JOURNAL OF VIROLOGY, Mar. 1997, p. 2072–2082 Vol. 71, No. 3
0022-538X/97/$04.00+0

Ultrastructural Analysis of the Replication Cycle of Pseudorabies Virus in Cell Culture: a Reassessment

HARALD GRANZOW,[1]* FRANK WEILAND,[2] ALICE JÖNS,[3] BARBARA G. KLUPP,[3] AXEL KARGER,[3] AND THOMAS C. METTENLEITER[3]

Institutes of Diagnostic Virology[1] and Molecular and Cellular Virology,[3] Friedrich-Loeffler-Institutes, Federal Research Centre for Virus Diseases of Animals, D-17498 Insel Riems, and Institute of Microbiology, Federal Research Centre for Virus Diseases of Animals, D-72076 Tübingen, Germany[2]

Received 25 October 1996/Accepted 10 December 1996

We reinvestigated major steps in the replicative cycle of pseudorabies virus (PrV) by electron microscopy of infected cultured cells. Virions attached to the cell surface were found in two distinct stages, with a distance of 12 to 14 nm or 6 to 8 nm between virion envelope and cell surface, respectively. After fusion of virion envelope and cell membrane, immunogold labeling using a monoclonal antibody against the envelope glycoprotein gE demonstrated a rapid drift of gE from the fusion site, indicating significant lateral movement of viral glycoproteins during or immediately after the fusion event. Naked nucleocapsids in the cytoplasm frequently appeared close to microtubules prior to transport to nuclear pores. At the nuclear pore, nucleocapsids invariably were oriented with one vertex pointing to the central granulum at a distance of about 40 nm and viral DNA appeared to be released via the vertex region into the nucleoplasm. Intranuclear maturation followed the typical herpesvirus nucleocapsid morphogenesis pathway. Regarding egress, our observations indicate that primary envelopment of nucleocapsids occurred at the inner leaflet of the nuclear membrane by budding into the perinuclear cisterna. This nuclear membrane-derived envelope exhibited a smooth surface which contrasts the envelope obtained by putative reenvelopment at tubular vesicles in the Golgi area which is characterized by distinct surface projections. Loss of the primary envelope and release of the nucleocapsid into the cytoplasm appeared to occur by fusion of envelope and outer leaflet of the nuclear membrane. Nucleocapsids were also found engulfed by both lamella of the nuclear membrane. This vesiculation process released nucleocapsids surrounded by two membranes into the cytoplasm. Our data also indicate that fusion between the two membranes then leads to release of naked nucleocapsids in the Golgi area. Egress of virions appeared to occur via transport vesicles containing one or more virus particles by fusion of vesicle and cell membrane. Our data thus support biochemical data and mutant virus studies of (i) two steps of attachment, (ii) the involvement of microtubules in the transport of nucleocapsids to the nuclear pore, and (iii) secondary envelopment in the *trans*-Golgi area in PrV infection.

Herpesviruses are large DNA-containing viruses which replicate in the nucleus of infected cells. On the basis of biological parameters supplemented by recent sequence data, the family *Herpesviridae* is divided into the subfamilies *Alpha-*, *Beta-*, and *Gammaherpesvirinae*. The majority of reports about herpesvirus replication concentrate on the human pathogenic alphaherpesviruses herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) or varicella-zoster virus (VZV). In addition, morphogenesis of the betaherpesvirus human cytomegalovirus (HCMV) has been studied in some detail (15, 42, 43). Although numerous ultrastructural analyses of the mechanism of multiplication of herpesviruses were performed (for reviews see references 38 and 44), partially combined with biochemical or immunological investigations, major steps in the infectious cycle are still unclear.

One of the controversial points concerns envelopment of nucleocapsids. Johnson and Spear (20) suggested that HSV-1 nucleocapsids are enveloped at the inner leaflet of the nuclear membrane which contains immature glycoproteins. Subsequently these virions are transported in the endoplasmic reticulum to the dictyosomes where glycoproteins are modified in situ as the enveloped virion transits through the Golgi stacks. In contrast, maturation of VZV (21) or pseudorabies virus (PrV) (6, 60) appeared to involve transit of nucleocapsids through the nuclear membrane by budding through the inner leaflet of the nuclear membrane followed by deenvelopment and release of nucleocapsids into the cytoplasm. Secondary envelopment was then proposed to occur in the *trans*-Golgi area at tubular vesicles containing terminally modified glycoproteins. The latter model was also proposed for HCMV (42, 43) and frog herpesvirus (50). Recent ultrastructural analysis of a specific PrV mutant lacking the UL3.5 protein provided additional evidence for this pathway (11).

Morphogenesis of PrV has been studied in ultrathin sections of tissues of infected animals (2, 6, 31, 32, 41) and in cultured cells (8, 10, 30, 46, 60). However, a comprehensive ultrastructural study of the various stages of intracellular PrV replication is still lacking. Here, we present an ultrastructural study of the major events in PrV replication and discuss the new results in light of existing data on herpesvirus multiplication.

MATERIALS AND METHODS

Cells and viruses. Madin Darby bovine kidney (MDBK), pig kidney (PSEK), and Vero cells were grown in minimum essential medium (Gibco) containing Earle's or Hank's salts and supplemented with 10% fetal calf serum (Gibco).

PrV strain Ka (PrV-wt) (22) was propagated in PSEK cells and purified on a 30 to 50% discontinuous sucrose gradient by using the procedure previously described by Klupp et al. (25).

Infection of cells. Confluent MDBK, Vero, or PSEK cells grown on petri dishes (60 mm diameter) or on glass supports (Lab-Tek chamber slides; Nunc, Naperville, Ill.) were incubated on ice for 15 min and were subsequently infected

* Corresponding author. Phone: 49-38351-7206. Fax: 49-38351-7151. E-mail: Harald.Granzow@rie.bfav.de.

with PrV-wt at a multiplicity of infection (MOI) of 100 or with gradient-purified PrV-wt at an MOI of 200 for adsorption/penetration experiments and an MOI of 1 to 2 for infection experiments. After an adsorption period of 1 h on ice the inoculum was quickly removed and replaced by fresh prewarmed medium. Cells were then incubated at 37°C in a 5% CO_2 atmosphere for different times. After three washes with phosphate-buffered saline (PBS), pH 7.2, cells were then processed for electron microscopy.

EM and immunolabeling of PrV. For routine electron microscopy (EM), noninfected and infected cell cultures in petri dishes (4, 5, 8, 10, 12, 14, 16, 18, 20, and 24 h postinfection [p.i.]) were fixed at different times after infection for 60 min with 2.5% glutaraldehyde buffered in 0.1 M Na-cacodylate (pH 7.2; 300 mOsmol) (Merck, Darmstadt, Germany). They were then scraped off the plate and pelleted by low-speed centrifugation and embedded in LMP agarose (Biozym, Oldendorf, Germany). Small pieces were postfixed in 1.0% aqueous OsO_4 (Polysciences Europe, Eppelheim, Germany) and stained with uranyl acetate. After stepwise dehydration in ethanol, the cells were cleared in propylene oxide, embedded in Epon 812 (Serva, Heidelberg, Germany), and polymerized at 59°C for 4 days.

After incubation (0, 30 s, 1, 2, 5, 10, 15, 20, 30, and 60 min) of chamber slides at 37°C, cells were fixed with 2.5% glutaraldehyde in PBS, pH 7.2, postfixed in 1% aqueous OsO_4, and processed in the chambers as for routine EM without embedding in agarose and clearing in propylene oxide.

The preembedding labeling of PrV after adsorption/penetration (0, 1, 5, 10, and 20 min of incubation at 37°C) was performed in chamber slides with a monoclonal antibody directed against PrV gE (58) and goat anti-mouse colloidal gold conjugate (GAM_{10}; BioCell, Cardiff, United Kingdom) as previously described (40). Fixation, dehydration (without clearing step in propylene oxide), and embedding was performed in the chambers as described above. Specificity of the reaction was controlled with a non-PrV-specific antibody.

Ultrathin sections, counterstained with uranyl acetate and lead salts, were examined with an electron microscope (Elmiskop 101; Siemens, Germany; Philips EM 400 T; Eindhoven, The Netherlands).

RESULTS

Initiation of infection. To analyze interactions between virus particles and cell surface, cells grown on chamber slides were incubated for 1 h on ice at an input MOI of 200 with sucrose gradient-purified PrV. The high MOI did not produce any toxic effect in the cell culture as deduced from control experiments using a lower MOI (data not shown). Immediately after replacing the inoculum with prewarmed medium (0 s) and after 30 s, or 1, 5, 10, 20, 30, 45, and 60 min cells were fixed in situ and after processing were cut perpendicular to their plane of growth. For an optimal visualization of the distance between virion and cell surface, ultrathin sections were tilted in the electron microscope. Although three different host cell lines of porcine, bovine, and primate origin were analyzed, overall results were similar. Therefore, representative electron micrographs are shown.

