

## Assembly of Vaccinia Virus: the Second Wrapping Cisterna Is Derived from the Trans Golgi Network

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Received 28 May 1993/Accepted 21 September 1993

During the assembly of vaccinia virus, the intracellular mature virus becomes enwrapped by a cellular cisterna to form the intracellular enveloped virus (IEV), the precursor of the extracellular enveloped virus (EEV). In this study, we have characterized the origin of this wrapping cisterna by electron microscopic immunocytochemistry using lectins, antibodies against endocytic organelles, and recombinant vaccinia viruses expressing proteins which behave as Golgi resident proteins. No labelling for endocytic marker proteins could be detected on the wrapping membrane. However, the wrapping membrane labelled significantly for a trans Golgi network (TGN) marker protein. The recycling pathway from endosomes to the TGN appears to be greatly increased following vaccinia virus infection, since significant amounts of endocytic fluid-phase tracers were found in the lumen of the TGN, Golgi complex, and the wrapping cisternae. Using immunoelectron microscopy, we localized the vaccinia virus membrane proteins VV-p37, VV-p42, VV-p21, and VV-hemagglutinin (VV-HA) in large amounts in the wrapping cisternae, in the outer membranes of the IEV, and in the outermost membrane of the EEV. The bulk of the cellular VV-p37, VV-p21, and VV-p42 were in the TGN, whereas VV-HA was also found in large amounts on the plasma membrane and in endosomes. Collectively, these data argue that the TGN becomes enriched in vaccinia virus membrane proteins that facilitate the wrapping event responsible for the formation of the IEV.

Vaccinia virus is the best-characterized member of the poxvirus family, which contains the largest and most complex animal viruses. The poxviruses have several unique features, two of which are particularly striking. First, they are DNA-containing viruses which, unlike other DNA-containing viruses, replicate and assemble in the cytoplasm of the host cell. Accordingly, these viruses encode their own machinery required for both DNA replication and transcription (40, 41). Second, the vaccinia virus makes different intracellular and extracellular forms which are both infectious (see below).

During the assembly of vaccinia virus, several different morphological forms can be identified: the immature virus (IV), the infectious, intracellular mature virus (IMV, previously referred to as the intracellular naked virus [57]), the intracellular enveloped virus (IEV), and the infectious, extracellular enveloped virus (EEV). The IVs are assembled at the viral factories, which are large, electron-dense structures enriched in viral DNA and proteins (7). These appear at 2 to 4 h postinfection. It was thought for a long time that the IV possesses only one membrane and that this membrane is synthesized de novo (7, 44). However, our recent data argue that the IV possesses two closely apposed membranes and that these cisternal membranes are derived from the intermediate compartment (57), which represents a compartment between the rough endoplasmic reticulum (ER) and the Golgi complex (27). The spherical IV matures into the brick-shaped IMV, which is characterized by two membranes. Subsequently, a wrapping event occurs in which the IMV becomes enveloped

by a cellular cisterna, thereby acquiring two additional membranes in one step (46, 47), giving rise to the four-membraned IEV. In the current model for virus egress, the IEV is believed to be released from the cell by fusion of its outermost membrane with the plasma membrane, thereby losing one membrane (47). The resulting EEV therefore possesses three membranes.

In this study, we have used endocytic fluid-phase tracers and antibodies specific for different marker proteins of intracellular membrane compartments to characterize the origin of the cellular cisterna which enwraps the IMV to form the IEV. Conventional Epon section analysis had suggested that these membranes are derived from the Golgi complex (7, 30, 31, 39). Our results support these earlier studies and specifically show that the IMV becomes enwrapped by a cisterna which is derived from the trans Golgi network (TGN). In uninfected cultured cells, the TGN is poorly accessible to fluid-phase tracers taken up by the living cell from the medium. However, in vaccinia virus-infected cells, we observed that the pathway from the endosomes to the TGN is greatly accentuated. In this study, we also show the subcellular localization of four vaccinia virus membrane proteins in vaccinia virus-infected cells by immunoelectron microscopy. These proteins are VV-p37 (37 kDa [30]), the VV-hemagglutinin (VV-HA, 85 kDa [54]), VV-p21 (21 kDa [9, 49]), and VV-p42 (42 kDa [11, 32, 61]). These proteins are present in the purified EEV but absent from IMV. VV-p42, VV-p37, and VV-p21 were shown to be essential for the formation of EEV (2, 9, 61). The current model for virus egress predicts that these envelope proteins are enriched in both outer membranes of the IEV. Therefore, in this analysis we also examined whether both outer membranes of the IEV can be labeled with antibodies to these viral proteins.

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## MATERIALS AND METHODS

**Cells and viruses.** The following cell lines were used: HeLa (ATCC CCL 10), RK<sub>13</sub> (rabbit kidney cells; ATCC CCL 37), J774 A.1 (mouse monocytes-macrophages; ATCC TIB 67), and NRK (normal rat kidney fibroblasts; ATCC CRL 1570). HeLa and RK<sub>13</sub> cell lines were cultured in Eagle's minimal essential medium with 10% heat-inactivated fetal calf serum and 1% nonessential amino acids, whereas J774 and NRK cells were grown in Dulbecco's minimal essential medium with 10% heat-inactivated fetal calf serum. The medium of all cells contained 100 U of penicillin per ml and 100 mg of streptomycin per ml, and cells were cultured at 37°C in 5% CO<sub>2</sub>.

Two wild-type vaccinia virus strains, IHD-J (International Health Department) and WR (Western Reserve), both kindly provided by B. Moss (National Institutes of Health, Bethesda, Md.), were used in our study. The following recombinant vaccinia viruses were also used. VV-G/PTV, which was provided by R. Compans (University of Alabama, Birmingham), expresses the G1/G2 glycoprotein of Punta Toro virus (PTV) under the control of the vaccinia virus 7.5 promoter (38). VV-MHV-M expresses the M glycoprotein of mouse hepatitis virus strain A59 (MHV) and was provided by J. Krijnse-Locker and P. Rottier (University of Utrecht, Utrecht, The Netherlands) (35, 36). To overexpress the human transferrin receptor (Tfr), VV-T7-infected cells (14) were transfected at 30 min postinfection with a plasmid encoding the human Tfr (C. Bucci and M. Zerial, European Molecular Biology Laboratory [EMBL], Heidelberg, Germany) (3). In addition, we used mutant vaccinia viruses vSI-14, in which the B5R open reading frame of the WR strain was deleted (61), and vRB12, in which the F13L open reading frame of WR was deleted (2). All viruses were routinely propagated and titered in HeLa and RK<sub>13</sub> cells as described previously (10). Subconfluent cells were infected in serum-free medium with 5 to 10 PFU of a crude vaccinia virus stock per cell and incubated for various times at 37°C in 5% CO<sub>2</sub>.

**Antibodies.** Mouse monoclonal antibody (MAb) 1H831 against VV-HA was prepared and characterized by Shida (54). Rat MAbs 15B6, 19C2, and 17C4, directed against the vaccinia virus envelope proteins VV-p37 and VV-p42, respectively, were prepared by the method of Galfre and Milstein (15). The antigens were prepared as described by Hiller and Weber (30). A mouse MAb directed toward the vaccinia virus envelope protein VV-p21 was provided by L. Payne (Virus Research Institute, Cambridge, Mass.) (49). Mouse MAbs directed to the G1 glycoprotein of PTV (38) were provided by J. Smith (U.S. Army Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md.). For localization of MHV-M, mouse MAb J 1.3 (13) and a rabbit antiserum recognizing the carboxy-terminal amino acids were provided by P. Rottier (University of Utrecht) (35). Affinity-purified rabbit anti-peptide sera specific for rab5 and for rab7 were kindly provided by M. Zerial (EMBL) (4). A rabbit antiserum specific for the mannose 6-phosphate receptor (MPR) (21) was provided by B. Hoflack (EMBL), and a rabbit antiserum specific for lgp120 (37) was provided by I. Mellman (Yale University, New Haven, Conn.). A mouse MAb specific for the human Tfr as well as the biotinylated lectins wheat germ agglutinin, concanavalin A, aleuria aurantia, ricin communis, and maackia amurensis were obtained from Boehringer (Mannheim, Germany). The lectins were visualized by using a mouse MAb to biotin (Boehringer) followed by a rabbit anti-mouse immunoglobulin G and protein A-gold.

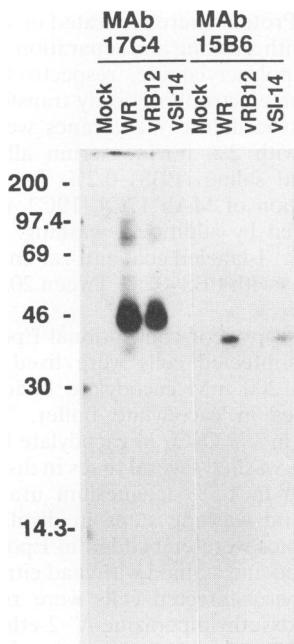
**Western blot (immunoblot) analysis.** At 18 h postinfection, cells were harvested and lysed in radioimmunoprecipitation

assay buffer (49). Proteins were separated on a sodium dodecyl sulfate 0.1% gel with stacking and separation portions containing 4 and 10% polyacrylamide, respectively. The resolved protein bands were electrophoretically transferred onto nitrocellulose. The nitrocellulose membranes were then sequentially incubated with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)-0.2% Tween 20 and then with a 1:500 dilution of MAb 17C4, 19C2, or 15B6 in PBS-Tween 20, followed by additional washing. The membranes were probed with <sup>125</sup>I-labeled goat anti-rat immunoglobulin G, washed three times with PBS-0.2% Tween 20, and analyzed by autoradiography.

**Electron microscopy.** For conventional Epon section analysis, vaccinia virus-infected cells were fixed for 1 h in 1% glutaraldehyde in 200 mM cacodylate buffer (pH 7.4) and washed four times in cacodylate buffer. Then cells were incubated for 1 h in 1% OsO<sub>4</sub> in cacodylate buffer containing 1.5% K<sub>3</sub>(Fe(CN)<sub>6</sub>), washed several times in distilled water, then incubated for 1 h in 1.5% magnesium uranyl acetate, and subjected to several washing steps in distilled water. After dehydration, samples were embedded in Epon. Ultrathin sections were prepared and stained with lead citrate. For cryosectioning, vaccinia virus-infected cells were rinsed twice with ice-cold N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (200 mM, pH 7.4) and then incubated with 25 mg of proteinase K per ml in HEPES until the cells detached (~2 min). The cell suspension was centrifuged at 1,000 rpm for 5 min in an Eppendorf microcentrifuge, the supernatant was removed, and the cell pellet was prefixed in 0.1% glutaraldehyde-4% paraformaldehyde in HEPES buffer for 1 h and then fixed overnight in fresh 8% paraformaldehyde in HEPES. To ensure that the proteinase K treatment did not cleave epitopes of surface antigens, in some experiments the cells were detached from the culture dish by scraping with a rubber policeman. Portions of fixed cell pellet were incubated for about 15 to 20 min in 2.1 M sucrose, frozen, and stored in liquid nitrogen. The cell pellets were cryosectioned, and the sections were labelled with primary antibodies followed by protein A-gold as described previously (18). When mouse or rat primary antibodies were used, we included an intermediate step with rabbit anti-mouse or rat antibodies before the protein A-gold step. The ultrathin sections were examined with a Zeiss EM 10 or a Phillips 400 electron microscope.

