Viral interference is sufficiently complete to be used to classify retroviruses into groups with envelope glycoproteins in common. All the ecotropic MuLVs, for example, fall into one interference group and, therefore, use the same cell surface receptors (for review see reference 29).

The basis of viral interference is the lack of sufficient free receptors to bind exogenous virions coming briefly into contact with the cell surface. If, however, a cell supporting the budding of progeny retrovirus particles is overlain by another cell infected with the same virus, the receptors at the surface of the overlying cell might, in principle, be free to associate with either the envelope glycoprotein molecules being inserted into their own plasma membrane or those clustered on the surface of the buds developing below. I have observed precisely this phenomenon in cultures of AtT20 cells. Some of the buds of both MuMTV and MuLV are bound by receptors and then invested by coated pits of contiguous cells. Such coated pits can invaginate, drawing with them the buds still attached to the surface of their parental cells by a narrow tube of the plasma membrane. At this stage these unique structures appear to be blocked such that completion of both budding and endocytosis is frustrated. These observations have implications for ideas about the mechanism of both receptor-mediated endocytosis and the budding of enveloped virus.

MATERIALS AND METHODS

Cell Culture: AtT20D16V cells were grown in Falcon plastic ware in Dulbecco's modified Eagle's medium (DME) supplemented with 3.5 gm/liter of glucose and 10% fetal calf serum or horse serum (Gibco Laboratories, Grand Island, NY). The cells were replated, following dispersion of clumps with 0.25% trypsin, 0.05% EDTA, every 4 to 5 days. Under these culture conditions the cell population doubles in ~40 h (23).

Electron Microscopy: Cells growing in 35-mm dishes were washed with Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS) and fixed at room temperature for 1 h in 2–4% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. They were then given three 10 min washes in 0.1 M sodium cacodylate buffer, pH 7.2, and postfixed in 2% OsO_4 in 0.1 M cacodylate buffer, pH 7.2, for 1 h. After three further washes in the buffer, the cells were incubated overnight at 4°C in 0.5% magnesium uranyl acetate (BDH Ltd., Poole, England) in water. They were then dehydrated in ethanol, detached from the dishes with propylene oxide, pelleted, and embedded in Epon. Thin sections were stained in lead citrate for 1 min before examination in Philips 300 or 400 electron microscopes. The blocked coated pits described here are relatively rare structures, occurring in about one in fifty of the cells examined in any one thin section.

Labeling with Cationized Ferritin: Cells in 35-mm dishes were washed free of medium and incubated at 4°C (1–4 h) or 37°C (1 h) in 100 μg/ml of cationized ferritin (5, 9) (Miles Laboratories Inc., Naperville, IL) in DME supplemented with 3.5 gm/liter of glucose but without serum or any other protein. The cells were then either fixed immediately or washed four times with DME plus glucose and then incubated in DME plus glucose until they were fixed as above. Cells incubated with cationized ferritin were not stained with magnesium uranyl acetate before embedding; instead thin sections were stained in 2% uranyl acetate in water for 10–15 min at room temperature and then for 1 min with lead citrate.

Immunoelectron Microscopy: Cells were fixed in 3% formaldehyde and 0.1% glutaraldehyde in Mg^{++}- and Ca^{++}-free PBS. They were then washed in PBS, scraped from the dishes, and either infiltrated with sucrose and frozen in liquid nitrogen (11) or infiltrated and embedded in Lowicryl K4M (2). I am greatly indebted to Dr. J. Roth (Biozentrum, Basel) for embedding the material in Lowicryl K4M. Thin sections of frozen or Lowicryl-embedded cells were cut and labeled with either an affinity-purified rabbit anticlathrin antiserum (13) generously provided by Dr. D. Louvard (Pasteur Institute, Paris) or with a rabbit antiserum against smooth muscle actin generously provided by Dr. T. Kreis (European Molecular Biology Laboratory, Heidelberg). After incubation with these antisera, sections were incubated with protein A conjugated to 7 nm gold. Finally the sections were contrasted with uranyl acetate or, in the case of the Lowicryl sections, uranyl acetate and lead citrate, before being examined.

RESULTS

Coated Pits Invest Retroviral Buds

Particles of the endogenous MuLV and MuMTV in AtT20 cells growing under the culture conditions described above can be seen in thin sections budding from the plasma membrane. The MuLV buds contain the crescent-shaped nucleoids which develop at the site of budding and are characteristic of C-type retroviruses. The MuMTV buds develop when spherical intracytoplasmic A-type particles, which are the presumptive nucleoids of the mature virions, move from deeper within the cytoplasm to beneath the plasma membrane.

Under our culture conditions AtT20 cells grow not as a monolayer but in clumps which over 4 to 5 days become many cells deep. As a result, the surfaces of most cells, particularly in dense cultures, are apposed to the surfaces of cells above and beneath, rather than being in direct contact with the substratum and the medium. The retroviral buds projecting from the surface of one cell are, therefore, often directly opposite a region of the plasma membrane of another cell. In this situation, coated pits in the plasma membrane of one cell sometimes develop around the budding MuLV and MuMTV virions of a contiguous cell. Fig. 1, *A* and *B*, shows

FIGURE 1 Viral buds in coated pits. MuLV C-type (*A*) and MuMTV A-type (*B*) retroviral buds at an early stage of development are associating with coated plasma membrane of contiguous cells. Note the close and regular apposition of the bud's envelope and the cell's membrane and the presence of material in the space between the two membranes, particularly in *B*. The clathrin coat (limits indicated by arrows) extends beyond the region of close association of the bud and the coated membrane; beyond this region of association the two membranes diverge. At a later stage of development the coated membrane follows the curvature of the developing bud (*C* and *D*). Note in *C* the prominent granules in the space between the envelope of the MuMTV bud and the coated pit. Note in *D* a second MuMTV A-type particle immediately below the budding A-type particle and also a coated vesicular profile (arrowhead). *E* shows an almost complete bud invested by a coated pit: the latter is surrounded by fibrillar cytoplasmic material which excludes ribosomes from the immediate vicinity of the coated pit. Having reached the stage shown in *E*, the coated pits begin to invaginate (*F*–*K*) drawing with them the bud attached by a stalk to its parental cell. Note the granules in the space between viral envelope and coated pit surface; also note that the cytoplasmic clathrin coat does not extend beyond the surface of the pit. Once the core of MuLV bud has developed into a complete sphere it is no longer possible to distinguish it by simple morphology from an MuMTV core. The buds in *H* and *I*, however, are clearly A-type particles. In *K*, the blocked coated pit has been sectioned above or below the plane of the pit's neck. This shows the fibrillar material which accumulates around these structures. *L* and *M* show transversely sectioned necks and stalks (arrowheads) appearing as concentric rings (cylinders) while the arrows indicate pits sectioned at different levels. Note that the stalks of the buds in cross section are smaller than ribosomes. Bars, 0.1 μm. (*A* and *B*) × 136,000; (*C*) × 115,000; (*D*) × 148,000; (*E*) × 102,000; (*F*) × 150,000; (*G*) × 98,500; (*H*) × 150,000; (*I*) × 145,000; (*J* and *K*) × 113,000; (*L* and *M*) × 115,000.