After 1 h at 0°C, virions were detected juxtaposed to the cell surface (Fig. 1A and B). The distance between virus envelope and cellular cytoplasmic membrane was determined by comparison with the size of visible viral surface projections to average between 12 and 14 nm. Immediately after the temperature shift (30 s), approximately 10 to 15% of virus particles were detected in a closer association with the cell membrane, with a distance averaging 6 to 8 nm (Fig. 1C; Table 1). One minute after temperature shift, virion envelope and cell membrane were in close contact, and fusion bridges could be detected, resulting in continuity between cellular and virion membranes (Fig. 1D). During these processes (Fig. 1E to G), viral tegument appeared unaltered without any recognizable dislocation or morphological transformation. After completion of the fusion event, tegument appeared to disperse in the cytoplasm with only remnants visible between cytoplasmic nucleocapsids and the fusion site (Fig. 1G).

By preembedding labeling with a monoclonal antibody against virion envelope glycoprotein E, rapid lateral movement of gE from the fusion site could be demonstrated as early as 1 min after initiation of penetration by temperature shift (Fig. 2A to D).

As early as 5 min after temperature shift numerous intracellular nucleocapsids were detected. Frequently, nucleocapsids were found in close proximity to cellular structures resembling microtubules by their typical morphology (24) (Fig. 2E and F). Although microtubules are known to depolymerize in the cold, they can be quickly restored within minutes at elevated temperature (57). At 5 min after temperature shift nucleocapsids were first observed juxtaposed to nuclear pores (Fig. 2G). Invariably, nucleocapsids at nuclear pores were oriented with one vertex pointing to the central granulum of the pore at a distance of 40 nm. Our data indicate that viral DNA appears to be released from morphologically intact nucleocapsids via the juxtaposed vertex region through the nuclear pore into the nucleoplasm (Fig. 2H and I). During this stage of infection, neither disassembly of nucleocapsids nor localization of nucleocapsids in the nucleus could be detected.

Morphogenesis of nucleocapsids. After hypertrophy of the nucleus, different stages of nucleocapsid assembly were detected, similar to that found in other herpesviruses (36, 51, 52). Crescent-shaped capsid structures with annular proteinaceous material, presumably consisting of scaffolding protein, arranged singly or in pseudocrystalline arrays, hexagonal capsids with annular protein, empty capsids, and capsids containing DNA were observed (Fig. 3A). At higher magnification, it appeared as if nucleic acid threads were associated with the vertices of the capsid (Fig. 3B). Concomitant with the detection of nucleocapsid assembly stages in the nucleus, the following events were simultaneously observed in all infected cells, irrespective of the incubation time after infection from 4 h p.i. onward.

Primary envelopment of nucleocapsids and exit from the nucleus. Primary envelopment of nucleocapsids was found to occur by budding through the inner leaflet of the nuclear membrane. Frequently, nucleocapsids were observed in intimate contact with the inner nuclear membrane, with the concomitant appearance of a sharp-bordered rim of electron-dense material at the predestined site of budding. Interestingly, surface projections were absent from the vaulting envelope. Between nucleocapsids and initial tegument, a clear halo was a distinctive feature in primary enveloped virions. This budding process resulted in release of enveloped monocapsidic virions into the perinuclear cisterna (Fig. 3C to F and Fig. 4A to C).

Ultrastructurally, two ways of putative nucleocapsid egress from the perinuclear cisterna were observed. First, budding capsids were found to be engulfed by the two leaflets of the nuclear membrane and after vesiculation they were detected in the cytoplasm surrounded by a bimembraneous structure (Fig. 3F and G). These virion-containing vesicles were observed in the vicinity of the Golgi apparatus where nucleocapsids appeared to be released by fusion of the inner (primary envelope) with the outer leaflet (vesicle membrane) (Fig. 3H). Second, nucleocapsids surrounded by a smooth envelope remained in the perinuclear cisterna (Fig. 4A to C) until released into the cytosol by fusion between the primary viral envelope and outer nuclear membrane (Fig. 4D). Both ways were observed in all sections studied but differed significantly in their frequency of detection (Table 1).

Naked intracytoplasmic nucleocapsids were detected adjacent to, within or partially wrapped by membranes in the Golgi area (Fig. 4E to G) which were most likely part of the *trans*-Golgi network (TGN). Comparing more than 100 virions in the perinuclear cisterna with more than 200 virions within Golgi vesicles in an optimal plane of section, this budding process was clearly distinguishable from primary envelopment and release of capsids from vesicles as described above. The decisive

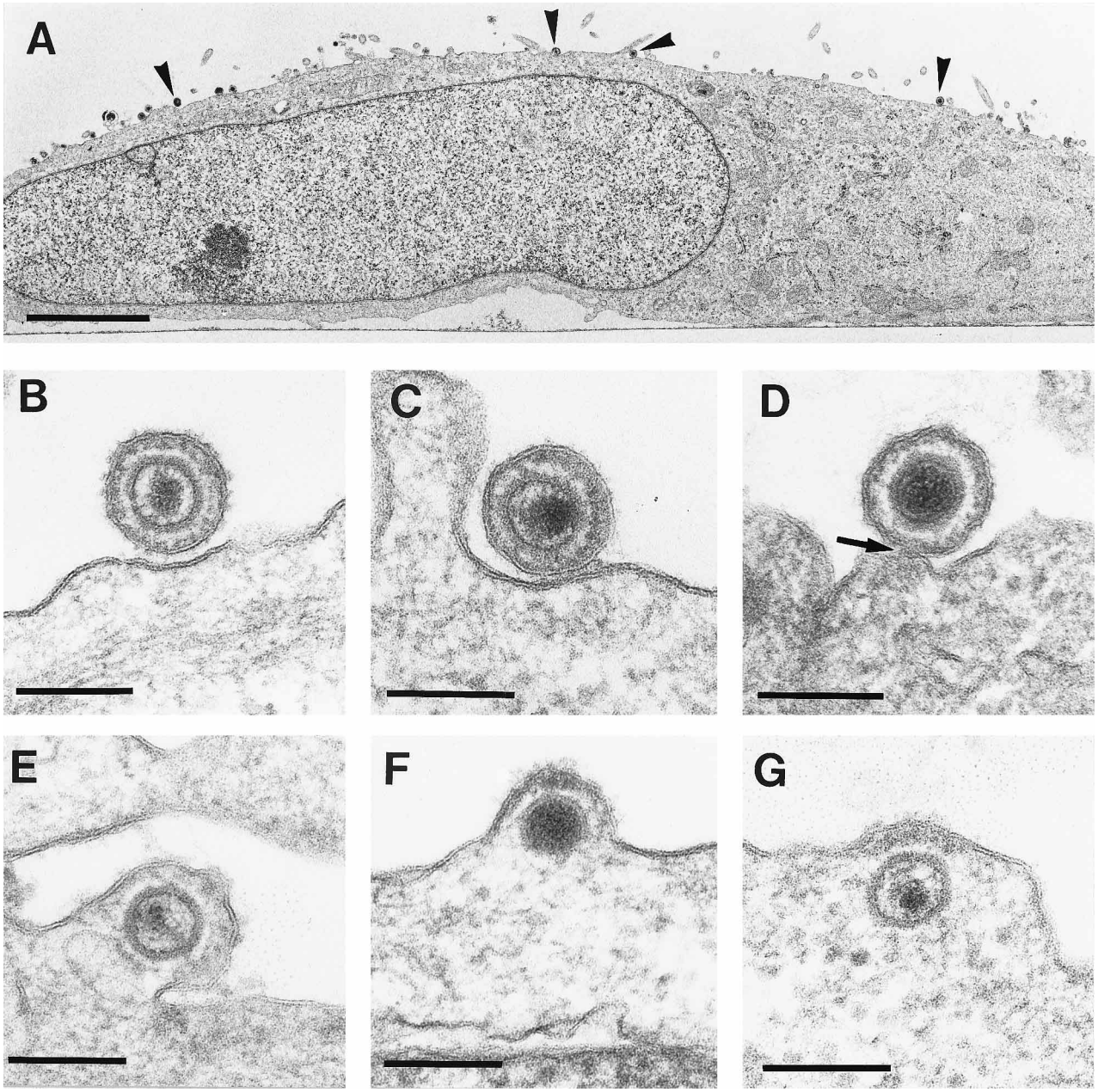

FIG. 1. Attachment and penetration of PrV. PrV virions (arrowheads) between microvilli adsorbed at the cell membrane of an MDBK cell (A). Initial attachment at the surface of a PSEK cell (B) as well as a closer association immediately after temperature shift from 0 to 37°C (C) is shown. In panel D penetration of a MDBK cell is initiated by formation of a fusion bridge (arrow) and completed by release of the nucleocapsid into the cytoplasm (E to G). All these stages (panels B to G) were detected as early as 1 min after temperature shift. Bars, 2.5 μm (A) or 150 nm (B to G).

difference is the visible presence of surface projections at the future virion envelope (Fig. 4F) and the addition of tegument material of less electron density than that of the material accumulated during budding into the nuclear cisterna (Fig. 4A to G).