For quantitation of immunogold labelling, electron micrographs were taken in a systematic fashion by moving the translation controls of the microscope in a set direction and photographing each structure of interest that came into view. The number of gold particles per area of the micrograph was estimated by using a stereological point counting procedure, and the density per linear trace of membrane was estimated by using intersection counts as described by Griffiths (18).

**Labelling of the endocytic pathway.** In most of the experiments, the endocytic pathway was labelled by adding either high-activity horseradish peroxidase (HRP; Serva, Heidelberg, Germany) at a final concentration of 10 mg/ml or BSA-gold (5 nm; final concentration, 20 to 30 units of optical density at 520 nm) to the medium of vaccinia virus-infected cells for different internalization time periods prior to fixation. For HRP studies, cells were fixed and washed in 200 mM cacodylate buffer, and the internalized HRP was visualized by incubation with 0.1% 3,3'-diaminobenzidine (Sigma) in 200 mM cacodylate buffer for 1 min followed by a 30-min reaction in 0.1% 3,3'-diaminobenzidine-0.01% H<sub>2</sub>O<sub>2</sub> in the dark at room temperature. The reaction was stopped by washing the mixture three times with 200 mM cacodylate buffer, and cells were then processed for Epon embedding.



**FIG. 1.** Western blot analysis of infected cell lysates probed with MAbs 17C4 and 15B6. RK<sub>13</sub> cells were infected with vaccinia virus strain WR, recombinant virus vSI-14, or recombinant virus vRB12 at a multiplicity of infection of 10. At 18 h postinfection, cells were harvested. Lysates of infected and uninfected cells were analyzed by Western blotting as described in Materials and Methods. The relative migration of molecular weight markers (in kilodaltons) is indicated. MAb 17C4 is specific for VV-p42, and MAb 15B6 recognizes specifically VV-p37.

For BSA-gold studies, 5-nm gold coupled to BSA was prepared as described by Slot and Geuze (56). After various internalization time periods, the cells were processed for cryosectioning as described above.

## RESULTS

**Characterization of MAbs directed against VV-p42 and VV-p37.** Rat MAbs were raised against VV-p42 and VV-p37, which are constituent proteins of the envelope of the EEV (46). To characterize these antibodies, RK<sub>13</sub> cells were infected either with the wild-type strain WR or with the mutant vSI-14, in which the B5R open reading frame coding for VV-p42 was deleted (61), or with the mutant vRB12, in which the F13L open reading frame coding for VV-p37 was deleted (2). Cell lysates which either contained all VV-proteins or lacked VV-p42 or VV-p37 were prepared. These cell lysates and lysates from mock-infected cells were analyzed by Western blotting with MAbs 15C6, 17C4, and 19C2. MAbs 17C4 (Fig. 1) and 19C2 (not shown) react with a 42-kDa protein which is absent from lysates of cells infected with vSI-14 (Fig. 1). MAb 15B6 reacts with a 37-kDa protein which is absent from lysates of cells infected with vRB-12 (Fig. 1). This result shows that MAb 15B6 specifically recognizes VV-p37, while MAbs 17C4 and 19C2 are specific for VV-p42.

**Immunocytochemical localization of vaccinia virus envelope membrane proteins in vaccinia virus-infected cells.** To localize the membrane proteins of the EEV, namely, VV-HA, VV-p21, VV-p37, and VV-p42, thawed cryosections of HeLa, J774, NRK, or RK<sub>13</sub> cells infected with the vaccinia virus strain WR or IHD-J were labelled with antibodies specific for these

proteins. Figure 2 shows the pattern of labelling for VV-HA, Fig. 3 shows the pattern for VV-p21, Fig. 4a to d show the patterns for VV-p42, and Fig. 4e and f show the patterns for VV-p37. The antibodies to all four viral membrane proteins labelled the four-membraned IEV. Examples are shown for VV-p21 (Fig. 3e and f), VV-p42 (Fig. 4b and c), and VV-p37 (Fig. 4f). None of the antibodies labelled either the two-membraned IMV (Fig. 2b and 3d) or the IV (Fig. 12c). In IEVs in which the two outer membranes were not closely apposed, it became evident that both of the outer membranes were labelled for all four viral marker proteins (Fig. 4b, c, and f).

As expected, VV-HA (Fig. 2a), VV-p37 (not shown), VV-p21 (not shown), and VV-p42 (Fig. 4d) were also found to be present on the outermost membrane of the three-membraned EEV, found outside the cells. The membranes of the cellular cisternae enwrapping the IMVs were strongly labelled with the antibodies to all four viral membrane proteins. Some representative examples are shown for VV-HA (Fig. 2c) and for VV-p21 (Fig. 3d). The antibodies specific for all four viral membrane proteins also gave significant labelling of the Golgi complex. Examples are shown for VV-p21 (Fig. 3b and c), VV-p42 (Fig. 4a), and VV-p37 (Fig. 4e). The amounts of labelling for VV-p42 (Fig. 4a), VV-p37 (Fig. 4f and 14a), and VV-p21 (Fig. 3a and e) on the plasma membrane and in endosomes were generally very low. However, in a few areas of the plasma membrane, patches of significant labelling were observed. Occasionally these patches were adjacent to EEV particles, as shown for VV-p42 in Fig. 4d. The presence of these patches on the plasma membrane can be explained in two ways, which are not mutually exclusive. First, these proteins could be transported via the constitutive secretory route from the TGN to the plasma membrane. Second, and more likely, these proteins were inserted into the plasma membrane following the fusion of the outermost membrane of the IEV with the cell surface during the release of progeny virus from the cell.

In addition to the labelling of the Golgi complex, the antibodies specific for VV-HA showed a strong labelling of the nuclear envelope (Fig. 2a) and the ER (not shown) as well as the plasma membrane (Fig. 2a) and endosomes (not shown). The labelling of the plasma membrane and of endosomes was much more intensive for VV-HA (Fig. 2b) than for the other three viral envelope proteins. The endosomes were identified by their characteristic morphology and by their content of internalized BSA-gold (see below).

VV-p42, VV-p37, and VV-p21 have been shown to play an important role in the wrapping of the IMV to form the IEV. If one of these proteins is deleted, the wrapping process is inhibited (2, 9, 61). We show here that these proteins localize to the wrapping membranes, the outer membranes of the IEV, the EEV, and the Golgi complex and in low amounts also to the plasma membrane and in endosomes. The fact that VV-p42, VV-p21, and VV-p37 localize to the Golgi complex, the plasma membrane, and endosomes suggests that the wrapping membranes could derive from any of these organelles. Therefore, we studied the wrapping event in more detail, using lectins, fluid-phase tracers for the endocytic pathway, as well as Golgi marker proteins.

**Lectin labelling in vaccinia virus-infected cells.** To characterize the cellular compartment involved in the wrapping of the IMV, we labelled thawed cryosections of vaccinia virus-infected cells with lectins. Oligosaccharides on glycoproteins and glycolipids undergo sequential modifications during transport from the ER to the TGN. These modifications are reflected in different specificities for lectins (reviewed in reference 18). For example, concanavalin A, which is specific for mannose, labels

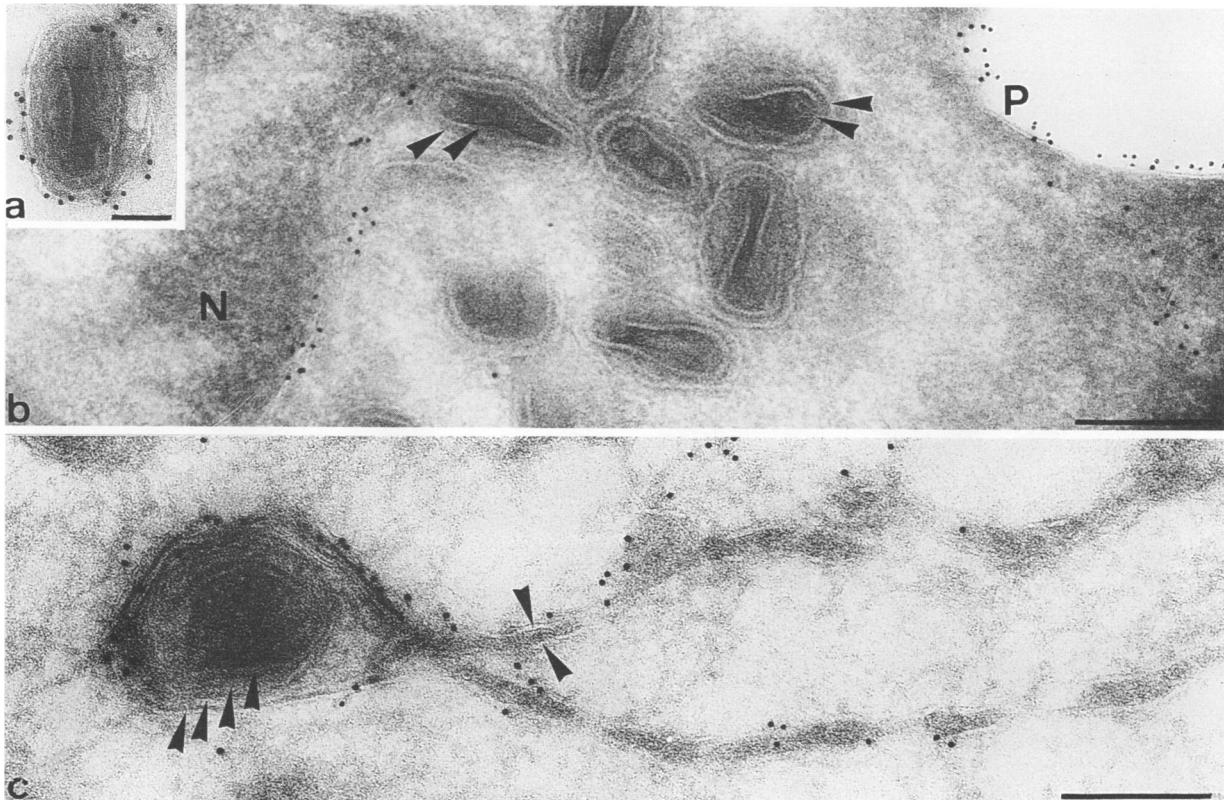


FIG. 2. Ultrathin cryosections of IHD-J-infected RK<sub>13</sub> cells labelled with antibodies specific for VV-HA. This protein is present on the nuclear envelope (b), the plasma membrane (b), the wrapping membranes (c) and the outermost membrane of the EEV (a). Note that the IMV particles which possess two membranes (arrowheads in panel b) are negative for VV-HA. The arrowheads in panel c point to the membranes of the wrapping cisterna and to the four membranes in the forming IEV. N, nucleus; P, plasma membrane. Bars, 100 nm (a) and 200 nm (b and c).

the ER, including the nuclear envelope and the entire Golgi stack. In contrast, wheat germ agglutinin specifically recognizes terminal *N*-acetylglucosamine and sialic acid and consequently binds only to oligosaccharides which have been exposed to sugar transferases in the Golgi complex. Whereas wheat germ agglutinin labels the whole Golgi stack, ricin communis, specific for galactose, maackia amurensis, specific for fucose, and aleuria aurantia, specific for sialic acid, label the more distal Golgi compartments on the trans aspect of the stack, as well as the TGN. It should be emphasized that all of these lectins may also label post Golgi compartments such as plasma membrane and endosomes, provided the lectin-binding sugar is not rendered inaccessible by addition of later sugars (18).