that these coated pits are formed by the association of patches of coated membrane with the developing viral envelope. As a result, the coated membrane begins to adopt the spherical contours of the bud, and the two membranes are separated by a uniform space 180–200 Å wide (Fig. 1, *C* and *D*). Electron-dense particles are frequently seen lying between the membranes of the coated pit and the viral envelope (Fig. 1, *C* and *F*); these may be images of complexes of the receptor with the viral envelope spike. Note that in the regions where the bud and coated membrane have not yet associated, these particles are not present and also that the gap between the two membranes is much larger (Fig. 1, *A* and *B*).

Retroviral Buds Are Invaginated

As the retroviral buds near completion and become spheres attached to the parental cells by a short stalk of plasma membrane, so the coated pits investing them also become progressively more spherical (Fig. 1, *D* and *E*). Since, however, the buds are still attached to their parental cells the coated pits around them cannot close and pinch off to yield free coated vesicles as do other coated pits in the same cells engaged in endocytosis (not shown). At this stage, the coated pits invaginate (Fig. 1, *F–K*) to depths of about 2,000–4,000 Å into the cytoplasm, drawing with them the still incompletely budded virion together with a very narrow tube that maintains the connection between the bud and its parental cell. This tubular stalk of membrane measured from longitudinal (Fig. 1, *G*, *I*, and *J*) and more accurately from transverse sections (Fig. 1, *L* and *M*) is only 225 ± 15 Å in diameter. The tubular neck of the coated pit which encases the stalk of the bud ranges in diameter from ~360 Å to ~500 Å. The coated pit itself is ~1,600–1,700 Å in diameter while the viral bud is 1,100–1,200 Å wide. Frequently, the cytoplasm immediately surrounding these coated pits contains unorganized fibrillar material which appears to exclude ribosomes (Figs. 1, *E* and *K*, and 2, *F–J*).

Blocked Coated Pits with Viral Buds Are Stable

Five lines of circumstantial evidence point to the conclusion that blocked coated pits are relatively stable structures which persist for hours rather than for the 1–2 min lifetime of normal coated pits (27).

SERIAL SECTIONS REVEAL CONNECTION TO PLASMA MEMBRANE: The probability of both the pit and its entire neck being included in any one single section is small. Usually the structure is cut at an angle to reveal either the pit and bud, with more or less of the neck and stalk (Fig. 1*M* and 2*H*), or the neck and stalk, cut longitudinally or transversely, but not the pit and bud (Fig. 1, *L* and *M*). By serial sectioning, however, the whole structure can be traced, as the two examples in Fig. 2 show. We have examined in serial sections more than 60 of these structures and all of them proved to have a neck and stalk connecting to the respective plasma membranes. If the coated pits detached to form free coated vesicles at a rate comparable to the rate of their invagination, detached vesicles, either coated or uncoated, containing a detached virus particle should have been seen. Since detached vesicles each enclosing a virion were never seen, these structures must be blocked such that neither endocytosis nor budding reach completion. All the detached coated vesicles in these preparations were normal and without virions.

CLUSTERS OF BLOCKED COATED PITS: Sometimes the blocked pits occur in clusters, especially at the rims of the cells (Fig. 3), a region that is analogous to the ruffing edges of more motile cells such as fibroblasts. Given the short average lifetime of normal coated pits (27), we interpret this clustering to indicate an accumulation of these structures over time rather than their synchronous formation. Note that in Figs. 2, *A–E*, and 3, the complex pits enclose more than one bud (see below).

PULSE-CHASE LABELING WITH CATIONIZED FERRITIN: Using cationized ferritin as an electron-dense label, pulse-chase experiments were done in an attempt to show that blocked coated pits formed during the tracer pulse were stable and remained labeled during the chase. First, to establish that cationized ferritin cannot simply diffuse down the necks of the blocked pits, 3–4-d-old AtT20 cell cultures were chilled to 4°C and then incubated with 100 μg/ml of cationized ferritin in medium without protein. Even after a 4-h incubation at 4°C, none of the blocked coated pits containing viral buds, which presumably had formed during the prior incubation at 37°C, were labeled (data not shown). Other cultures were allowed to bind and endocytose the label (100 μg/ml) for 1 h at 37°C. Some of the cultures were then fixed; others were very thoroughly washed and incubated in protein-free medium in the absence of the label for a further 2 h and then fixed. After a 1-h exposure at 37°C to cationized ferritin, the ligand could be seen in normal coated pits, in tubular endosomal structures close to the cell surface, and in lysosomes deeper within the cytoplasm. In addition, after labeling at 37°C, in contrast to 4°C, cationized ferritin was incorporated into blocked coated pits containing viral buds (Fig. 4, *A* and *B*). Furthermore, after a 2-h chase some of the blocked pits were still labeled with ferritin even on pairs of cells whose plasma membranes were free of label (Fig. 4*C*). The simplest interpretation of images such as that in Fig. 4*C* is that the blocked pits were labeled as they formed during the 1-h pulse of cationized ferritin and remained stable throughout the 2-h chase, during which cationized ferritin, bound to other parts of the plasma membranes of the cells, was cleared by endocytosis.

MORE THAN ONE A-TYPE PARTICLE IN BLOCKED PITS: Sometimes two or three MuMTV A-type particles are processively invaginated by a single coated pit structure (Fig. 5). Serial sections of such structures (Figs. 2 and 3) show that the A-type particles are connected to one another and to their parental cell by a sheath of the latter's plasma membrane, which, between the particles and in the neck of the coated pit, becomes tubular (Fig. 5, *B* and *C*). Significantly, only the spherical parts of the membrane of the coated pit which are directly surrounding the viral buds are coated. The tubular regions in the neck and between the viral particles are not coated.