Virus egress. After putative secondary envelopment, morphologically mature virions were located either as single ones in vesicles (Fig. 4H) or as multiple virions in larger vacuoles. The latter most probably arose by fusion of smaller vesicles. Virions were released by fusion of the vesicle/vacuole membrane with the cell membrane in an exocytotic process (Fig. 5A and B). At the peak of virus production, the cytoplasm of

TABLE 1. PrV particle distribution during virus replication

% PrV particles at the cell surface		% Budding at nuclear membrane		% Cytoplasmic nucleocapsids 12–18 h p.i.		
Distance 12-14 nm	Distance 6-8 nm	Through both lamella	Only through the inner lamella	Naked	Partially enveloped	Enveloped
100[a]	0	<10	>90	~45	~15	~40
85–90[b]	10–15					

[a] After adsorption at 4°C without temperature shift.
[b] After adsorption at 4°C and 1 min of incubation at 37°C.

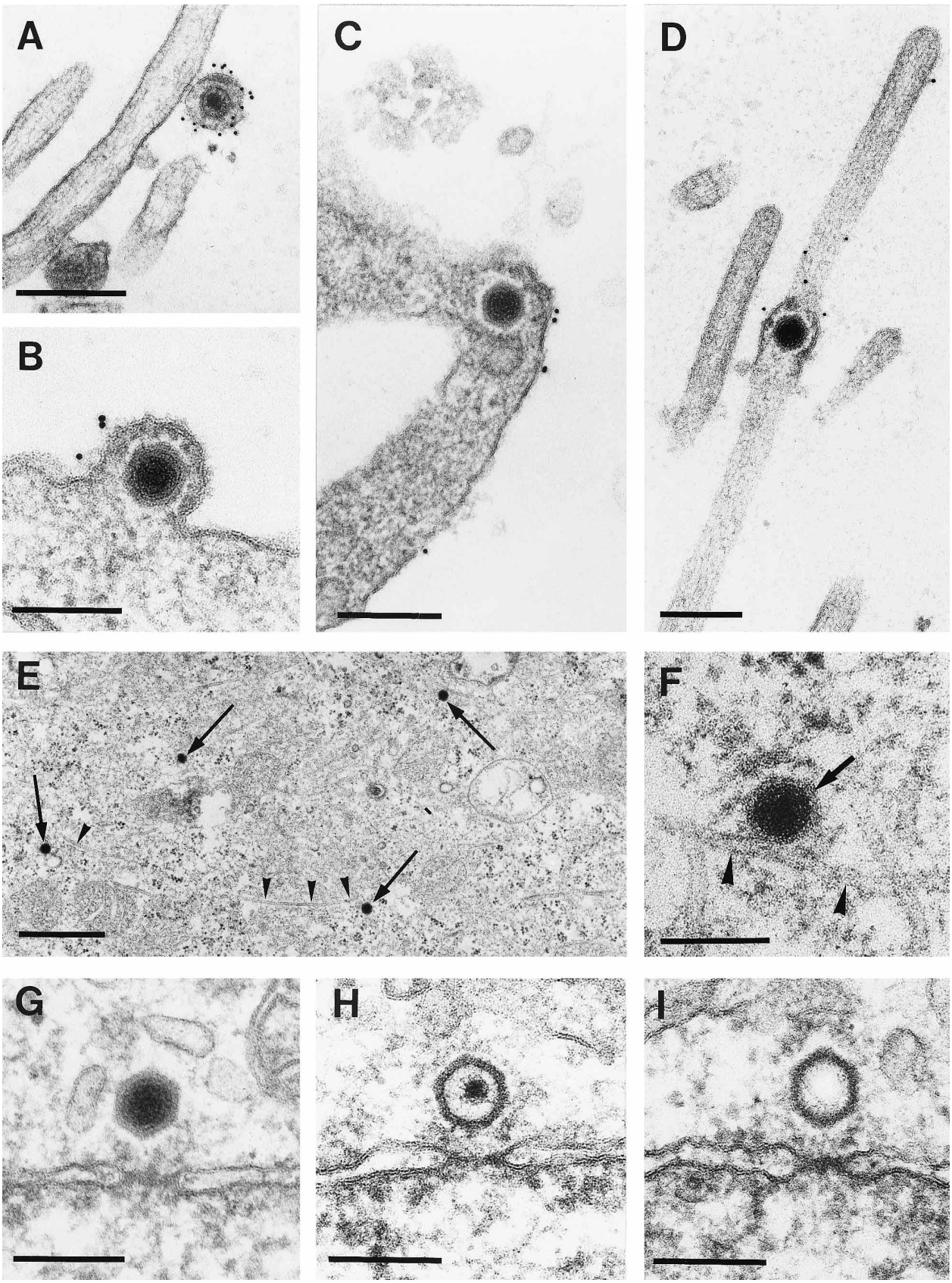

FIG. 2. Glycoprotein labeling and uncoating of penetrated PrV. Glycoprotein E distribution during the penetration event was detected by specific gold (GAM_{10}) labeling of the virus envelope of particles adsorbed at a PSEK cell microvillus (A) or fusing with the cell surface (B). Subsequently, lateral dispersion of gE was detected by labeling the cell surface (C) or microvillus membrane of PSEK cells after fusion was completed (D). (E and F) Released intracytoplasmic nucleocapsids (arrows) were regularly found close to microtubules (arrowheads). (G to I) Within 5 min after temperature shift nucleocapsids were detected at nuclear pores. Bars, 300 nm (A), 150 nm (B and F to I), 200 nm (C and D), or 750 nm (E).