All of the lectins that we tested were found to bind to the cellular membranes which enwrap the IMVs (Fig. 5a) and to the two outer membranes of the IEV (Fig. 5b to d). The outermost membrane of the EEV also showed a strong lectin labelling (not shown). The IMVs were negative for all of the lectins (Fig. 5a, c, and d).

The labelling of the two outer membranes of the IEV with lectins specific for sugars that are added in late Golgi structures suggests that the wrapping cisterna originates from either a late Golgi or a post Golgi compartment.

**Endocytic fluid-phase tracers have free access to the wrapping cisterna of the IMV.** The lectin labelling data as well as the phospholipid analysis of purified IEV and EEV (57) argue that the cisterna that wraps around the IMV originates from either a late Golgi or a post Golgi compartment such as an endocytic organelle. The latter can easily be labelled by

allowing cells to internalize fluid phase tracers such as HRP or colloidal gold conjugated to BSA-gold added to the cell culture medium. Therefore, vaccinia virus-infected cells were allowed to internalize these tracers for various time periods prior to fixation. HRP was visualized by using the diaminobenzidine cytochemical reaction before embedding in Epon. BSA-gold can be directly visualized in thawed cryosections, which can be additionally labelled with antibodies against viral and cellular proteins.

When cells were incubated for periods longer than 15 min with HRP or 30 min with BSA-gold (which is a less sensitive marker), these tracers were seen in various endosomal structures, as expected. However, these markers were also quite evident in many of the wrapping cisternae (Fig. 6b to g). The early endosome compartment is operationally defined as structures which label with fluid-phase tracers internalized for no longer than 5 min (20). When the HRP internalization time in vaccinia virus-infected cells was reduced to 5 min, the HRP reaction product was seen in a very small percentage of the wrapping cisternae (Fig. 6f).

In general, in many cell types, neither the reaction product of HRP nor any BSA-gold can be found in any of the Golgi cisternae when cells are incubated with fluid-phase tracers for periods of up to 2 h (24). In contrast, when vaccinia virus-infected cells were incubated with HRP for up to 2 h, a strong reaction product of HRP was observed in one or two cisternae on the trans side of the Golgi complex (Fig. 6a). In some images, we found the HRP reaction product throughout all Golgi cisternae (Fig. 6b). BSA-gold, internalized for longer

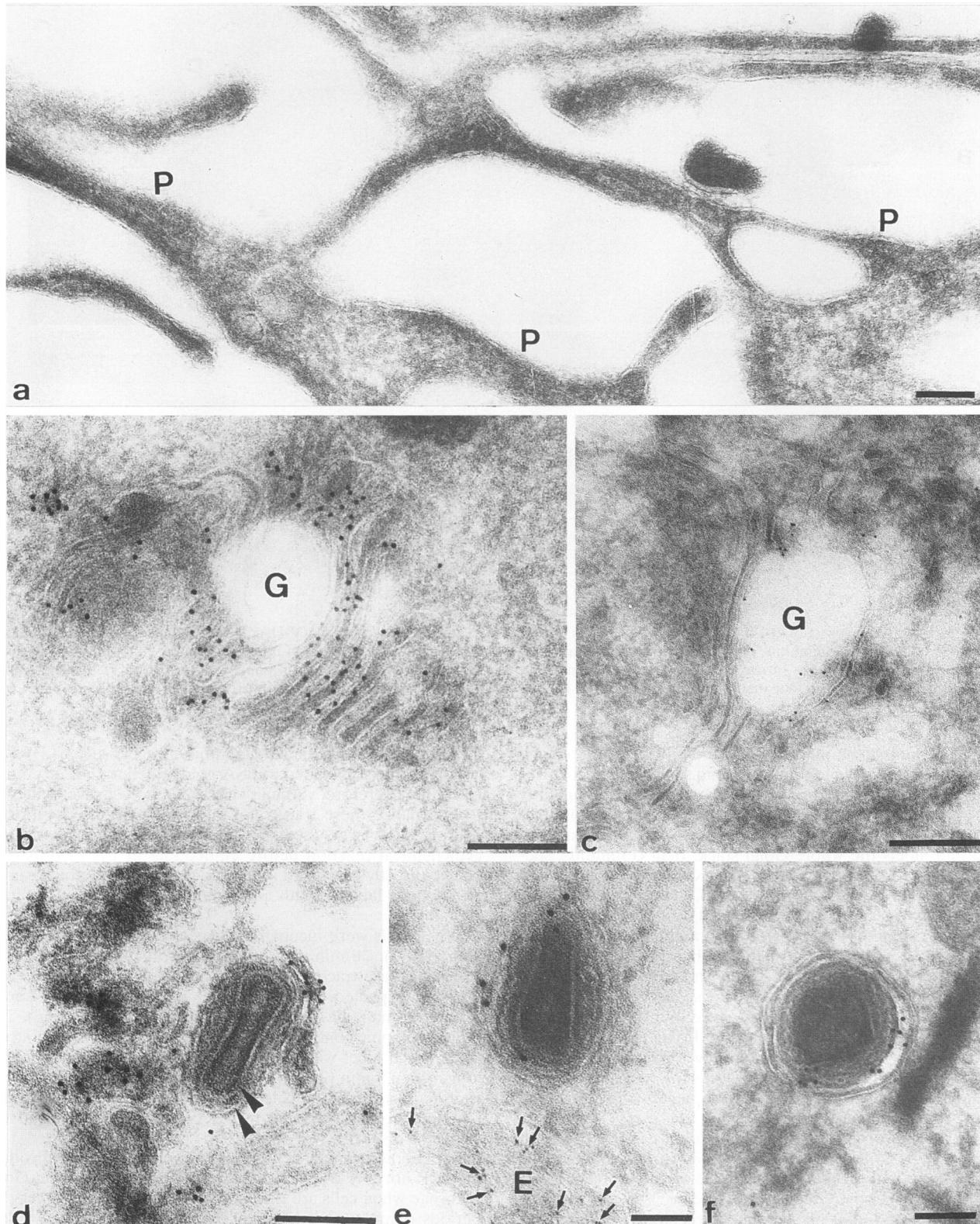


FIG. 3. Ultrathin cryosections of IHD-J-infected RK<sub>13</sub> cells labelled with a MAb specific for the viral envelope protein VV-p21. This protein is enriched in the Golgi apparatus (b). Following treatment with cycloheximide for 3 h, VV-p21 is localized predominantly on one side of the Golgi complex, presumably the trans/TGN side (c). The wrapping cisternae (d) and the two outermost membranes of the IEV (e and f) are also positive for VV-p21. Note that in panel d, the wrapping is not complete. The arrowheads in panel d denote the two membranes of the IMV. The plasma membrane (a) as well as endosomes containing internalized BSA-gold (e, small arrows) are essentially devoid of labelling; one gold particle is indicated in panel a. G, Golgi complex; P, plasma membrane; E, endosomes. Bars, 300 nm (a), 200 nm (b to d), and 100 nm (e and f).

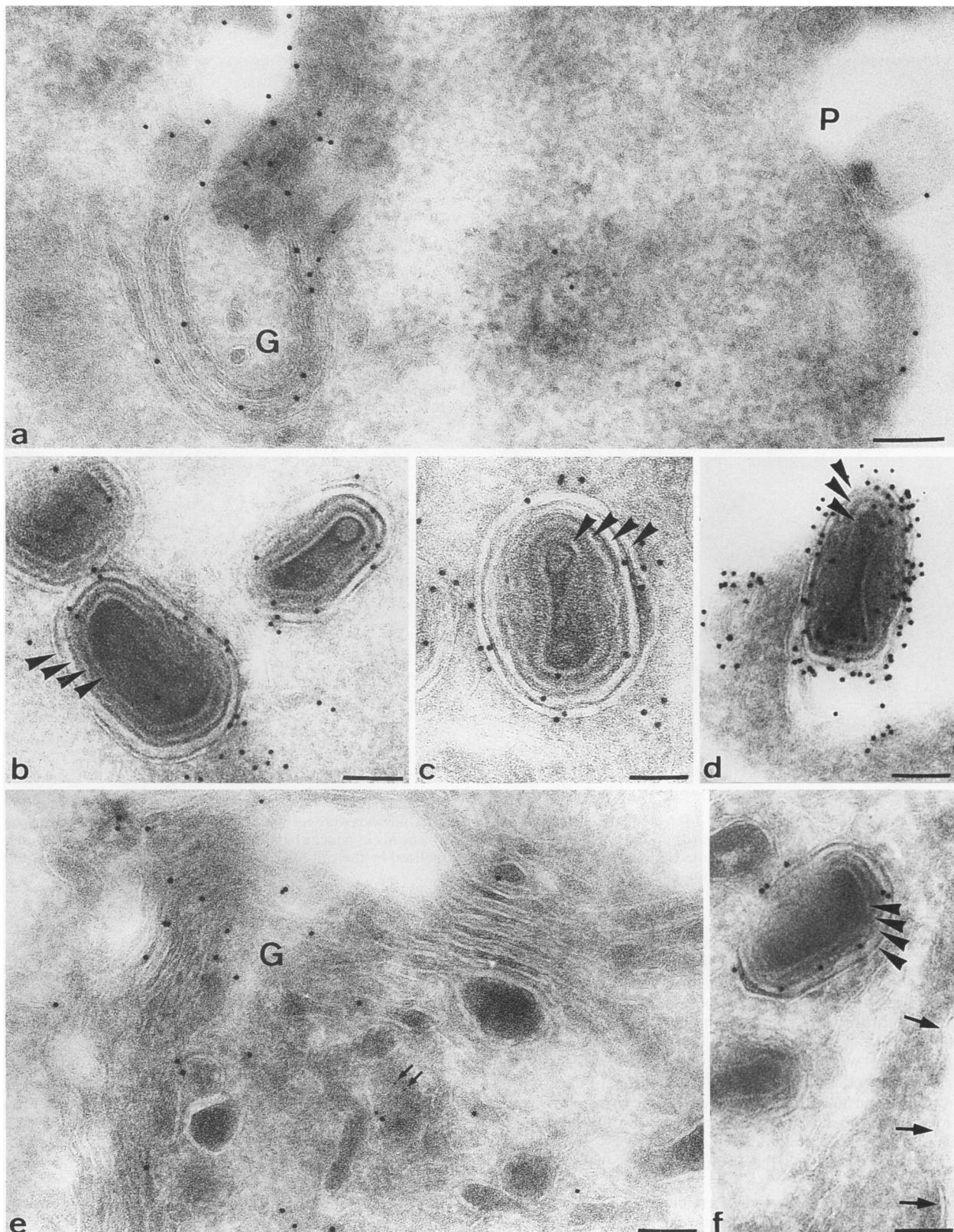


FIG. 4. Ultrathin cryosection of IHD-J-infected RK<sub>13</sub> cells labelled with antibodies specific for the vaccinia virus envelope proteins VV-p42 (a to d) and VV-p37 (e and f). MAb 17C4 specific for VV-p42 labels the Golgi complex (a), the outer two membranes of the IEV (b and c), and the envelope of the EEV (d). While the plasma membrane has generally a low level of labelling (a), patches of very high labelling are sometimes seen (d). The four membranes of the IEV (b, c, and f) and the three membranes of the EEV (d) are denoted by arrowheads. MAb 15B6 specific for VV-p37 labels the Golgi complex (e) and the two outer membranes of the IEV (f). The large arrows in panel f point to the plasma membrane, which was unlabelled by antibodies to VV-p37, while the small arrows in panel e denote internalized BSA-gold (5 nm) in the Golgi region. G, Golgi complex; P, plasma membrane. Bars, 200 nm (a) and 100 nm (b to f).