Only cytoplasmic MuMTV A-type particles can give rise to these complex pit structures. MuLV C-type particles cannot form them because their cores develop gradually at the site of budding rather than being delivered as fully formed spheres to below the plasma membrane. Since the budding of enveloped viruses requires the progressive association of the viral core with plasma membrane in which viral envelope glycoproteins are accumulated to the exclusion of other cellular membrane proteins, it must be a gradual process. Therefore, the complex blocked coated pits with three MuMTV buds

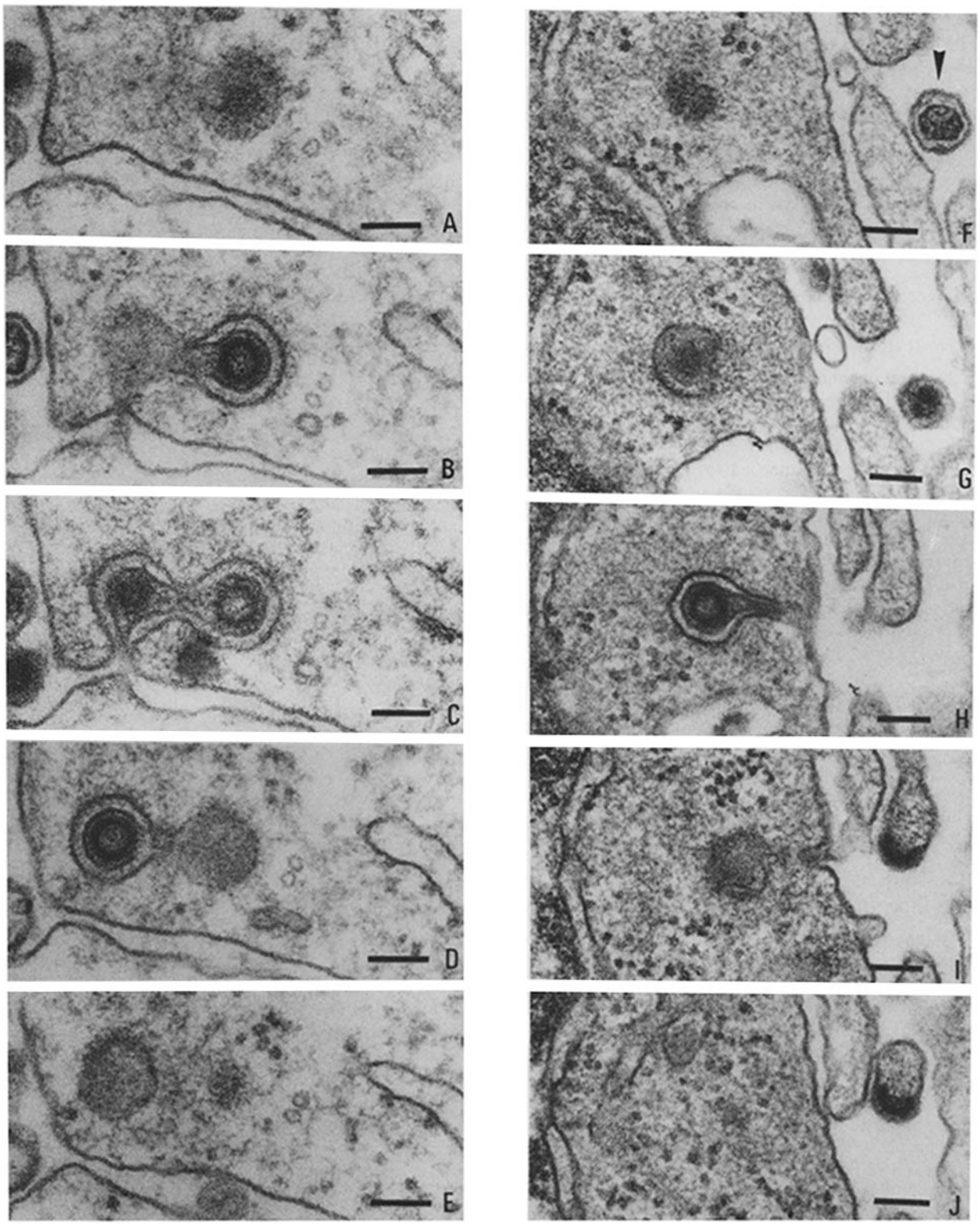

FIGURE 2 Serial sections ~400 Å thick of blocked coated pits. *A–E* show two A-type particles within a complex coated pit. *F–J* show a blocked coated pit connecting to the tip of a thin projection of a second cell. Note the fibrillar material around the blocked coated pits and in Fig. 2 *F* the extracellular virion (arrowhead) with a mature nucleoid. Bars, 0.1 μm. (*A–E*) × 105,000; (*F–J*) × 95,000.

must have persisted at least for the length of time required to produce that many viral buds.

A-type particles never bud in rows from regions of the plasma membrane facing extracellular spaces or the medium. There they bud only singly. In other words, this processive budding appears to be a consequence of the invagination of the first bud into a coated pit and, as a result, its failure to detach from the parental cell.