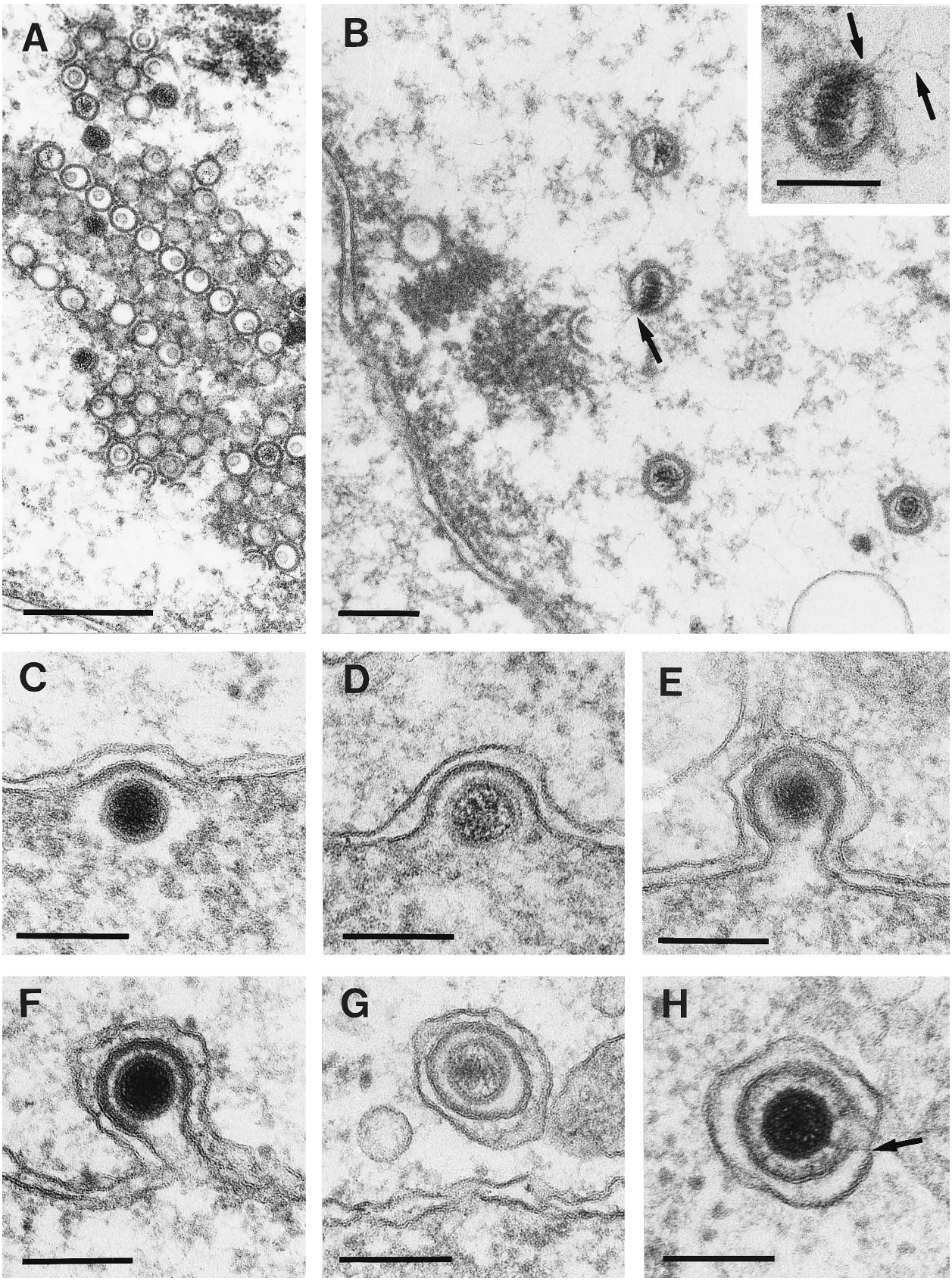

FIG. 3. Intranuclear maturation of nucleocapsids and egress from the nucleus. Crescent-shaped capsids with an annular protein structure, empty capsids, and DNA-containing nucleocapsids were arranged as pseudocrystals (A) or singly (B) within the nucleus of Vero cells. At higher magnification, structures resembling nucleic acid threads (arrows) were found in contact with vertices of capsids (B, inset). Budding through both membranes of the perinuclear cisterna (C to E) and vesiculation at the outer leaflet of the nuclear membrane (F and G) are shown in PSEK cells, as is a putative fusion event (arrow) between intravesicular virion envelope and vesicle membrane to release nucleocapsids into the cytoplasm (H). Bars, 500 nm (A), 150 nm (B to H), or 100 nm (inset).

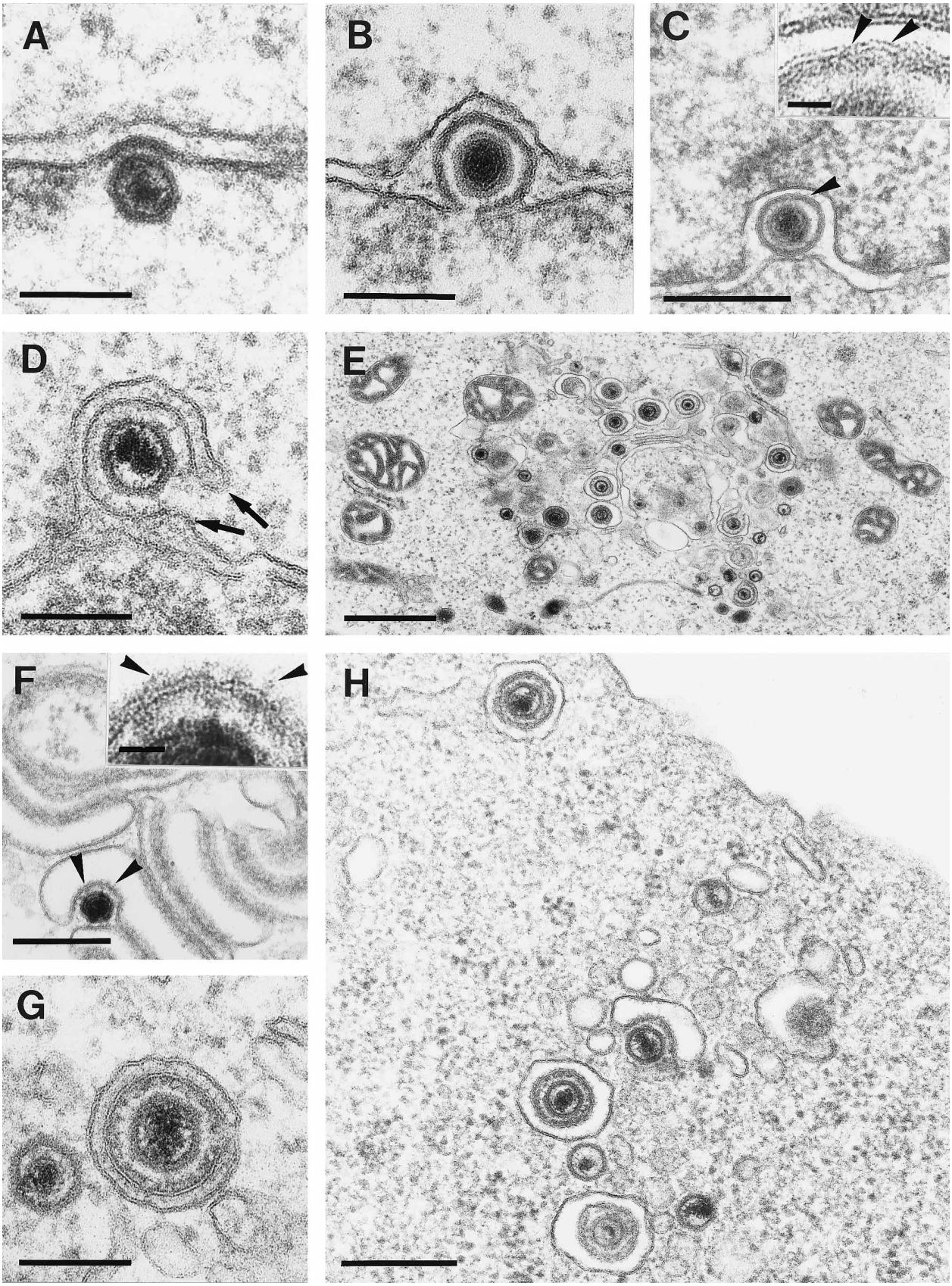

FIG. 4. Egress of virions from the nucleus. Nucleocapsids were also observed budding into the perinuclear cisterna of MDBK (A) and PSEK cells (B and C). Direct fusion (arrows) between virion envelope and outer leaflet of the perinuclear membrane in a Vero cell was sometimes observed (D). Naked nucleocapsids were detected in the Golgi area of PSEK cells adjacent to, within, or wrapped by membranes of tubular vesicles (F to H). Note the difference in appearance of the envelope surface (arrowheads) in virions in the perinuclear cisterna (C, inset) compared to virions after final envelopment (F, inset). Bars, 150 nm (A, B, D, and G), 750 nm (E), 300 nm (C, F, and H), or 25 nm (insets).