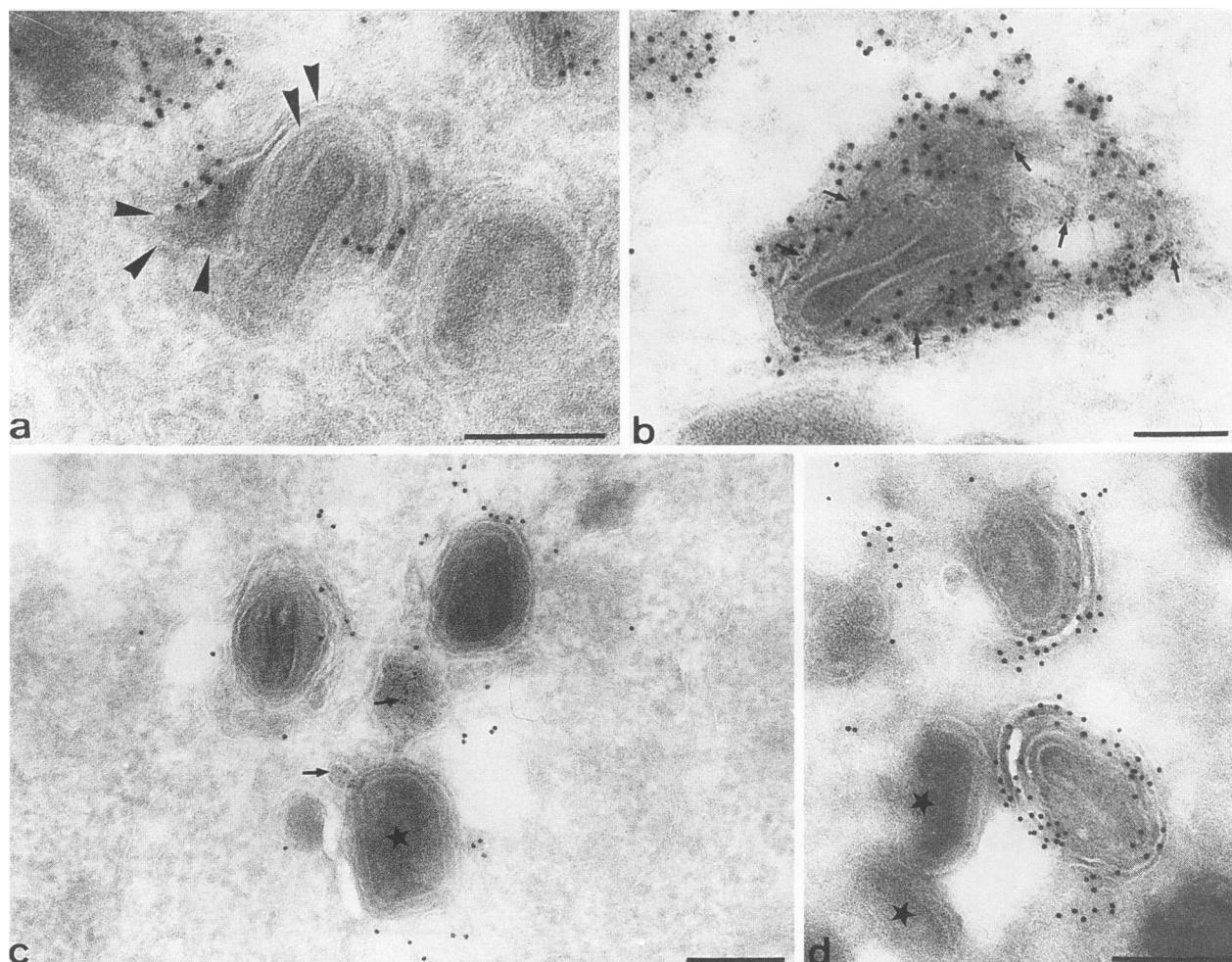


FIG. 5. Ultrathin cryosections of IHD-J-infected RK<sub>13</sub> cells labelled with lectins. Glycans were recognized by ricin communis (a and b), maackia amurensis (c), and wheat germ agglutinin (d) on the wrapping membranes (arrowheads in panel a) and on the outer two membranes of the IEV (b to d). The small arrows (b and c) denote BSA-gold (5 nm) which was internalized by the cell for 2 h prior to fixation. The stars denote IMV which are not labelled by any of the lectins. Bars, 200 nm.

than 30 min, could also be observed in low but significant amounts in one cisterna mainly on the trans side of the Golgi stack, presumably the TGN (see Fig. 9a, 11a, and 13a). Vaccinia virus infection thus appears to induce an abnormally high rate of transport of endocytic tracers to the trans Golgi cisternae.

Although the wrapping cisterna was clearly accessible to fluid-phase tracers, these data did not enable us to determine whether this cisterna belonged to an endocytic organelle or the late Golgi or TGN. Therefore, the next series of experiments was focused on the detection of either endocytic or Golgi and TGN marker proteins in the membranes of the wrapping cisternae.

**Are the wrapping membranes of endocytic or Golgi or TGN origin?** An additional complication for the analysis of the origin of the membrane cisterna which wraps the IMV to form the IEV was the consistent observation of IMV particles within the lumen of endosomes (Fig. 7c and d). The endosomes were identified by their characteristic morphology, showing a reticulum of tubular, cisternal, and vesicular elements (19, 26), by their content of internalized BSA-gold, and also by labelling with antibodies specific for endosomal marker proteins (Fig. 7c

and d). One explanation for this phenomenon could be that these virions were released from the cells and taken up again via the endocytic pathway. However, further experiments will be needed to explain how these virions find their way into the endosomes and what their fate is. The important point for our analysis here was to be able to distinguish between the wrapping event per se and virions that were found within the lumen of endosomes. Therefore, we searched for profiles in which the wrapping event was not complete. In this case, a part of the virus was free in the cytoplasm (Fig. 2c, 3d, 5a, 6c to f, 7a and b, 8c, 9d, and 11b).

To elucidate in more detail the possible role of the endocytic pathway in the wrapping process, antibodies against well-characterized marker proteins of early and late endosomes as well as lysosomes were used. Thawed cryosections of vaccinia virus-infected cells were labelled with different peptide antibodies specific for the two small GTP-binding proteins rab5 and rab7. rab5 was shown to be enriched on the cytoplasmic domains of early endosomes and on the plasma membrane (4), whereas rab7 is predominantly present on the membranes of late endosomes (4, 52). While the early and late endocytic organelles in vaccinia virus-infected cells were found to be

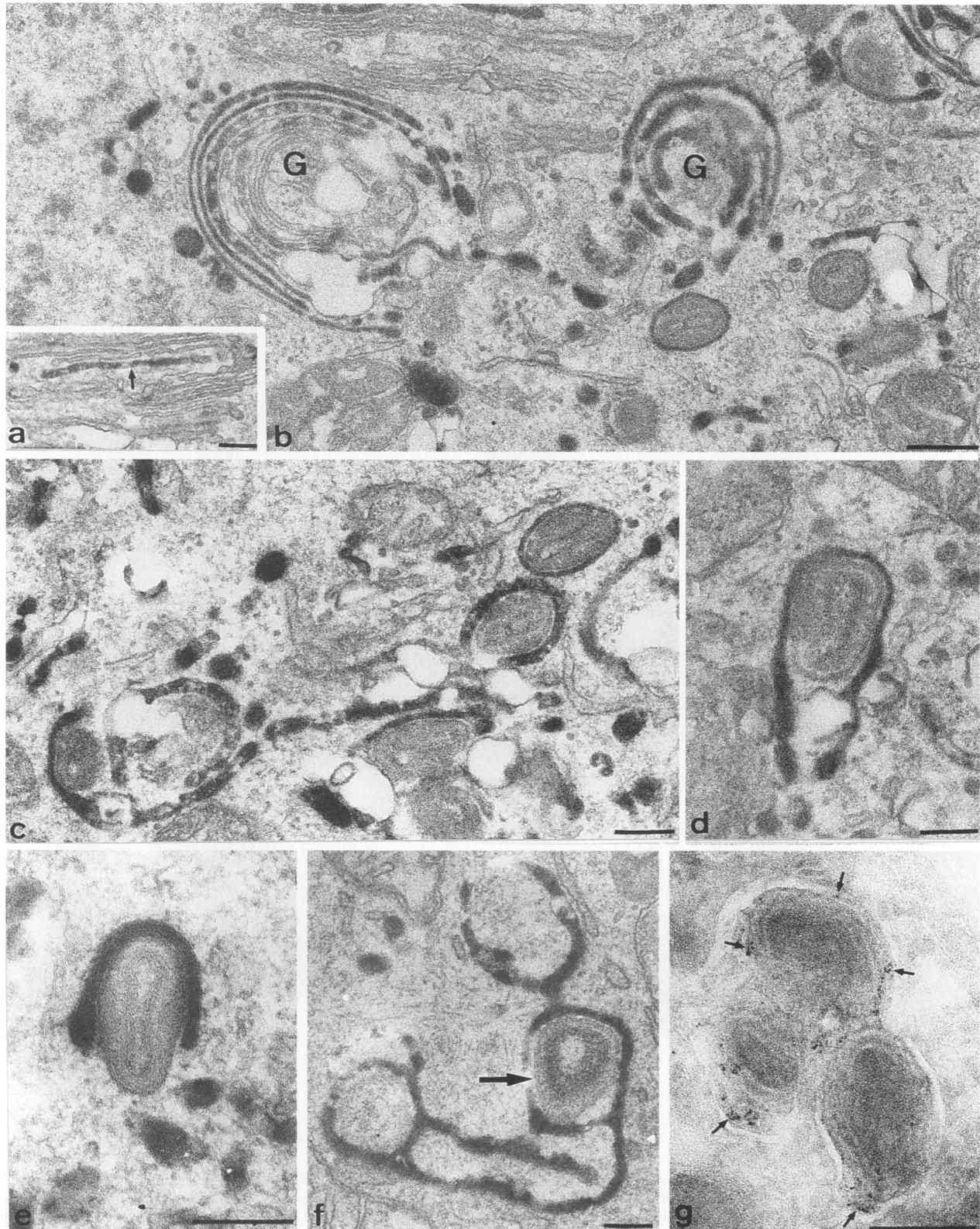


FIG. 6. Ultrathin sections showing the distribution of internalized HRP or BSA-gold in vaccinia virus-infected cells. WR-infected HeLa cells (a to e) and IHD-J-infected RK<sub>13</sub> cells (f and g) were allowed to internalize HRP (a to e, and g) or for 5 min (f) from the medium prior to fixation and then subjected to either embedding in Epon or freezing and ultrathin sectioning. The lumen of the wrapping membranes contain the reaction product of HRP (b to f) and internalized BSA-gold (g). Note that following vaccinia virus infection, HRP can also reach the Golgi complex (a and b). The more common situation is shown in panel a, where only one cisterna (presumably the TGN) reacts for HRP. In panel b, the reaction product of HRP fills a number of Golgi cisternae (f and g). When the internalization time was restricted to 5 min, a time at which in uninfected cells only early endosomes are labelled with fluid-phase tracers, a few wrapping membranes with the HRP reaction product can be found (f). The arrow denotes an IMV which appears to become enwrapped. G, Golgi complex. Bars, 300 nm (b) and 200 nm (a and c to g).