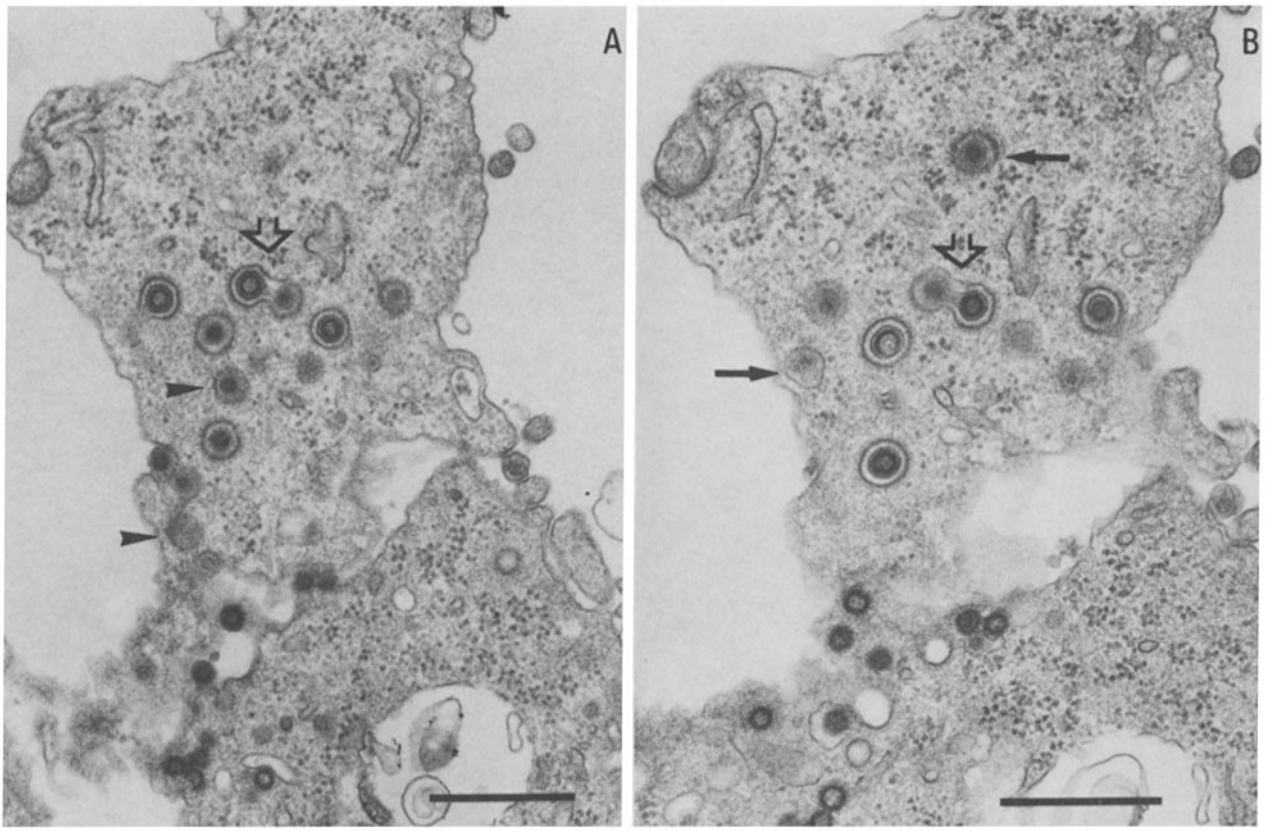

FIGURE 3 Micrographs of two consecutive sections ~600 Å thick in a ribbon of 10 sections showing the leading edges of two contiguous cells. Note several blocked coated pits at the rim of one cell and numerous MuMTV A-type particles at the rim of the contiguous cell. The blocked coated pits indicated by arrowheads in *A* are not included in *B*; conversely, that arrowed in *B* is not included in *A*. Note the complex pit containing two viral buds (open arrow). The whole ribbon of 10 sections, of which these are two, contained a cluster of at least 17 blocked coated pits. In this particular case, all the pits were in one cell and the A-type particles in the other. In general, however, it is not the case that one cell donates the buds to the pits of its neighbor, but rather that each cell along its surface has coated pits enclosing a neighbor's buds and its own buds in its neighbor's pits. The polarity indicated in this figure is fortuitous. Bars, 0.5 μm. (*A*) × 40,000; (*B*) × 44,000.

BLOCKED COATED PITS INVOLVING LYSED CELLS: AtT20 cultures, especially when dense, contain some lysed cells and much cellular debris accumulates in the medium. In thin sections of such cultures, one sometimes sees blocked coated pits involving a lysed cell that has lost most of its cytoplasm and a normal cell (Fig. 6). Assuming that the cells lysed before fixation rather than during it, images such as those in Fig. 6, *A* and *B*, provide further circumstantial evidence that the blocked coated pits are stable.

Clathrin Coat of Blocked Coated Pits Is Stable

The blocked coated pits at all stages of development, including those that have invaginated, have at least a partial coat on their cytoplasmic face. Using immunogold labeling procedures, this coat can be labeled (Fig. 7) with an affinity-purified anticlathrin antiserum (13). There is, however, no clathrin detectable with this antibody lining the necks of the pits (Fig. 7), which in conventional thin sections do not appear to be coated.

Many of the micrographs (Figs. 1 *K*, 2 *F–J*, 3, and 6) show that the blocked coated pits and their necks are surrounded by extensive finely fibrillar material without ordered structure following the fixation procedures used. This material is not labeled with the anticlathrin antibody and I have also failed to label it by immunoelectron microscopy with a rabbit antiserum against smooth muscle actin. In this context, it is significant that exposure of AtT20 cells to cytochalasin B, a drug known to inhibit actin gelation and microfilament function (22, 28), at concentrations up to 40 μg/ml for 1 h at 37°C does not significantly change the structure of the blocked coated pits, of their necks, or of the associated fibrillar material (not shown). This drug also has little effect on the endocytosis of Semliki Forest virus by baby hamster kidney cells (15).

Viral Cores Fail to Mature

In all of the several hundred blocked coated pits that have been examined, the core of the viral bud had in cross section an annular structure (Figs. 1, 2, 3, and 5, for example). This indicates that the final steps in the maturation of the viral cores into compact mature nucleoids is blocked. By contrast, fully budded progeny virions lying free in the medium or extracellular spaces have the condensed uniformly electron-dense nucleoids characteristic of mature retrovirions (Figs. 2 *F* and 8). This establishes that the cores of the endogenous MuLV and MuMTV of AtT20 cells are capable of completing their morphological maturation but only after they have detached from their parental cells.