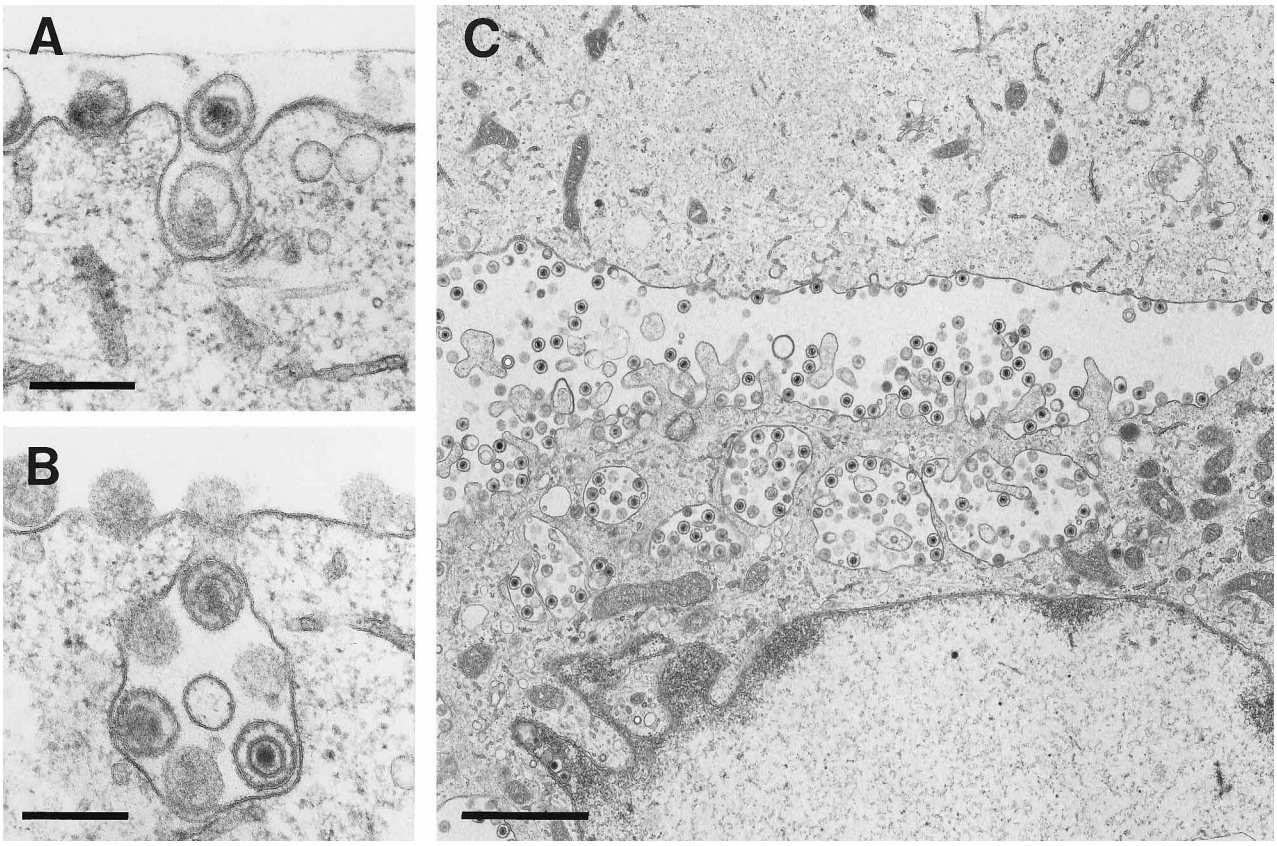

FIG. 5. Exocytosis of PrV. Release of newly produced viral progeny from PSEK cells by exocytosis either singly (A) or via vacuoles containing multiple virions (B) resulted in dramatic alterations of the cell surface (C). Bars, 250 nm (A and B) or 1.5 μm (C).

infected cells harbored numerous virus-containing vacuoles and the cell surface was covered with released virions (Fig. 5C).

Oddities. Besides the above-described stages, the following deviations were observed. Whereas penetration by direct fusion between virion envelope and cell membrane was predominant from 1 to 20 min after temperature shift, at later times virions were also detected juxtaposed to coated pits as well as in stages of viropexis in coated vesicles independent of the MOI used (Fig. 6A and B). These morphologically intact virus particles accumulated in cytoplasmic vesicles. Naked DNA-containing nucleocapsids without morphological signs of degradation were observed in the vicinity of these virus-containing vesicles in the cytoplasm (Fig. 6C). However, fusion stages between intravesicular virions and vesicle membranes have not been detected so far.

We also observed that either empty capsids or capsids still containing the annular scaffolding protein structure were enveloped by budding at the inner nuclear membrane similar to mature nucleocapsids. Defective virions were released into the cytoplasm by fusion at the outer nuclear membrane; were observed adjacent to, within, or partially wrapped by membranes at tubular vesicles in the area of the putative TGN, and appeared to be released by exocytosis (Fig. 6D to F). Such events were observed in all infected cells but with a frequency of less than 1% in relation to normal nucleocapsid maturation. These findings indicate a default mechanism of virion egress which also functions, although not very efficiently, with defective capsids.

DISCUSSION

In this study, interaction of PrV strain Ka with host cells was analyzed by EM to obtain a comprehensive picture of the main stages of viral replication. Cells of porcine, bovine, and primate origin were analyzed, but cell-specific differences were not detected. Therefore, the replication stages discussed below appear to be similar if not identical in these cells.

Two distinct stages of attachment of virions to the cell surface were detected in our analyses, characterized by different distances between the virion envelope and target cell. Whereas in initial binding virions are separated from the cell surface by approximately 12 to 14 nm, in a second stage this distance was reduced to 6 to 8 nm. A biphasic attachment has previously been shown to occur in PrV (23) by measuring stability of virion binding in the presence of exogenous heparin. We hypothesize that the two morphologically distinct stages of attachment correlate with the different sensitivity toward exogenous heparin.

Penetration by herpesviruses occurs by a pH-independent direct fusion between envelope and cell membrane (12, 19, 35, 48, 49, 53, 61). This entry mechanism is fast and was found to occur within 1 min after temperature shift. These data correlate with results from penetration studies using citrate inactivation of extracellular virus to assess the rate of entry. In PrV, 50% of input PFU are protected from citrate inactivation after approximately 5 to 10 min (34). Since it is unclear how long virions remain sensitive to inactivation, biological and ultrastructural data match reasonably well.

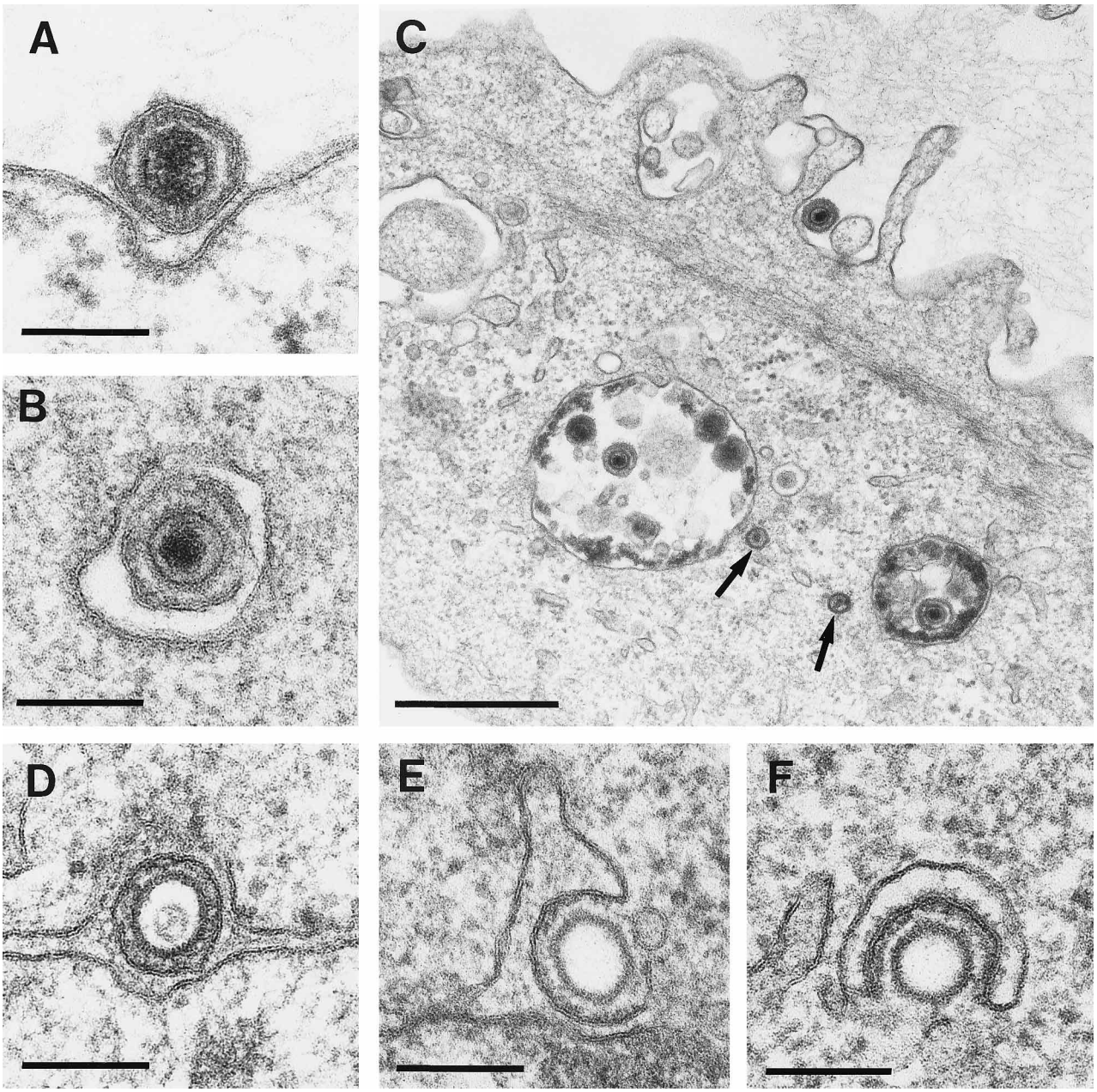

FIG. 6. Oddities. At 20 min after temperature shift single virions were seen at coated pits (A), within coated vesicles (B), and in lysosomes (C) of MDBK cells. Nucleocapsids (arrows) were detectable in direct proximity to lysosomes (C). Capsids still containing scaffolding protein, as well as empty capsids, were found enveloped in the perinuclear cisterna (D), released from the cisterna (E), and putatively reenveloped in the *trans*-Golgi area (F) of Vero cells, similar to mature nucleocapsids. Bars, 150 nm (A and B and D to F) or 750 nm (C).