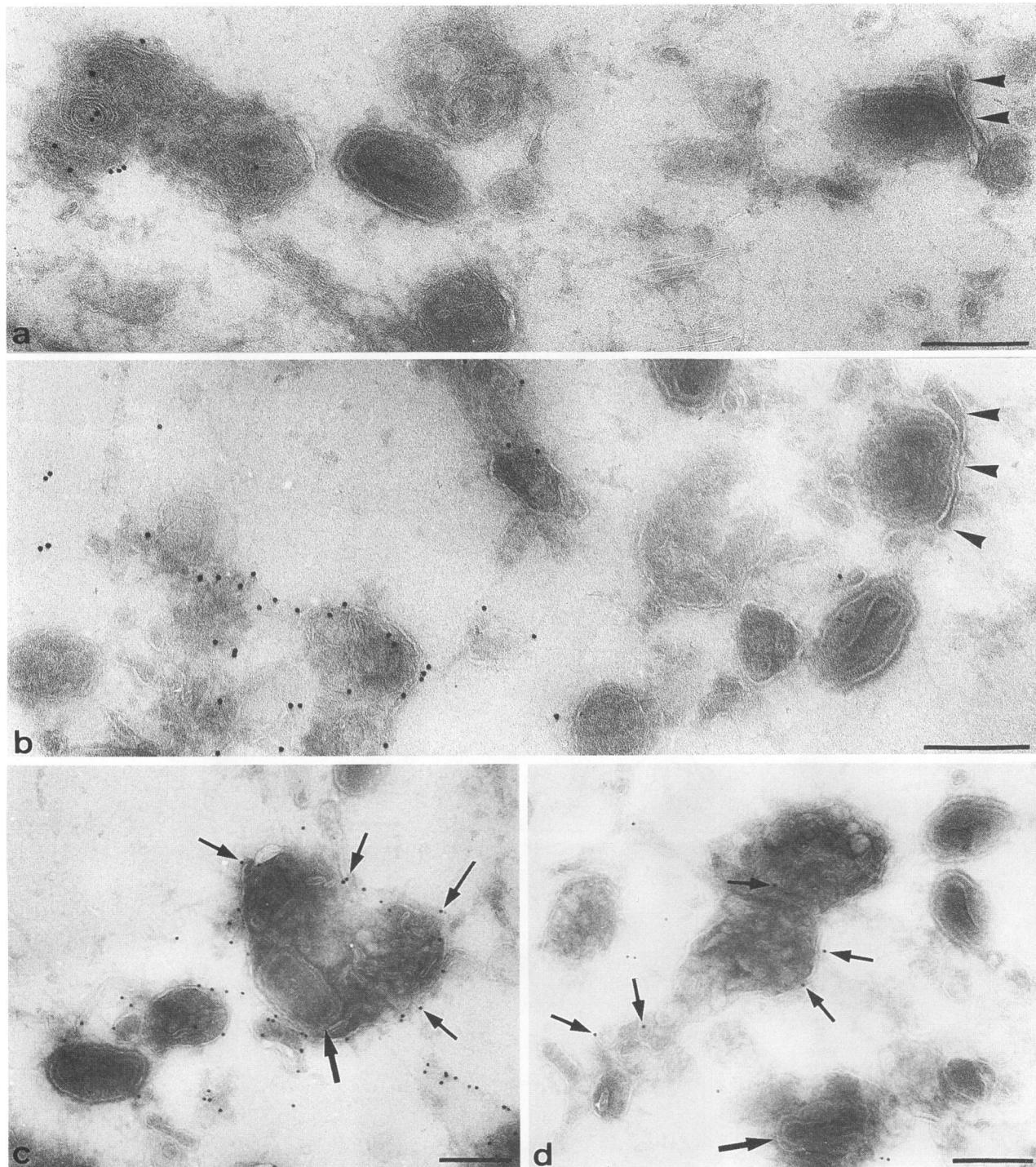


FIG. 7. Ultrathin cryosections of IHD-J-infected NRK cells labelled with antibodies specific for MPR (a), lgp120 (b and c), or rab7 (d). The wrapping membranes were consistently negative for lgp120 (a), MPR (b), and rab7 (not shown). Arrowheads in panels a and b denote the wrapping membranes. Note that the endocytic organelles (asterisks) are positive for MPR (a), lgp120 (b and c), and rab7 (d). Thin arrows denote the specific labelling for these markers on endocytic organelles; thick arrows point to IMV particles in endosomes (c and d). Bars, 200 nm.

positive for rab5 (not shown) and rab7 (Fig. 7d), respectively, there was no evidence for the presence of these marker proteins on the membranes of the wrapping cisternae.

The cation-independent MPR and a family of membrane

glycoproteins, known as lgps or lamps, have been shown to be enriched in late endosomes and lysosomes (16, 21, 34). Therefore, we labelled cryosections of vaccinia virus-infected cells with antibodies specific for these proteins. While late endocyti-

structures were strongly labelled by these markers (Fig. 7a to c), there was no significant labelling on the membranes enwrapping the IMV (Fig. 7a and b).

The Tfr complex is known to recycle mainly between the early endosomes and the plasma membrane and to a lesser extent between the late endosomes and the plasma membrane (see reference 59 for a review). The bulk of the intracellular Tfr complex is localized in the early endosomes (33). Thawed cryosections of vaccinia virus-infected cells overexpressing the human Tfr were labelled with antibodies specific for human Tfr. The majority of label was found either on the plasma membrane or in early endosomes, identified by the uptake of BSA-gold for 5 min. However, we could find no evidence for the presence of the Tfr on the membranes of the wrapping cisternae (not shown).

Collectively, these data argue that the membranes responsible for wrapping the IMVs do not originate from either early or late endosomes or lysosomes. In the next series of experiments, we therefore focused on the detection of Golgi and TGN marker proteins on the wrapping cisternae.

In most cell types, endogenous Golgi and TGN marker proteins are present in low concentrations, which is reflected in a relatively low immunogold labelling. In order to have Golgi marker proteins in high concentrations, we used two recombinant vaccinia viruses that express proteins with the characteristic features expected of bona fide Golgi or TGN marker proteins.

The first recombinant vaccinia virus (VV-G/PTV) expresses the G1/G2 protein complex of PTV. When expressed by vaccinia virus, the G1/G2 protein complex is localized to the Golgi stack. The N-linked oligosaccharides of the G1/G2 protein complex acquire endoglycosidase H resistance but no neuraminidase sensitivity (5, 6). Since sialic acid is added to N-linked glycoproteins in the TGN, it appears that these proteins reach only the trans cisternae of the Golgi complex, not the TGN (23). In VV-G/PTV-infected cells, the Golgi stacks were found to be strongly labelled with an antibody specific for G1 (Fig. 8a). In some sections, one unlabelled cisterna could be seen adjacent to the labelled cisternae (Fig. 8b). This unlabelled cisterna probably represents the TGN. However, we could not detect any significant label with the antibody against G1 on the membranes enwrapping the IMV (Fig. 8b to d).

The second recombinant vaccinia virus (VV-MHV-M) expresses the M protein (formerly referred to as E1) of MHV. When expressed in vaccinia virus, the M protein acquires TGN modifications and behaves as a late Golgi/TGN resident protein colocalizing with the TGN marker protein TGN 38 (23, 35, 36). Therefore, HeLa cells were infected with VV-MHV-M and also allowed to internalize BSA-gold (5-nm size) for up to 2 h before fixation. When cryosections from these samples were labelled with antibodies specific for the M protein, only very low amounts of labelling could be detected on the plasma membrane (Fig. 9a, 11a, and 13a) and in endosomes, defined by their characteristic morphology and by their high concentration of internalized BSA-gold (Fig. 9a and 13a). However, a strong labelling for the M protein was seen on the membranes of a variable number of cisternae on the trans side of the Golgi stack (Fig. 9a) as well as on the membranes in continuity with the wrapping cisternae of the IMV (Fig. 11b). In addition, low but significant amounts of the internalized BSA-gold were observed in the TGN (Fig. 9a, 11a, and 13a). The outer two membranes of the IEV, often containing variable amounts of BSA-gold (Fig. 9c), also showed a low labelling for the M protein (Fig. 9b to d). The majority of the EEV particles in the extracellular space were negative for the M protein. On a few

EEV particles, a small amount of labelling for the M protein could be detected (not shown). Further biochemical studies are needed to determine whether significant amounts of the M protein, which is obviously not a normal structural protein of vaccinia virus, are indeed incorporated into the IEV and/or EEV.

These results argue that the IMV wrapping cisterna derives from the TGN. Following vaccinia virus infection, the transport of fluid-phase endocytic markers into the TGN appears to be significantly increased in comparison with uninfected cells, resulting in the incorporation of these endocytic tracers into the wrapping cisterna.

**Clathrin-like coated buds are associated with the wrapping membranes.** Within the Golgi/TGN complex, clathrin-coated buds are exclusively found in the TGN (22, 25, 43). Clathrin can be visualized in Epon sections, as it forms distinct bristles or spikes. Upon examination of Epon sections of RK<sub>13</sub> cells infected for 8 h with vaccinia virus strain IHD-J, we found clathrin-like coated vesicles budding from a membrane cisterna which was enwrapping the IMV (Fig. 10). This observation further supports the immunocytochemical data showing that the wrapping cisterna originates from the TGN.

**Do vaccinia virus envelope proteins behave as TGN resident proteins?** If the TGN is involved in wrapping, it would be expected that one or more of the viral envelope proteins would be significantly enriched in the TGN, in effect behaving as TGN resident proteins. To address this point, vaccinia virus-infected cells were incubated in normal medium for 8 h and then further incubated for 1 to 3 h in the presence of cycloheximide in order to chase the viral membrane proteins to their final destination in the cell. Following this treatment, VV-p42, VV-p37, and VV-p21 (Fig. 3c) were localized predominantly on one side of the Golgi stack, presumably the TGN. Even after 3 h of cycloheximide treatment, the amounts of labelling for these three viral proteins found on the plasma membrane and in endosomes were not increased in comparison with untreated cells (see below).

A double-labelling analysis was performed to determine whether the four viral envelope proteins colocalize with the MHV-M and, if they do, whether they maintain this colocalization upon long incubation periods with cycloheximide. Therefore, HeLa cells infected with the recombinant VV-MHV-M were incubated in normal medium for 8 h and then further incubated for 1 to 3 h in the presence of cycloheximide. The endocytic organelles were labelled in these cells by adding BSA-gold (5 nm) to the culture medium for 2 h prior to fixation. Thawed cryosections of these preparations were double labelled with antibodies specific for MHV-M (visualized by 9-nm gold-protein A) and with each of the antibodies specific for VV-p42 (Fig. 11a to c), VV-p21 (not shown), VV-HA (Fig. 11d), and VV-p37 (Fig. 12 and 13). The vaccinia virus proteins were visualized with 15-nm gold particles. Regardless of whether the cells were treated with cycloheximide, the M protein colocalized with the vaccinia virus envelope proteins on the wrapping membranes, as shown for VV-p42 in Fig. 11b, and on the outer membranes of the IEV (Fig. 11c and d, 12a to c, and 13b to d). VV-p42 (Fig. 11a), VV-p37 (Fig. 12a to c and 13a), VV-p21 (not shown), and VV-HA (not shown) also colocalized with the M protein predominantly on one side of the Golgi complex. Of the four viral envelope proteins that we examined, the only one that did not seem to be predominantly enriched in the TGN was VV-HA. After 3 h of cycloheximide treatment, it was still localized in high concentrations on the plasma membrane and in endosomes, although significant amounts nevertheless colocalized with the M protein in the Golgi complex (not shown).