DISCUSSION

The observations reported here establish that in cultures of

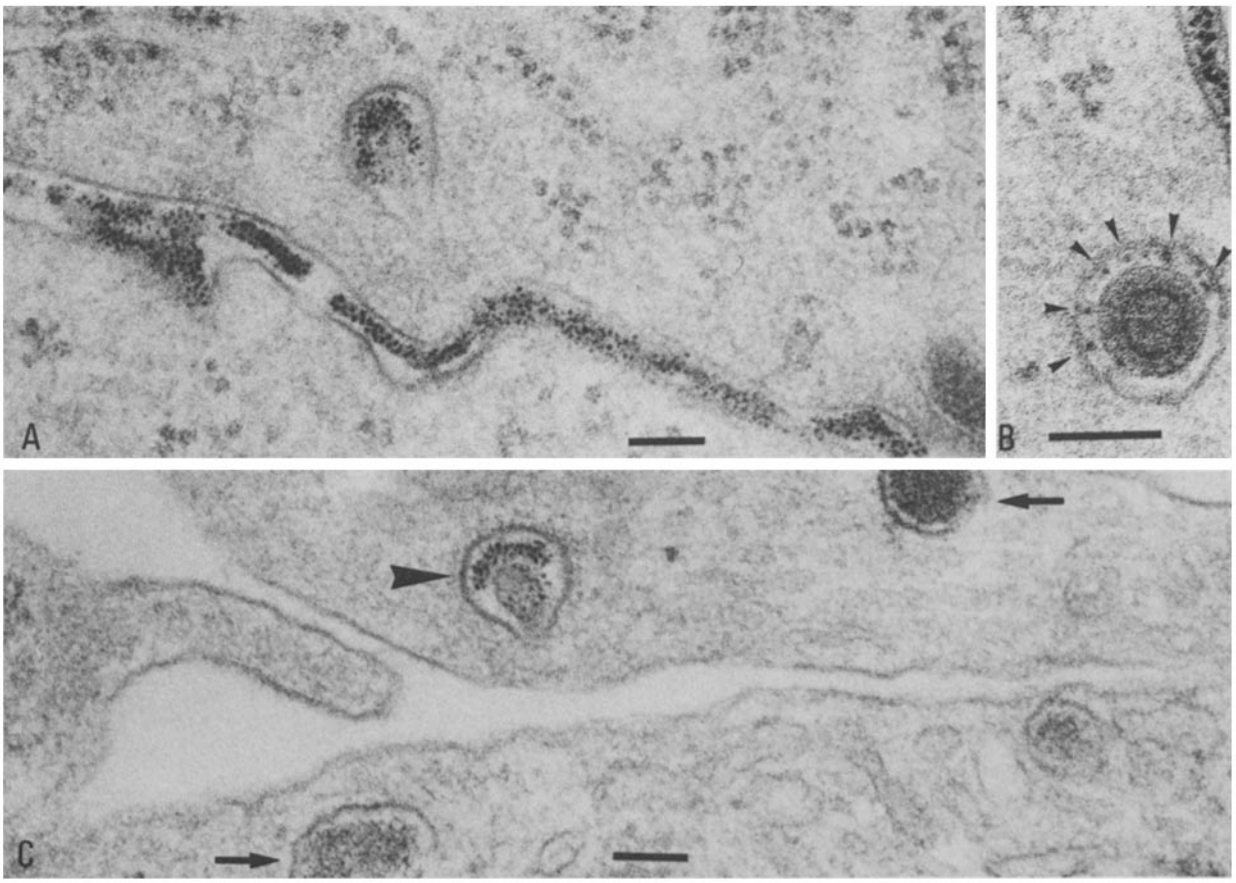

FIGURE 4 Blocked coated pits labeled with cationized ferritin. *A* and *B* show pits labeled with cationized ferritin during a 1-h pulse of the tracer (100 μg/ml) at 37°C followed by immediate fixation. Note that the label lines the plasma membranes of the cells as well as being within the blocked coated pits. *B* shows particles of ferritin (arrowheads) in the space between the envelope of the bud and the outer surface of the coated pit. *C* shows a labeled blocked coated pit (arrowhead) but the adjacent plasma membranes are not labeled with ferritin. These cells were exposed to the label for 1 h at 37°C, then washed thoroughly and incubated in the absence of label for a 2-h chase period. ACTH containing secretory granules are indicated by arrows. Bars, 0.1 μm. (*A* and *C*) × 100,000; (*B*) × 150,000.

AtT20 cells budding particles of the endogenous MuLV and MuMTV are sometimes invested by coated pits of contiguous cells. These coated pits are able to invaginate to depths of up to 4000 Å, drawing with them into the cytoplasm the viral buds which remain attached by narrow tubular stalks running down the extended necks of the pits to the plasma membrane of their parental cells. Further steps in both endocytosis and viral budding are blocked and these unique structures appear to be stable for at least a few hours. Blocked coated pits are relatively rare; in any one thin section they may be present in <5% of the cells and so they are easily overlooked. On the other hand, their properties raise several interesting points concerning endocytosis and viral budding which justify searching thin sections for them.

Viral Interference and Blocked Pits

Can the initial stages of receptor-mediated endocytosis of retroviral buds by cells infected with the same viruses be reconciled with viral interference, which prevents a cell from taking up virus particles with the same envelope glycoproteins as those specified by the resident retrovirus? Since I have never observed the endocytosis of free progeny MuLV or MuMTV particles, which accumulate in large numbers in the extracellular spaces in clumps of AtT20 cells, it appears that as far as free virions are concerned these cells exhibit classical interference. I suggest that two differences between a bud and a free virion are crucial and can account for the partial endocytosis of the former despite interference and the failure of the latter to be endocytosed because of interference. These differences are the respective mobilities and surface curvatures of a bud and a virion. The surface of a bud, especially at its earliest stages, has little curvature, whereas a virion is a 1,200-Å sphere; furthermore, a bud is immobile compared to an extracellular virion moving by random motion. Because of viral interference, most receptors in the plasma membrane of AtT20 cells will be saturated. Presumably, when an extracellular virion touches the cell surface, too few free receptors are present in the small area of contact to bind to the envelope spike glycoprotein of the particle and anchor it before it moves. The virion will, therefore, never be endocytosed. By contrast, a bud emerging from a contiguous cell presents initially a surface of low curvature in which a cluster of envelope spike glycoprotein molecules is progressively enlarging, and this structure is relatively immobile. In this situation, any free receptors in the larger area of contact can bind to the bud's surface and, since the bud is relatively immobile, more receptors can steadily be recruited to the site. The binding of receptors to the clustered envelope glycoprotein molecules necessarily clusters the receptors and this could act as a signal