Using a preembedding immunogold labeling technique we were able to show rapid lateral movement of virion glycoproteins from the fusion site. As early as 1 min after temperature shift, label was found dispersed from the presumed fusion site, indicating significant fluidity of the cytoplasmic membrane next to the fusion site. Whether the physical parameters of the cell membrane are influenced by the translocated viral glycoproteins is unclear at present.

At late stages in infection, independently of the MOI, virions were also detected at coated pits, inside coated vesicles, and inside structures resembling secondary lysosomes, recognizable by remnants of different cellular cytosis processes in comparison to primary lysosomes with their electron-dense homogeneous content (24). Naked nucleocapsids were found in close proximity to lysosomes. No viral remnants were detected inside lysosomes, as would have been expected if virions were degraded by lysosomal enzymes. Receptor-mediated endocytosis has been observed for HSV-1 and has been proposed to represent a dead-end way of penetration which does not lead to productive infection (4). In addition, Chinese hamster ovary (CHO) cells, which exhibit a high resistance to PrV infection, have been demonstrated by EM to take up virus by endocytosis. However, productive infection did not ensue (45). Since viropexis appears to be a rather late event in our studies, it is

unclear whether it is able to contribute to viral replication even if it could lead to productive infection.

During attachment and penetration, virions showed morphological alterations only at the point of contact with the cell membrane. Disassembly of tegument or dislocation of the nucleocapsid as found in HSV-1 (13) was not observed. This discrepancy could result from the different preparation of samples. In our study, no centrifugation step was included between infection and fixation, which could distort cell-associated unfixed virions. Alternatively, both viruses differ in this respect. After fusion only tegument remnants were detectable between cell membrane and translocated nucleocapsid. The rest of the tegument presumably dispersed into the cytoplasm rather quickly.

The mechanism of transport of nucleocapsids to nuclear pores is largely unclear. Intracytoplasmic virion transport processes have been suggested to be mediated by elements of the cytoskeleton (29), and interaction of microtubules with reovirus (1), adenovirus (7, 56), rotavirus (17), and presumably HSV-1 (47) has been described. PrV nucleocapsids were frequently observed in close contact to microtubular structures, which might imply a role for microtubules in transport of nucleocapsids to the nuclear pore. Inhibitors of microtubular function have been shown to affect axonal flow of HSV-1 in rat sensory neurons in vitro (26, 39), yielding further evidence for the role of the cytoskeleton in intracellular virion transport.

At the nuclear pore, DNA-containing and empty capsids were detected at a distance of approximately 40 nm from the central granulum. Nucleocapsids were invariably oriented with one vertex toward the nuclear pore and appeared morphologically intact. Stages of nucleocapsid disassembly as found for HSV-1 (3) were not observed, presumably because this process is too fast to be detectable in static electron micrographs. Since nucleocapsids are directed with one vertex toward the nuclear pore it can be assumed that DNA is released via the vertex region. This is supported by the findings of Newcomb and Brown (37), who induced extrusion of DNA from HSV-1 nucleocapsids and suggested that penton sites in the vertex area may open transiently to permit DNA exit.

Intranuclear capsid morphogenesis appeared to follow the well-known pattern of herpesvirus nucleocapsid formation (16, 27, 28, 36, 38), and A-, B-, and C-type capsids were detected. Intermediate assembly stages appeared as paired arcs of proteinaceous material, semicircles, and finally two concentric circles presumably consisting of capsomers and scaffolding protein. Integration of DNA into maturing nucleocapsids seems to proceed via the vertex regions as previously described for HSV-1 (37) which contrasts with the situation in murine cytomegalovirus in which coiled spider-like DNA complexes were integrated into coreless capsids with orifices (59). All stages of nucleocapsid formation and maturation were found distributed in the nucleoplasm or within pseudocrystals. Since nuclei of necrotic cells after virus replication lack pseudocrystals, we hypothesize that these are transient structures which dissolve during replication to release individual capsids.

In HSV-1, packaging of shorter than full-length genomic DNA fragments was described (55). However, these capsids did not become enveloped. We show here that envelopment and egress of incomplete PrV particles can regularly occur, thereby probably contributing to the relatively high particle to PFU ratio in extracellular virion preparations.

Egress of nucleocapsids from the nucleus appeared to involve primary envelopment at the inner nuclear membrane. Since these stages were observed only rarely we assume that the envelopment process is rapid, perhaps comparable to the kinetics of membrane fusion during penetration. So far, published reports indicate differences in the egress of HSV-1, VZV, or PrV nucleocapsids from the nucleus. Results in HSV-1 favor a model that includes envelopment at the inner nuclear membrane and translocation of enveloped virions through the exocytic pathway. Here, glycoproteins are proposed to be modified in situ during transit of enveloped virions through the Golgi apparatus (5, 9, 54). In contrast, data for VZV and PrV suggest deenvelopment at the outer nuclear membrane, translocation of nucleocapsids to the *trans*-Golgi area, and reenvelopment during which nucleocapsids are wrapped by tubular vesicles (14, 60, 62). As shown here, surface projections on enveloped virions inside the perinuclear cisterna were absent, and the tegument was homogeneous, sharp bordered, and electron dense. In contrast, during secondary envelopment the proposed future virion envelope, i.e., the luminal side of the invaginated tubular vesicle, contained clearly visible surface projections which resembled those found on intact extracellular virions. In addition, the tegument was enlarged and less electron dense than after primary envelopment. Therefore, there were visible differences in virion morphology after primary and secondary envelopment. Our data thus support a deenvelopment/reenvelopment pattern of virion maturation, which has been proposed for PrV and VZV (6, 14, 60, 62) and which has been substantiated by analysis of a replication-deficient PrV mutant defective in synthesis of the UL3.5 protein (11). That PrV and VZV very closely resemble one another is increasingly evident and is supported by sequence homology data which separate HSV and VZV-PrV into different genera within the alphaherpesviruses (33). Our proposal of a deenvelopment/reenvelopment pathway is consistent with previous ultrastructural data regarding PrV infection of rat brain (6). These authors also propose primary envelopment at the nuclear membrane followed by release of nucleocapsids into the cytoplasm and secondary envelopment in the *trans*-Golgi area. In contrast to our data which imply transit through the nuclear membrane including vesiculation or fusion at the outer lamella of the nuclear membrane, Card et al. (6) demonstrated deenvelopment at the perinuclear cisterna and the endoplasmic reticulum near the TGN after passage through trabeculae. This difference might be due to the situation in the experimentally infected animal compared to our cell culture systems. A mode of maturation similar to that of PrV and VZV has also been proposed for HCMV (42, 43). Whether these different ways are mutually exclusive or are indicative of multiple pathways for herpesvirus egress is unclear at present. A cell-type dependence in this respect was demonstrated for VZV (18). However, we were unable to detect differences using cells from either pigs, cattle, or monkeys.

There is general agreement that mature herpesvirus particles are released by exocytosis. In this study, the number of virions in single exocytic vesicles increased in a time-dependent manner. At first only single intravesicular virions were detected, whereas later in the infectious cycle multiple virions could be found inside a single vesicle. Exocytosis of virions with aberrant nucleocapsids also seems to occur.