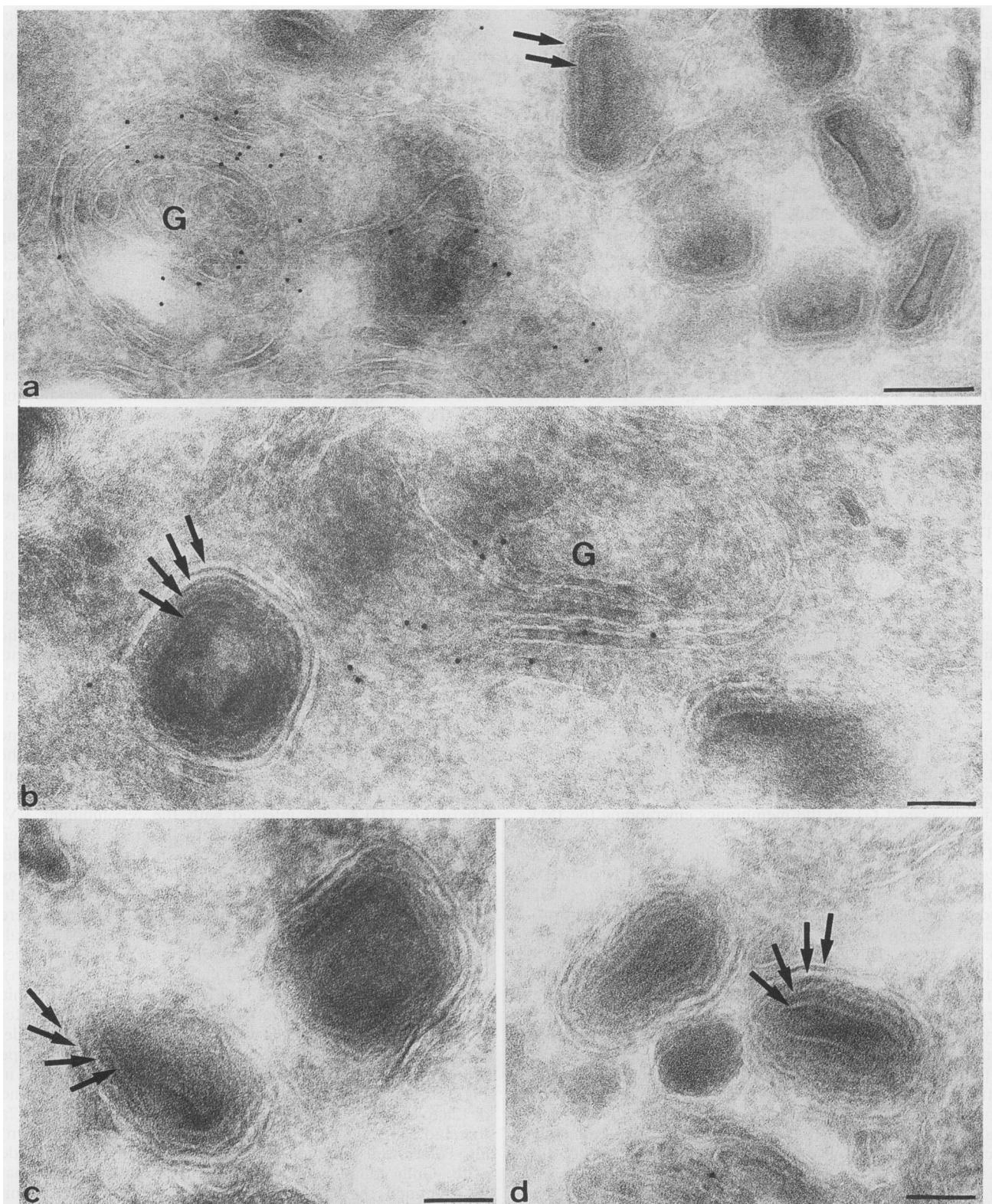


FIG. 8. Ultrathin cryosections of RK<sub>13</sub> cells infected with the recombinant vaccinia virus expressing the G1/G2 protein of PTV. Antibodies specific for the G1 protein label the Golgi complex (a and b) but not the wrapping membranes (b to d). The two arrows in panel a denote the two unlabelled membranes of the IMV. Note the absence of labelling on the IEV in panels b to d. The four membranes are indicated by arrows. G, Golgi complex. Bars, 200 nm (a) and 100 nm (b to d).

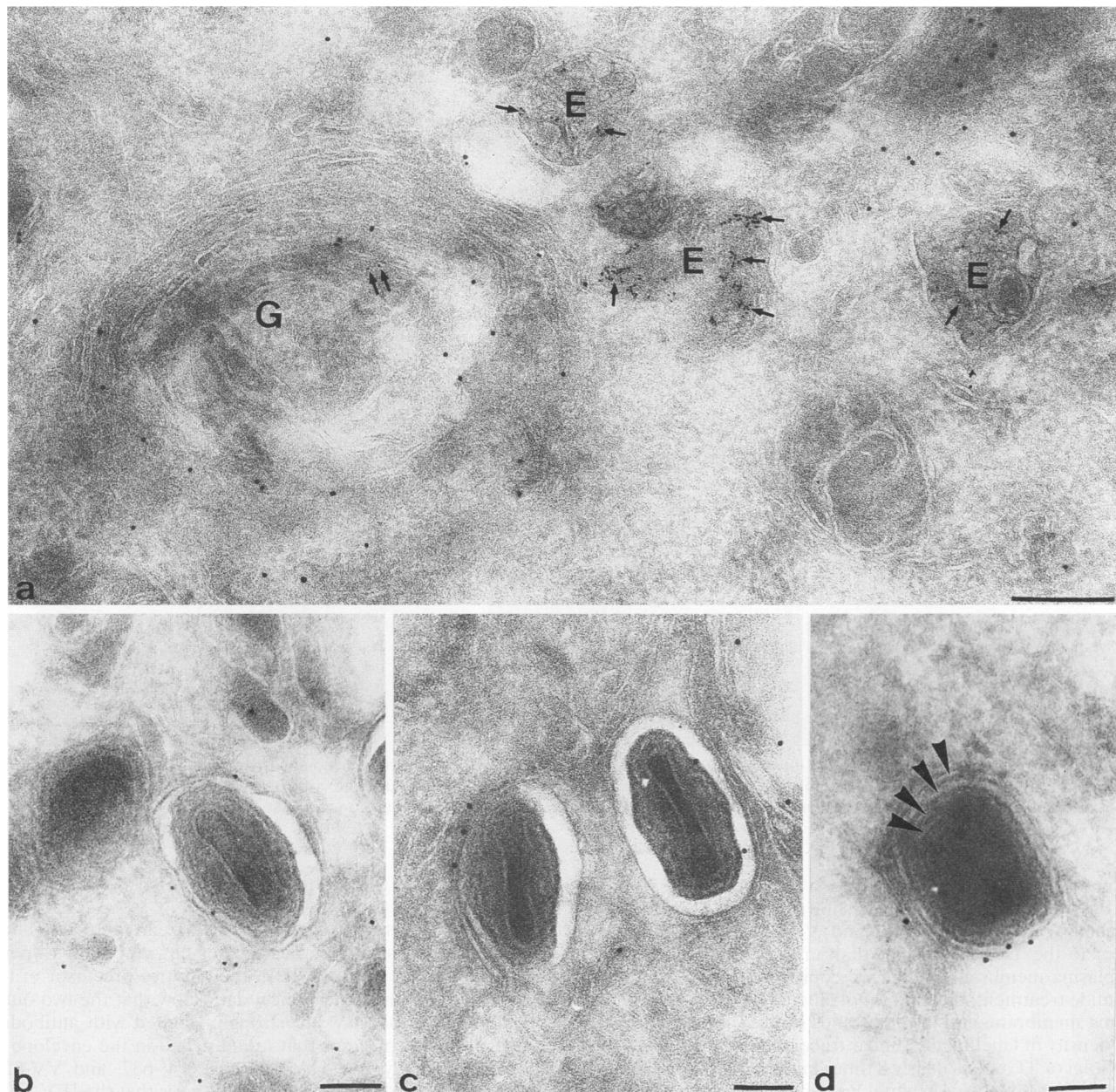


FIG. 9. Ultrathin cryosections of HeLa cells infected with the recombinant vaccinia virus expressing MHV-M. Antibodies specific for the MHV-M label mainly the trans/TGN side of the Golgi complex (a), while no label is associated with the plasma membrane or with endosomes (a). The small arrows denote internalized BSA-gold in endosomes and in the TGN region. Some of the MHV-M protein is also detected on the wrapping membranes and on the outer two membranes of the IEV (b to d). The arrowheads in panel d point out the four membranes of the IEV. G, Golgi complex; E, endosomes. Bars, 200 nm (a) and 100 nm (b to d).

To document the subcellular localization of VV-p42 and VV-p37 more precisely, the labelling associated with the Golgi complex, the plasma membrane, and endosomes was quantified. The TGN was defined as a structure containing high concentrations of the MHV-M protein. It should be noted that in this experiment, it was not possible to distinguish between trans Golgi cisternae and the TGN. The quantitation of the labelling was carried out in two steps. First, the density of labelling of VV-p42 or VV-p37 over the micrograph area of TGN or endosomes was determined. According to stereologi-

cal theory, this relates to the density of label per volume of organelle (18). For the plasma membrane, which is a two-dimensional entity, a volume parameter cannot be determined. Therefore, in the second part of the analysis, the density of label over the linear trace of the plasma membrane was compared with that found in systematically selected parts of the TGN (possibly including trans Golgi cisternae), where the membranes were cut perpendicularly and were distinct. Thus, the density of labelling per linear micrometer of membrane could be determined by relating the number of gold particles to



FIG. 10. Ultrathin sections of an IHD-J-infected RK<sub>13</sub> cell embedded in Epon. An IMV particle becomes enwrapped in the TGN region of the Golgi complex. The large arrowheads denote the two membranes of the cisterna wrapping around the virus particle. Note the clathrin-like coated bud associated with the wrapping membrane (small arrowheads). G, Golgi complex; IV, immature virus. Bar, 200 nm.

the number of intersections between the membrane profiles and the lines of a lattice grid. As shown in Table 1, the density of labelling for both VV-p42 and VV-p37 was significantly higher in the TGN (or Golgi) than in the endosomes and on the plasma membrane. When we compared 1- and 2-h cycloheximide treatment, the amount of labelling for VV-p37 on the plasma membrane and in endosomes showed little difference. The density of labelling per linear trace of membrane profile in the Golgi or TGN was nearly 8 times higher for VV-p37 and 20 times higher for VV-p42 than it was on the plasma membrane. Collectively, these data argue that in vaccinia virus-infected cells, VV-p42, VV-p37, and VV-p21, like MHV-M, are significantly enriched in the TGN.