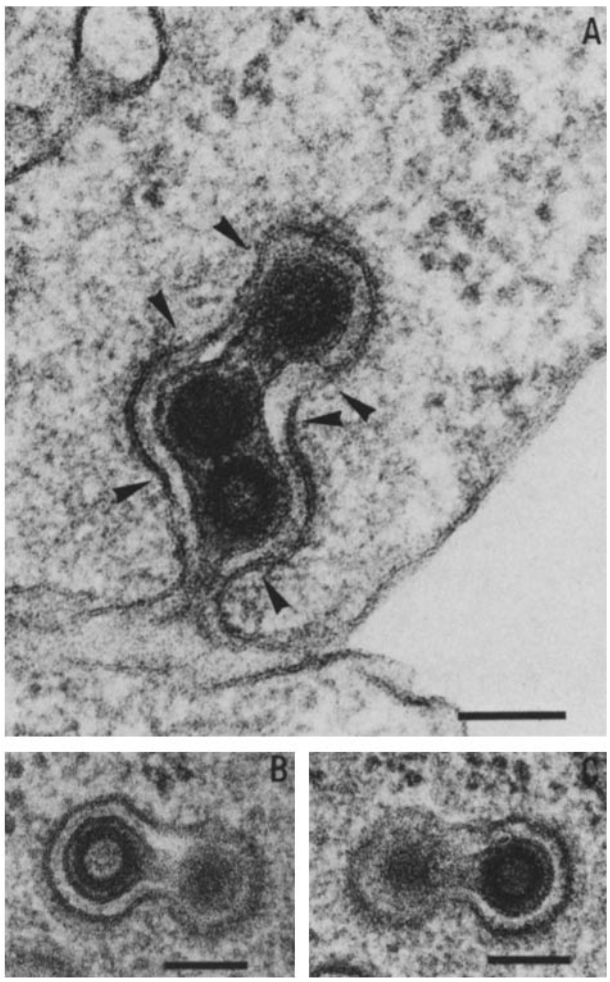

FIGURE 5 Complex blocked coated pits. *A* shows three A-type MuMTV particles within a single coated pit structure. This figure emphasizes that coated pits can enlarge to accommodate large ligands such as a row of virions. The arrows indicate regions of the pit's cytoplasmic surface which are coated; note that diffuse and particulate material is present between the bud's envelopes and the outer surface of the pit in these same regions. Complex coated pits would arise when small clusters of A-type particles (as shown in Fig. 1, *D* and *I*) bud in succession. *B* and *C* show at higher magnifications parts of Fig. 3, *A* and *B*, with two A-type particles in a complex pit. The connection to the plasma membranes of the two cells involved was traced in other sections of the series. Note that the two buds are connected by a tubular region similar to the stalk of single buds in coated pits and that the clathrin coat is restricted to the surfaces of the pit enclosing the buds. Bars, 0.1 μm. (*A*) × 140,000; (*B* and *C*) × 110,000.

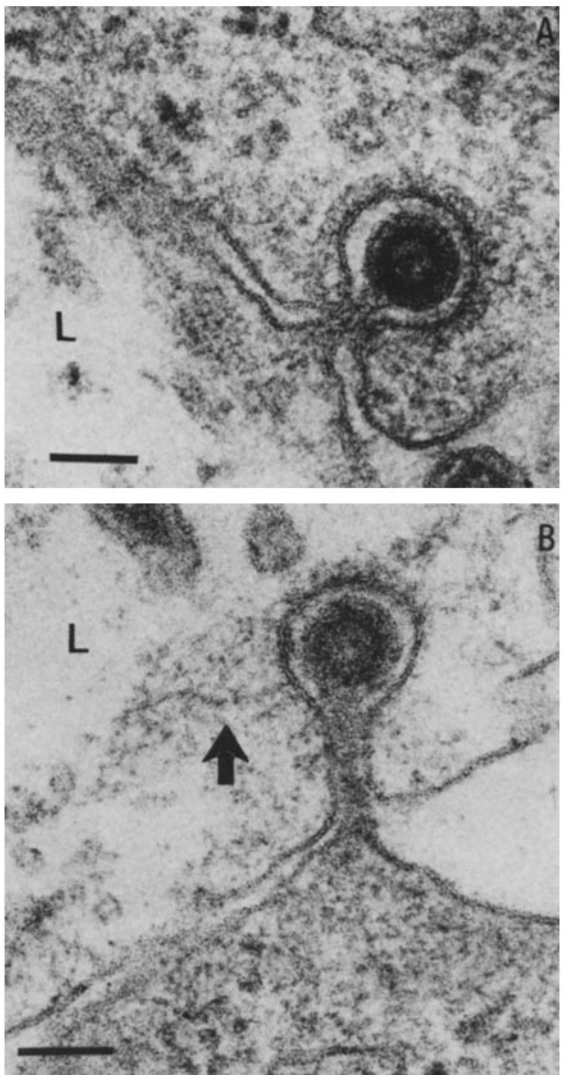

FIGURE 6 Blocked coated pits involving lysed cells. *A* shows a bud emerging from a cell that has lysed (*L*) in a coated pit of a normal cell. *B* shows the converse situation with the coated pit in the lysed cell (*L*). Note the fibrillar material (arrow) still associated with the coated pit. Bars, 0.1 μm. (*A*) × 115,000; (*B*) × 125,000.

to trigger the polymerization of clathrin on the cytoplasmic face of the membrane. Fig. 1, *A* and *B*, clearly shows that initially the area of membrane that is coated on its cytoplasmic face significantly exceeds the area of intimate association between the bud's envelope and the coated membrane. Possibly, therefore, once initiated by clustered receptors, the polymerization of clathrin spreads well beyond the nucleation site delimited by the receptor cluster. However, as the bud develops, so the apposed coated membrane progressively follows the bud's contours. This presumably reflects the recruitment of more free receptors to the coated area and their association with the bud's developing envelope. The end result is a viral bud completely invested by a coated pit. This resolves the paradox and reconciles these observations with the phenomenon of viral interference. In short, the viral bud represents a cluster of ligand free to move in only two dimensions, the plane of the cell membrane, while a virion is free to move in three dimensions. As a result, mobile receptors in an adjacent plasma membrane are exposed to the ligands of a bud for longer times than they are exposed to the ligands of a free virion, and therefore the former but not the latter are enclosed in coated pits.

Distribution of Clathrin

On the blocked pits, the clathrin coat is restricted to the spherical pit surface and does not extend to the tubular necks; the same is true of pits with necks in fibroblasts (17, 19, 25, 30). Moreover, in the complex pits with two or three A-type particles, the clathrin coat is restricted to each sphere of membrane surrounding a particle and does not cover the tubular regions between particles. In short, the distribution of the clathrin coat parallels the expected distribution of recep-