Our data provide the first comprehensive ultrastructural analysis of PrV replication in cultured cells, which leads us to propose the replicative events shown in a diagrammatic fashion in Fig. 7. Although we are well aware of the problems inherent in ultrastructural analyses, e.g., the difficulty to deduce a dynamic process from static electron micrographs as well as the inability to differentiate biologically irrelevant observations from those important for the infectious cycle, our data correlate well with biological/biochemical studies of viral mutants defective at various stages of the viral replication cycle

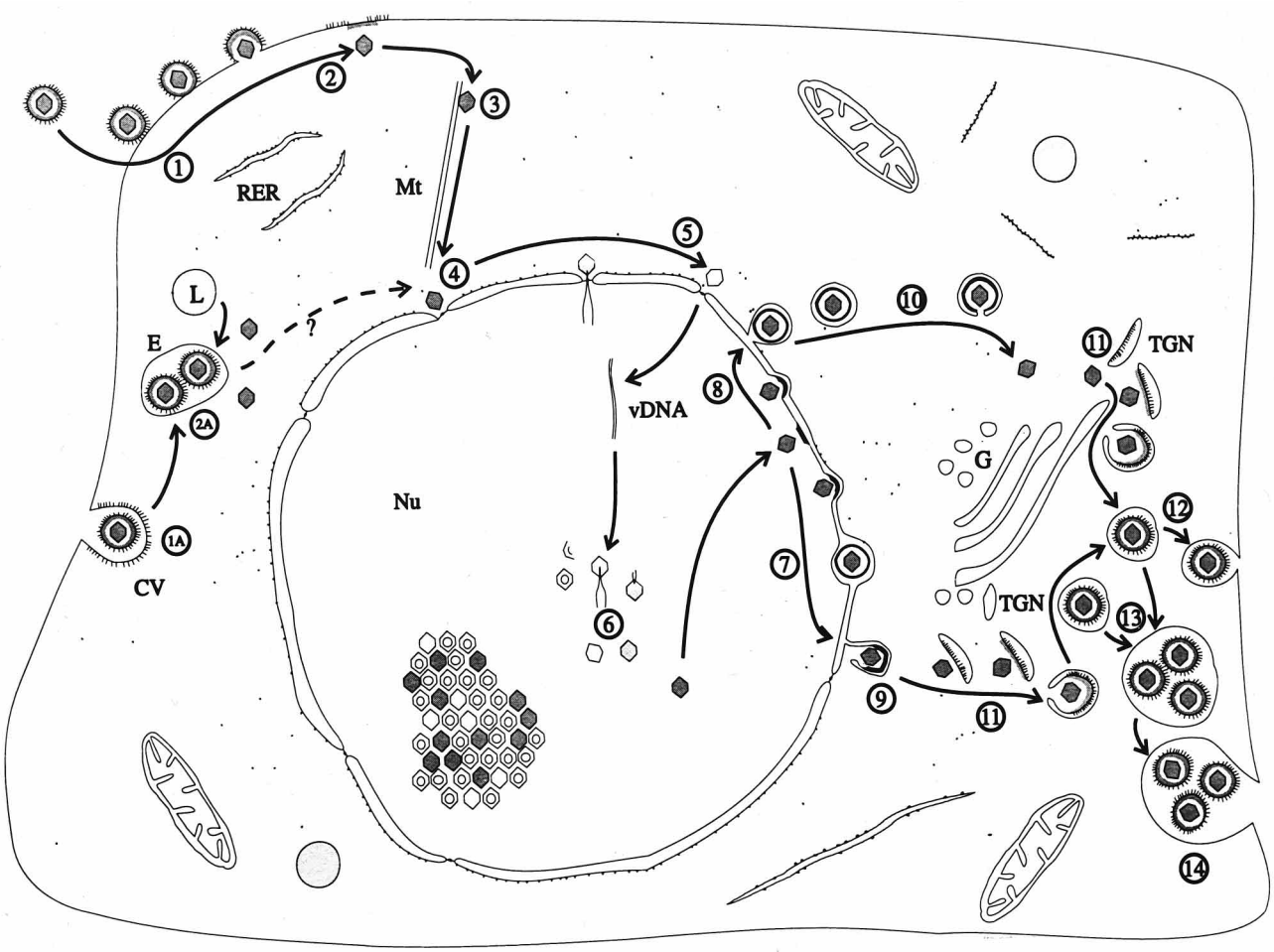

FIG. 7. Diagram of proposed stages of PrV replication in cell culture. Coated vesicle (CV), endosome (E), Golgi apparatus (G), lysosome (L), microtubule (Mt), nucleus (Nu), rough endoplasmic reticulum (RER), TGN, and viral DNA (vDNA) are depicted. Arrows indicate the proposed route of parental and progeny virions deduced from the electron micrographs. The question mark denotes lack of data for biological relevance of virion endocytosis at later time points after temperature shift.

and thus contribute to our understanding of herpesvirus replication.

ACKNOWLEDGMENTS

The authors thank J. Schmidt for skillful preparation of the diagram of replicative events, C. Möller for excellent technical assistance in electron microscopical preparations, and H. Stephan and W. Kramer for photographic assistance.

REFERENCES

1. **Babiss, L. E., R. B. Luftig, J. A. Weatherbee, R. R. Weihing, U. R. Ray, and B. N. Fields.** 1979. Reovirus serotypes 1 and 3 differ in their in vitro association with microtubules. J. Virol. **30:**863–874.
2. **Baskerville, A.** 1972. Ultrastructural changes in the pulmonary airways of pigs infected with a strain of Aujeszky's disease virus. Res. Vet. Sci. **13:**127–132.
3. **Batterson, W., D. Furlong, and B. Roizman.** 1983. Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of viral reproductive cycle. J. Virol. **45:**397–407.
4. **Campadelli-Fiume, G., M. Arsenakis, F. Farabegoli, and B. Roizman.** 1988. Entry of herpes simplex virus in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. J. Virol. **62:**159–167.
5. **Campadelli-Fiume, G., F. Farabegoli, S. DiGaeta, and B. Roizman.** 1991. Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. J. Virol. **65:**1589–1595.
6. **Card, J. P., L. Rinaman, R. B. Lynn, B.-H. Lee, R. P. Meade, R. R. Miselis, and L. W. Enquist.** 1993. Pseudorabies virus infection of the rat nervous system: ultrastructural characterization of viral replication, transport, and pathogenesis. J. Neurosci. **13:**2515–2539.
7. **Dales, S., and Y. Chardonnet.** 1973. Early events in the interaction of adenoviruses with HeLa cells. IV. Association with microtubules and the nuclear pore complex during vectorial movement of the inoculum. Virology **56:**465–483.
8. **Darlington, R. W., and L. H. Moss.** 1968. Herpesvirus envelopment. J. Virol. **2:**48–55.
9. **DiLazzaro, C., G. Campadelli-Fiume, and M. R. Torrisi.** 1995. Intermediate forms of glycoconjugates are present in the envelope of herpes simplex virions during their transport along the exocytic pathway. Virology **214:**619–623.
10. **Felluga, B.** 1963. Electron microscopic observations on pseudorabies virus development in a line of pig kidney cells. Ann. Sclavo **5:**412–424.
11. **Fuchs, W., B. G. Klupp, H. Granzow, H.-J. Rziha, and T. C. Mettenleiter.** 1996. Identification and characterization of the pseudorabies virus UL3.5 protein, which is involved in virus egress. J. Virol. **70:**3517–3527.
12. **Fuller, A. O., and P. G. Spear.** 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. Proc. Natl. Acad. Sci. USA **84:**5454–5458.
13. **Fuller, A. O., and W.-C. Lee.** 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. J. Virol. **66:**5002–5012.
14. **Gershon, A. A., D. L. Sherman, Z. Zhu, C. A. Gabel, R. T. Ambron, and M. D. Gershon.** 1994. Intracellular transport of newly synthesized varicella-zoster virus: final envelopment in the *trans*-Golgi network. J. Virol. **68:**6372–6390.