## DISCUSSION

The goal of this study was to characterize in more detail how the vaccinia virus IMV form becomes enwrapped by a membrane cisterna to form the IEV, the putative precursor of the EEV. Our immunocytochemical data show that the two outer membranes of the IEV are strongly labelled with antibodies specific for four proteins that are enriched in the envelope of the EEV, namely, VV-HA, VV-p42, VV-p37, and VV-p21. This result is consistent with the hypothesis that the IEV is the precursor of the EEV. The IEV was also labelled significantly with lectins that recognize sugars known to be added late in the Golgi complex but was negative for the pre-TGN Golgi marker

TABLE 1. Quantitation of VV-p37 and VV-p42 labelling in cells infected with the recombinant vaccinia virus expressing MHV-M

Protein	Duration (h) of cycloheximide treatment	Density of 15-nm immunogold/ $\mu\text{m}^2 \pm \text{SD}^a$		Density of 15-nm immunogold/ $\mu\text{m} \pm \text{SD}^b$	
		TGN or Golgi <sup>c</sup>	Endosomes <sup>d</sup>	TGN or Golgi	Plasma membrane
VV-p37	1	87 $\pm$ 17.8	22 $\pm$ 4.5	1.74 $\pm$ 0.25	0.2 $\pm$ 0.05
	2	74 $\pm$ 10.5	22 $\pm$ 8.4		
VV-p42	1	86 $\pm$ 11.6	12 $\pm$ 9.6	2 $\pm$ 0.26	0.09 $\pm$ 0.03

<sup>a</sup> Density of gold per area of micrograph over the structure.

<sup>b</sup> Density of gold particles per linear trace of membrane profile. Note that this cannot directly compare with the values given per square micrometer.

<sup>c</sup> Defined as membranes enriched in MHV-M (10-nm gold). In this experiment, it is not possible to unequivocally distinguish between TGN and Golgi elements.

<sup>d</sup> Defined as organelles containing high concentrations of BSA-gold and low concentrations of MHV-M (10-nm gold). These structures can easily be recognized by their morphological characteristics.

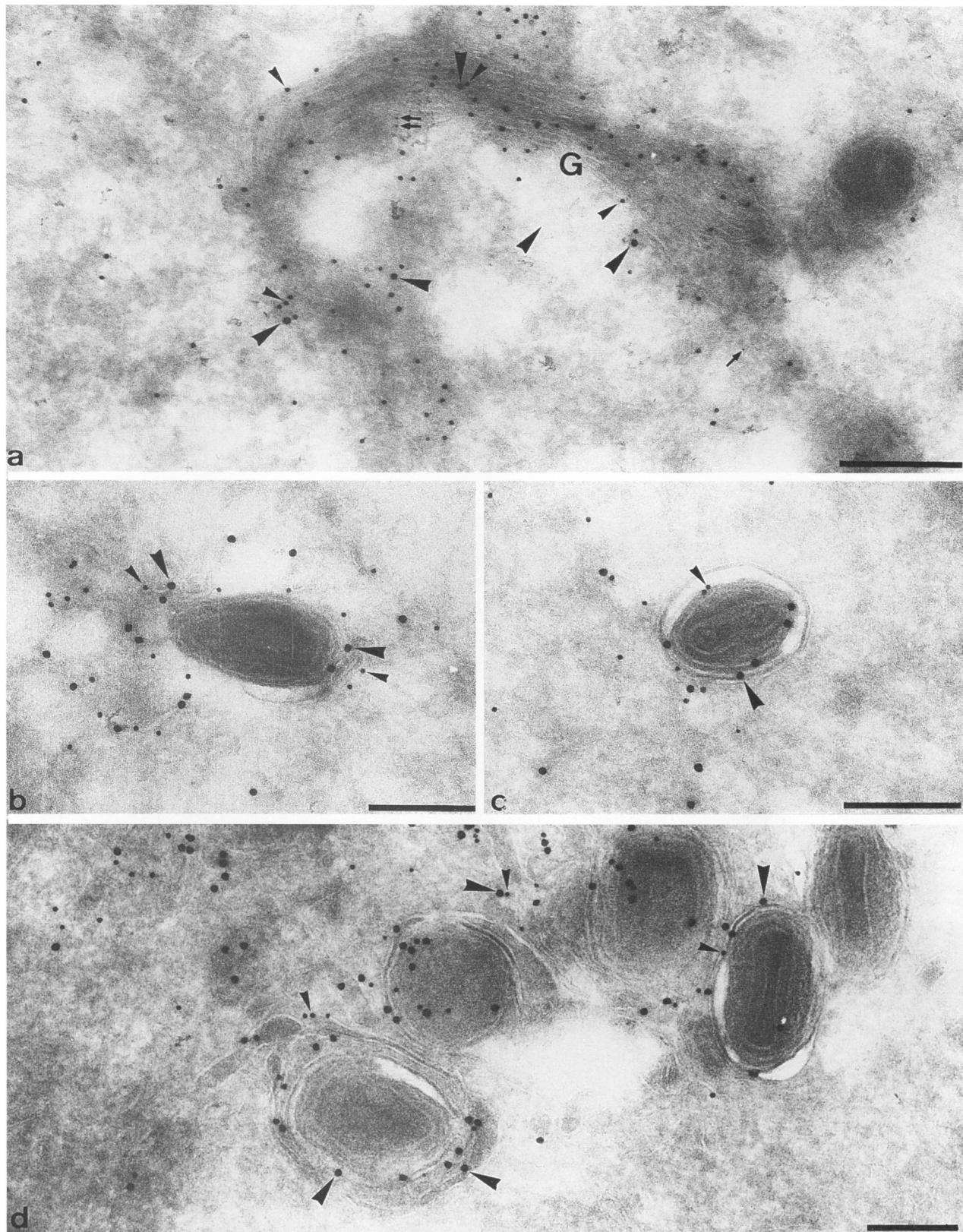


FIG. 11. Ultrathin cryosections of HeLa cells infected with the recombinant vaccinia virus expressing MHV-M. Prior to fixation, the endocytic organelles were labelled by internalization of BSA-gold (5 nm; small arrows) for 2 h and incubated for an additional 1 h with cycloheximide. The sections were double labelled with antibodies specific for MHV-M (9-nm gold particles; small arrowheads) (a to d) and for VV-p42 (MAb 19C2; a to c) or VV-HA (d) were visualized with 15-nm gold particles (large arrowheads). VV-p42 colocalizes with MHV-M on the wrapping membranes (b to d). Internalized BSA-gold can also be found in the Golgi region (a, small arrows). G, Golgi complex. Bars, 250 nm (a) and 200 nm (b to d).

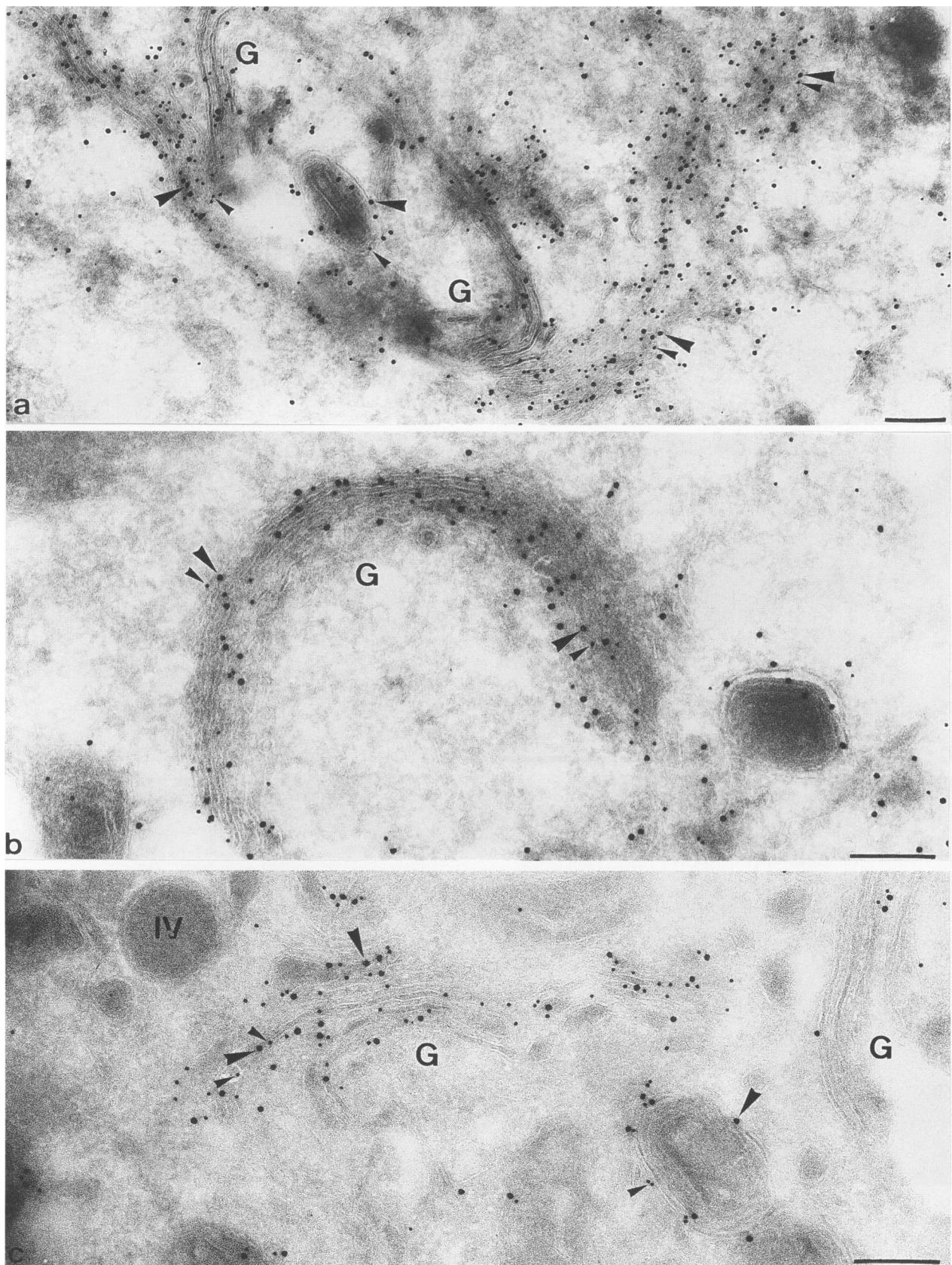


FIG. 12. Ultrathin cryosections of HeLa cells infected with the recombinant vaccinia virus expressing MHV-M, incubated for 1 h with cycloheximide prior to fixation and double labelled with antibodies specific for MHV-M (9-nm gold particles; small arrowheads) and VV-p37 (15-nm gold particles; large arrowheads). MHV-M and VV-p37 colocalize in the Golgi complex (a to c). A small amount of MHV-M colocalizes with VV-p37 on the outer two membranes of the IEV (a to c). G, Golgi complex; IV, immature virus. Bars, 200 nm.