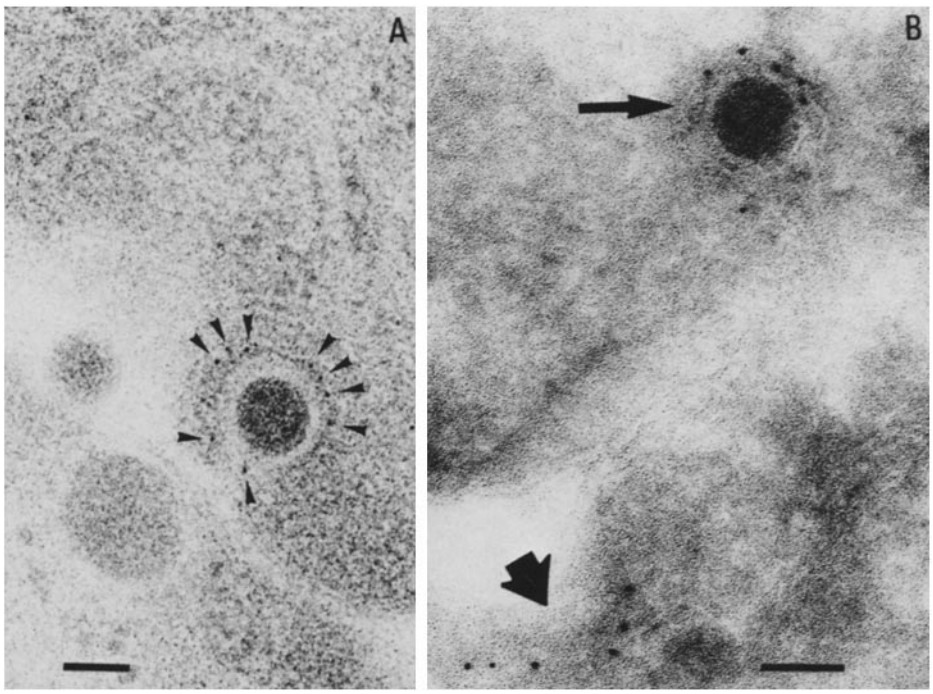

FIGURE 7 Immunogold labeling of clathrin. *A* shows a blocked coated pit in a section of cells embedded in Lowicryl K4M and labeled with an affinity-purified anticlathrin antibody and 7 nm gold. The labeling (indicated by arrowheads) is restricted to the cytoplasmic surface of the coated pits. The section was contrasted with uranyl acetate and lead citrate. *B* shows a cryosection labeled with the same antibody and stained with uranyl acetate. The clathrin labeling is restricted to the cytoplasmic surface of the blocked (arrow) and normal coated pits (wider arrow). Bars, 0.1 μm. (*A*) × 90,000; (*B*) × 15,000.

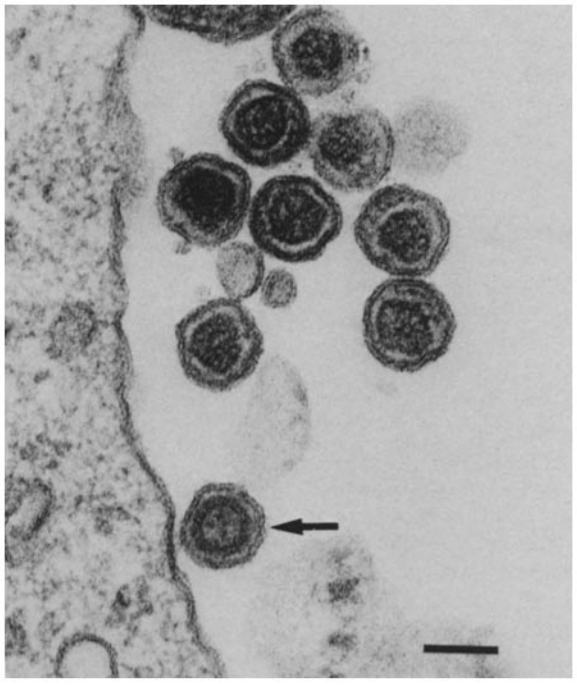

FIGURE 8 A cluster of completely budded extracellular retrovirions close to the surface of an AtT20 cell. Note that the core of the arrowed virion has the immature form. Those of the other virions are mature and condensed. Bar, 0.1 μm. × 95,000.

tor-envelope spike glycoprotein complexes. In most of the micrographs, electron-dense particles line the outer face of the coated pit membrane. These granules are more prominent in pits enclosing MuMTV A-type buds than MuLV C-type buds and, significantly, MuMTV virions have more prominent envelope spikes than MuLV virions (for review see reference 10). This granular material, which is usually closer to the membrane of the pit than the envelope of the bud, does not, as far as is discernible, extend down the necks of the pits; it could, therefore, be aggregates of receptor-envelope spike glycoprotein complexes. If this interpretation is correct, it is interesting that the clathrin coat initially extends beyond the region containing these complexes (Fig. 1, *A* and *B*), but once the bud is fully invested, the complexes on one side of the pit membrane and clathrin on the other often have similar limits of distribution (for example, Fig. 1, *F–H*; also, Fig. 5). The coincidence between dense granules on the extracellular side of a coated pit membrane and clathrin on the cytoplasmic side in chick oocytes endocytosing lipoprotein-like particles has been commented on by Perry and Gilbert (18). The clustered receptors bound to ligand may, therefore, place an upper limit on the final distribution of the clathrin coat.

Alternatively, the absence of a clathrin coat from the tubular regions of the blocked coated pits might be the result of steric factors. Perhaps the clathrin lattice cannot accommodate the bend at the junction of the spherical pit surface and the tubular pit neck.

Uncoupling of Pit Detachment from Invagination

Normally, the detachment of coated pits to generate free coated vesicles, the uncoating of the vesicles, and movement of the vesicles deeper into the cytoplasm are rapid processes whose precise sequence is difficult to determine. The structures described here could be considered as a blocked intermediate of this normally very rapid set of events. The mere existence of the blocked coated pits establishes that the step of pinching off a pit to form a free vesicle is not an absolute prerequisite of the movement of a pit deeper into the cytoplasm. Since coated pits with necks in connection with the plasma membrane have also been reported by others (17, 19, 25, 30), one might suggest that in general invagination shortly precedes pinching off during normal endocytosis. Of course the fact that the blocked coated pits in AtT20 cells are stable structures unable to complete endocytosis also raises the possibility that whenever and wherever a pit, regardless of its contents, moves into the cytoplasm before detaching from

the plasma membrane, that pit is blocked. Alternatively, the structures described here may be blocked because of the failure of the viral bud to detach.

That the blocked coated pits in AtT20 cells always have at least a partial clathrin coat on the spherical surfaces enclosing the viral bud strongly suggests that they cannot be uncoated by the recently discovered uncoating enzyme (20). Pinching off a pit to yield a coated vesicle, presumably accompanied by a change in the conformation of the clathrin coat, may therefore be an essential prerequisite of uncoating.