15. **Gibson, W.** 1993. Molecular biology of human cytomegalovirus, p. 303–329. *In* Y. Becker and G. Darai (ed.), Frontiers of virology, vol. 2. Molecular aspects of human cytomegalovirus diseases. Springer-Verlag, Heidelberg, Germany.
16. **Gibson, W., and B. Roizman.** 1974. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10:**1044–1052.
17. **Granzow, H., H. Schirrmeier, J. Beyer, and E. Lange.** 1988. Morphologische Studien bei Virusinfektionen des Darmtraktes—Virusreplikation und Zytopathologie in Zellkulturen und Enterozyten beim Ferkel. I. Ultrastruktur des nichtinfizierten Darmepithels und bei Rotavirusinfektion. Arch. Exp. Vet. Med. **42:**558–570.
18. **Harson, R., and C. Grose.** 1995. Egress of varicella-zoster virus from the melanoma cell: a tropism for the melanocyte. J. Virol. **69:**4994–5010.
19. **Huang, A. S., and R. R. Wagner.** 1966. Penetration of herpes simplex virus into human epidermoid cells. Proc. Soc. Exp. Biol. Med. **116:**863–869.
20. **Johnson, D. C., and P. G. Spear.** 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43:**1102–1112.
21. **Jones, F., and C. Grose.** 1988. Role of cytoplasmic vacuoles in varicella-zoster virus glycoprotein trafficking and virion envelopment. J. Virol. **62:** 2701–2711.
22. **Kaplan, A. S., and A. Vatter.** 1959. A comparison of herpes simplex and pseudorabies virus. Virology **7:**394–407.
23. **Karger, A., and T. C. Mettenleiter.** 1993. Glycoproteins gIII and gp50 play dominant roles in the biphasic attachment of pseudorabies virus. Virology **194:**654–664.
24. **Kleinig, H., and P. Sitte.** 1986. Zellbiologie, 2. Aufl. Gustav Fischer Verlag, Stuttgart, Germany.
25. **Klupp, B. G., N. Visser, and T. C. Mettenleiter.** 1992. Identification and characterization of pseudorabies virus glycoprotein H. J. Virol. **66:**3048–3055.
26. **Kristensson, K., E. Lycke, M. Roytta, B. Svennerholm, and A. Valne.** 1986. Neuritic transport of herpes simplex virus in rat sensory neurons in vitro. Effects of substances interacting with microtubular function and axonal flow. J. Gen. Virol. **67:**2023–2028.
27. **Liu, F., and B. Roizman.** 1991. The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. J. Virol. **65:**5149–5156.
28. **Liu, F., and B. Roizman.** 1993. Characterization of the protease and other products of amino-terminus-proximal cleavage of the herpes simplex virus 1 U_L26 protein. J. Virol. **67:**1300–1309.
29. **Luftig, R. B.** 1982. Does the cytoskeleton play a significant role in animal virus replication? J. Theor. Biol. **99:**173–191.
30. **McCracken, R. M., and J. K. Clarke.** 1971. A thin-section study of the morphogenesis of Aujeszky's disease virus in synchronously infected cell cultures. Arch. Ges. Virusforsch. **34:**189–210.
31. **McCracken, R. M., and C. Dow.** 1973. An electron microscopic study of Aujeszky's disease. Acta Neuropathol. (Berlin) **25:**207–219.
32. **McCracken, R. M., J. B. McFerran, and C. Dow.** 1973. The neural spread of pseudorabies virus in calves. J. Gen. Virol. **20:**17–28.
33. **McGeoch, D. J., and S. Cook.** 1994. Molecular phylogeny of the Alphaherpesvirinae subfamily and a proposed evolutionary timescale. J. Mol. Biol. **238:**9–22.
34. **Mettenleiter, T. C.** 1989. Glycoprotein gIII deletion mutants of pseudorabies virus are impaired in virus entry. Virology **171:**623–625.
35. **Morgan, C., H. M. Rose, and B. Mednis.** 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. **2:**507–516.
36. **Newcomb, W. W., and J. C. Brown.** 1991. Structure of the herpes simplex virus capsid: effects of extraction with guanidine hydrochloride and partial reconstitution of extracted capsids. J. Virol. **65:**613–620.
37. **Newcomb, W. W., and J. C. Brown.** 1994. Induced extrusion of DNA from the capsid of herpes simplex virus type 1. J. Virol. **68:**433–440.
38. **Nii, S.** 1992. Electron microscopic study on the development of herpesviruses. J. Electron Microsc. **41:**414–423.
39. **Penfold, M. E. T., P. Armati, and A. L. Cunningham.** 1994. Axonal transport of herpes simplex virions to epidermal cells: evidence for a specialized mode of virus transport and assembly. Proc. Natl. Acad. Sci. USA **91:**6529–6533.
40. **Pietschmann, S. M., E. H. Hausmann, and H. R. Gelderblom.** 1989. Immunogold labeling of viruses in situ, p. 255–284. *In* M. A. Hayat (ed.) Colloidal gold—principles, methods and applications, vol. 2. Academic Press, Inc., San Diego, Calif.
41. **Pol, J. M. A., F. Wagenaar, and A. Gielkens.** 1991. Morphogenesis of three pseudorabies virus strains in porcine nasal mucosa. Intervirology **32:**327–337.
42. **Radsak, K., H. Kern, B. Reis, M. Reschke, T. Mockenhaupt, and M. Eickmann.** 1995. Human cytomegalovirus—aspects of viral morphogenesis and of processing and transport of viral glycoproteins, p. 295–312. *In* G. Barbanti-Brodano (ed.) DNA tumor viruses: oncogenic mechanisms. Plenum Press, New York, N.Y.
43. **Radsak, K., M. Eickmann, T. Mockenhaupt, E. Bogner, H. Kern, A. Eis-Hübinger, and M. Reschke.** 1996. Retrieval of human cytomegalovirus glycoprotein B from the infected cell surface for virus envelopment. Arch. Virol. **141:**557–572.
44. **Roizman, B., and A. E. Sears.** 1996. Herpes simplex viruses and their replication, p. 2231–2295. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, vol. 2, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
45. **Sawitzky, D., H. Hampl, and K.-O. Habermehl.** 1990. Entry of pseudorabies virus into CHO cells is blocked at the level of penetration. Arch. Virol. **115:**309–316.
46. **Smid, B., L. Valicek, and A. Sabo.** 1981. Morphogenesis of Aujeszky's disease virus in pig lung macrophage cultures. Acta Vet. Brno **50:**79–87.
47. **Sodeik, B., M. W. Ebersold, and A. Helenius.** 1996. Microtubule and dynein mediated transport of incoming HSV-1 capsids to the nucleus, abstr. 174. 21st Herpesvirus Workshop, July 27–August 2. Northern Illinois University, De Kalb, Ill.
48. **Spear, P. G.** 1993. Entry of alphaherpesviruses into cells. Semin. Virol. **4:** 167–180.
49. **Spear, P. G., M. Wittels, A. O. Fuller, D. WuDunn, and R. Johnson.** 1988. Herpes simplex virus: pathway of entry into cells, p. 163–175. *In* R. W. Compans, A. Helenius, and M. B. A. Oldstone (ed.), Cell biology of viral entry, replication and pathogenesis. Alan R. Liss, New York, N.Y.
50. **Stackpole, C. W.** 1969. Herpes type-virus of frog renal adenocarcinoma. I. Virus development in tumor transplants maintained at low temperatures. J. Virol. **4:**75–87.
51. **Tatman, J. D., V. G. Preston, P. Nicholson, R. M. Elliott, and F. J. Rixon.** 1994. Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. J. Gen. Virol. **75:**1101–1113.
52. **Thomsen, D. R., W. W. Newcomb, J. C. Brown, and F. L. Homa.** 1995. Assembly of the herpes simplex virus capsid: requirement for the carboxyl-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes. J. Virol. **69:**3690–3703.
53. **Topilko, A., and S. Michelson.** 1994. Hyperimmediate entry of human cytomegalovirus virions and dense bodies into human fibroblasts. Res. Virol. **145:**75–82.
54. **Torrisi, M., R., C. DiLazzaro, A. Pavan, L. Pereira, and G. Campadelli-Fiume.** 1992. Herpes simplex virus envelopment and maturation studied by fracture label. J. Virol. **66:**554–561.
55. **Vlazny, D. A., A. Kwong, and N. Frenkel.** 1982. Site specific cleavage packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full length viral DNA. Proc. Natl. Acad. Sci. USA **79:** 1423–1427.
56. **Weatherbee, J. A., R. B. Luftig, and R. R. Weihing.** 1977. Binding of adenovirus to microtubules. II. Depletion of high molecular-weight microtubule-associated protein content reduces specificity of in vitro binding. J. Virol. **21:**732–742.
57. **Wehland, J., M. C. Willingham, and I. V. Sandoval.** 1983. A rat monoclonal antibody reacting specifically with the tyrosylated form of α-tubulin. I. Biochemical characterization, effects on microtubule polymerization in vitro, and microtubule polymerization and organization in vivo. J. Cell Biol. **97:** 1467–1475.
58. **Weiland, E., and B. G. Klupp.** Unpublished data.
59. **Weiland, F., G. M. Keil, M. J. Reddehase, and U. H. Koszinowski.** 1986. Studies on the morphogenesis of murine cytomegalovirus. Intervirology **26:** 192–201.
60. **Whealy, M. E., J. P. Card, R. P. Meade, A. K. Robbins, and L. W. Enquist.** 1991. Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J. Virol. **65:**1066–1081.
61. **Wittels, M., and P. G. Spear.** 1990. Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. Virus Res. **18:**271–290.
62. **Zhu, Z., M. D. Gershon, Y. Hao, R. T. Ambron, C. A. Gabel, and A. A. Gershon.** 1995. Envelopment of varicella-zoster virus: targeting of viral glycoproteins to the *trans*-Golgi network. J. Virol. **69:**7951–7959.