Construction of the Neck of the Pits

A striking feature of the overall morphology of the blocked coated pits is the narrowness, uniform diameter, and straightness of their necks. The invagination of a viral bud still attached to its parental cell by a short stalk does not wedge open the neck of the pit. Instead the stalk of the bud elongates into a very narrow tube running down the length of the neck. Apparently, something acting like a sphincter or collar at the site where the coated pit joins the plasma membrane tightly constricts both the neck of the pit and the stalk of the bud, and the constriction is maintained as the coated pit invaginates to a depth of some 3,000 Å drawing with it bud and stalk. Unfortunately the micrographs give no clue as to the nature of the constriction; they simply imply its existence. The straightness of the neck of these pits compared to the necks of coated pits in other cells (17, 19, 25, 30) may well result from the presence of the concentric tubes of plasma membrane.

Failure of Budding to Complete

The existence of these blocked coated pits indicates that the formation of the coated pit and its subsequent invagination must be faster than the completion of viral budding. Once the bud is invaginated neither endocytosis nor budding go to completion. The stalk of the bud may prevent detachment of the coated pit, but why does budding fail to complete? Little is known about the molecular interactions involved in the budding of retroviral particles (for reviews see references 7 and 8). However, by analogy with other enveloped viruses that bud from the plasma membrane (for reviews see references 8 and 21), the essential step is probably the association of one or more proteins in the shell of the viral core with the cytoplasmic tails of the transmembrane spike anchoring protein (7, 8, 16). As budding proceeds, these viral envelope glycoprotein molecules cluster in the plasma membrane at the site of the bud and their interaction with viral core proteins wraps the membrane around the core. Normally budding is completed by a membrane fusion event when the plasma membrane is completely constricted at the base of the bud. In the blocked coated pits, budding fails to go to completion, the membrane fusion event is prevented when the bud is at the end of its narrow stalk within the pit. If the parental cell's plasma membrane is fluid enough to be drawn out into a tube up to 2,000 Å long but only 255 ± 15 Å in diameter, it should certainly be fluid enough to continue to wrap around the spherical viral core within the pit. Rather, I suggest that the failure of budding to go to completion is the result of an exclusion of viral envelope glycoproteins from the very narrow stalk. For steric reasons, it is highly unlikely that a lipid bilayer with a radius of curvature as small as 110–120 Å could accommodate any transmembrane proteins. When lipid vesicles are produced by spray-freezing pure egg lecithin, the smallest obtained have a radius of 100–120 Å (26). The narrow stalk of the buds may well, therefore, exclude all membrane proteins and act as an effective barrier preventing further envelope glycoprotein molecules from reaching the bud.

Failure of Viral Cores to Mature

Normally the final stage in the maturation of retrovirus particles occurs extracellularly, after their detachment from the parental cell. Over a period of time measured in hours rather than seconds, the spherical cores with an outer shell and electron-lucent center compact to yield the smaller, more uniformly electron-dense mature nucleoid; concomitant with this morphological change in the virions of murine retroviruses, the final steps in the cleavage of the viral core proteins from their polypeptide precursors take place within the virion (6–8). Significantly, the cores of invaginated buds in blocked coated pits do not undergo this final morphological change; they invariably retain the immature structure. This implies that detachment of a bud is a necessary prerequisite of the final biochemical and morphological maturation of its core. That being the case, perhaps a change in the environment within the virion (for example, a change in the ionic milieu) is essential to trigger this maturation, and perhaps this change can only occur after virions are totally isolated from the parental cell cytoplasm and no longer buffered by it. The fact that this final step in maturation is delayed until after detachment is also consistent with, and indeed essential to, models of budding which attribute the driving force to associations between core proteins and transmembrane proteins.

Processive Endocytosis of A-type Particles

A-type particles never bud in rows from the free surfaces of AtT20 cells into extracellular spaces; there they only bud singly. On the other hand, examples have been shown here of two or three A-type particles within one coated pit structure. Birdwell et al. (3) reported that in chick embryo fibroblasts infected with Sindbis virus, rows of over 100 viral cores project from the cell surfaces in fine processes. They proposed, to account for this multiple budding at restricted sites, that either the plasma membrane is more fluid at these sites or that the initiation of one bud alters the membrane so that it acts "as a center of nucleation to attract other nucleocapsids." In the case of AtT20 cells, the restriction of rows of A-type particles to coated pit structures suggests that it is the invagination of the first particle into the coated pit which induces the chain to form. Once invaginated, the first bud cannot detach. Any other A-type particle in the immediate vicinity can then move into the opening of the stalk connecting the first particle to the parental cell and begin to bud. Clustering of the envelope glycoprotein on the second bud would then induce a second coated pit around it and so on for a third cycle (Fig. 5). I have never seen more than three A-type buds in one complex pit. This upper limit may simply reflect the fact that A-type particles in AtT20 cells occur either singly or in only small clusters of two to five when they lie immediately below the plasma membrane.

Future Studies

The blocked coated pits described here do not occur often enough to make attempts at their isolation worthwhile; further

studies will, therefore, be restricted to immunoelectron microscopic approaches. With the appropriate antibodies, it should be possible to answer at least some of the questions these structures pose; for example, whether or not viral envelope glycoproteins are present in the tubular membrane stalks of the buds and which cytoskeletal proteins, if any, are present in the fibrillar cytoplasmic material that is frequently present around the coated pits.

If these structures are blocked such that neither budding nor endocytosis can be completed, what is their ultimate fate? Do they persist indefinitely as a novel form of intracellular junction or are they eventually eliminated? Preliminary observations suggest that at least some blocked coated pits end up in secondary lysosomes or autophagic vacuoles near the cell surface in which cationized ferritin also accumulates. How the blocked coated pits enter these structures and how they are detached from the plasma membranes remains to be elucidated.

It would also be interesting to examine carefully other cells supporting the replication of retroviruses to determine how general these blocked coated pits are. Given the very many electron microscopic studies of cells infected with retroviruses, it is surprising that such remarkable structures have not been previously reported. Their formation probably depends critically on factors such as the extent of viral budding, the efficiency of viral interference, and the growth of the cells in clumps.

It gives me pleasure to thank my colleagues, and in particular S. Fuller, G. Griffiths, L. Roman, K. Simons, and G. Warren, for their advice, criticism, and suggestions during this work. S. Fuller and L. Roman also criticized the manuscript. I am especially indebted to S. Tooze for culturing the cells and for advice and suggestions throughout. D. Louvard and T. Kreis generously provided antibodies. A Ohlsen, U. Reuter-Carlson, and R. Back gave me materials for the electron microscopy as well as advice. I. Benner typed the manuscript with skill and patience.

Received for publication 24 June 1985, and in revised form 10 July 1985.

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