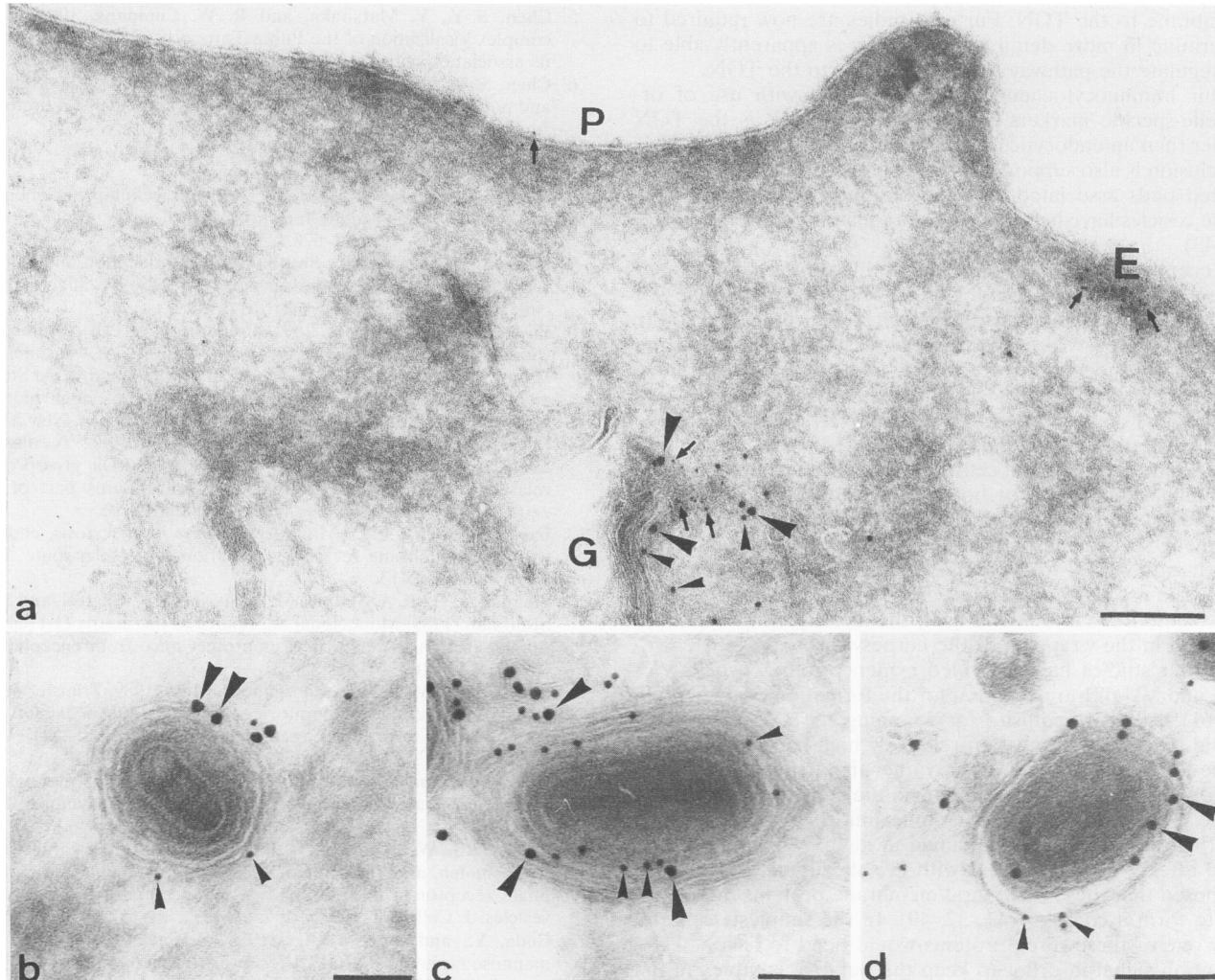


FIG. 13. Ultrathin cryosections of HeLa cells infected with the recombinant vaccinia virus expressing MHV-M. Prior to fixation, cells were incubated for 3 h with cycloheximide, and BSA-gold (5 nm) was internalized for the last 2 h before fixation. Double labelling with antibodies specific for MHV-M (9-nm gold particles; small arrowheads) and VV-p37 (15-nm gold particles; large arrowheads) show colocalization in the Golgi complex (a) and on the two outer membranes of the IEV (b to d). Note that internalized gold particles (small arrows) colocalize with MHV-M and VV-p37 in the Golgi complex (a). The plasma membrane is free of MHV-M and VV-p37 label. The small arrows denote internalized gold in an endosome and in the region of the Golgi complex. A few BSA-gold particles occasionally adhere to the plasma membrane. E, endosome; G, Golgi complex; P, plasma membrane. Bars, 200 nm (a and d) and 100 nm (b and c).

protein PTV-G1. These data are in agreement with the results of a lipid analysis (57) showing that the lipid composition of purified IEV and EEV is similar to that of late Golgi compartments or plasma membrane in containing relatively high amounts of sphingomyelin and phosphatidylserine but low amounts of phosphatidylinositol. In conjunction with our previous immunocytochemical data on the formation of the IMV (57), this finding argues strongly that the membranes involved in the wrapping event are derived not from the rough ER, the intermediate compartment, or early Golgi structures but rather from a late Golgi or a post Golgi compartment.

When labelling endocytic organelles with the fluid-phase tracers HRP and BSA-gold, we were initially surprised to find that significant amounts of these tracers appeared to have access to the lumen of the wrapping cisternae, suggesting that the wrapping cisternae might derive from an endocytic or

ganelle. However, the data also show that following vaccinia virus infection, significant amounts of fluid-phase endocytic tracers are rapidly transported to the TGN and in many cases even to the whole Golgi stack. It is well established that a pathway exists from either early or late endosomes (or both) to the TGN (8, 17, 42). However, in most cell types, this pathway is a minor one compared with the pathway from early to late endosomes or with the recycling pathway from early endosomes to the plasma membrane (24). Even when a tracer is bound to the plasma membrane, only small amounts appear to reach the TGN after internalization (60). In contrast, in regulated secretory cells following a burst of exocytosis, significant levels of endocytic markers, and in particular of membrane-bound markers, can be easily detected in the TGN (12, 28, 29, 45). Therefore, at least some cell types are able to regulate the amount of membrane traffic from the plasma

membrane to the TGN. Further studies are now required to determine in more detail how the virus is apparently able to up-regulate the pathway from endosomes to the TGN.

Our immunocytochemical data obtained with use of organelle-specific markers clearly indicate that it is the TGN rather than an endocytic organelle which wraps the IMV. This conclusion is also supported by the observation of clathrin-like coated buds associated with the wrapping membrane, since these vesicles have been shown to originate from the TGN (22, 25, 43).

Recently, Tooze et al. (58) reported that internalized fluid-phase markers have access to the wrapping membranes of vaccinia virus as well as cytomegalovirus, which belongs to the family of herpesviruses. That study was based solely on the fluid-phase endocytic tracers HRP and BSA-gold combined with conventional Epon section analysis. Despite the fact that the authors also observed internalized HRP throughout the Golgi cisternae in vaccinia virus-infected cells, but not in cytomegalovirus-infected cells, the authors concluded that the wrapping membranes for both viruses derive from the early endosomal compartment. From the data presented here using organelle-specific markers, we feel confident of our conclusion that it is the TGN which wraps around the IMV to form the IEV. The use of organelle-specific markers will also be essential to determine more definitively the identity of the organelle involved in the wrapping of the herpesviruses.

Earlier studies have provided evidence that VV-p37, VV-p42, and VV-p21 are essential for the formation of EEV (2, 9, 53, 61). As a prerequisite for wrapping the IMV, the TGN would be expected to retain relatively high concentrations of these EEV membrane proteins rather than transporting them rapidly to the plasma membrane via the constitutive secretory route. Our experiments with cycloheximide show that all three viral marker proteins are retained in high levels in the TGN, even after 3 h of treatment with cycloheximide. It has been proposed that these three viral membrane proteins may interact to form a complex (11, 32, 49). In the simplest scenario, only one of these three proteins would need to have a TGN retention signal in order to keep the putative complex in the TGN. The partners would then be retained indirectly by being part of the protein complex. Studies are now under way to express these viral proteins independently by transfection in uninfected cells in order to test this retention hypothesis (62).

#### ACKNOWLEDGMENTS

We thank the following people for providing recombinant vaccinia viruses, vaccinia virus deletion mutants, and antibodies: C. Bucci, M. Zerial, L. Payne, R. Blasco, B. Hoflack, I. Mellman, J. Fleming, J. Smith, R. Compans, J. Krijnse-Locker, and P. Rottier. We also thank B. Moss, B. Doms, and J. Burkhardt for helpful suggestions and discussions and for critical reading of the manuscript.

#### REFERENCES

- Anderson, R. G. W., and R. K. Pathak. 1985. Vesicles and cisternae in the trans Golgi apparatus of human fibroblasts are acidic compartments. *Cell* **40**:635–643.
- Blasco, R., and B. Moss. 1991. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-dalton outer envelope protein. *J. Virol.* **65**:5910–5920.
- Bucci, C., R. G. Parton, I. H. Mather, H. Stunnenberg, K. Simons, B. Hoflack, and M. Zerial. 1992. The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell* **70**:715–728.
- Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP-binding proteins to exocytic and endocytic compartments. *Cell* **62**:317–329.
- Chen, S.-Y., Y. Matsuoka, and R. W. Compans. 1991. Golgi complex localization of the Punta Toro virus G2 protein requires its association with the G1 protein. *Virology* **183**:351–365.
- Chen, S. Y., Y. Matsuoka, and R. W. Compans. 1991. Assembly and polarized release of Punta Toro virus and effects of brefeldin A. *J. Virol.* **65**:1427–1439.
- Dales, S., and B. G. T. Pogo. 1981. Biology of poxviruses. *Virology monographs*. Springer-Verlag, Vienna.
- Duncan, J. R., and S. Kornfeld. 1988. Intracellular movement of two mannose 6-phosphate receptors: return to the Golgi apparatus. *J. Cell Biol.* **106**:612–628.
- Duncan, S. A., and G. L. Smith. 1992. Identification and characterization of an extracellular envelope glycoprotein affecting vaccinia virus egress. *J. Virol.* **66**:1610–1621.
- Earl, P. L., and B. Moss. 1991. Preparation of cell cultures and vaccinia virus stocks, p. 16.16.1–16.16.7. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. Greene Publishing Associates and Wiley Interscience, New York.
- Engelstadt, M., S. T. Howard, and G. L. Smith. 1992. A constitutively expressed vaccinia gene encodes a 42-kDa glycoprotein related to complement control factors that forms part of the extracellular virus envelope. *Virology* **188**:801–810.
- Farquhar, M. G. 1983. Multiple pathways of exocytosis, endocytosis and membrane recycling: validation of a Golgi route. *Fed. Proc.* **42**:2407–2413.
- Fleming, J. O., R. A. Shubin, M. A. Sussman, N. Casteel, and S. A. Stohlman. 1989. Monoclonal antibodies to the matrix (E1) glycoprotein of mouse hepatitis virus protect mice from encephalitis. *Virology* **168**:162–167.
- Fuerst, T. R., M. P. Fernandez, and B. Moss. 1989. Transfer of the inducible lac repressor/operator system from *Escherichia coli* to a vaccinia virus expression vector. *Proc. Natl. Acad. Sci. USA* **86**:2549–2553.
- Galfre, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* **73B**:1–46.
- Geuze, H. J., W. Stoorvogel, G. J. Strous, J. W. Slot, J. Zijderhand-Bleekemolen, and I. Mellman. 1988. Sorting of mannose 6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. *J. Cell Biol.* **107**:2491–2501.
- Goda, Y., and S. R. Pfeffer. 1988. Selective recycling of the mannose 6-phosphate/IGF-II receptor to the *trans* Golgi network *in vitro*. *Cell* **55**:309–320.
- Griffiths, G. 1993. Fine structure. *Immunocytochemistry series*. Springer-Verlag, Heidelberg.
- Griffiths, G., R. Back, and M. Marsh. 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. *J. Cell Biol.* **109**:2703–2720.
- Griffiths, G., and J. Gruenberg. 1991. The arguments for pre-existing early and late endosomes. *Trends Cell Biol.* **1**:5–9.
- Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* **52**:329–341.
- Griffiths, G., S. Pfeiffer, K. Simons, and K. Matlin. 1985. Exit of newly synthesized membrane proteins from the trans cisterna of the Golgi complex to the plasma membrane. *J. Cell Biol.* **101**:949–964.
- Griffiths, G., and P. Rottier. 1992. Cell biology of viruses that assemble along the biosynthetic pathway. *Semin. Cell Biol.* **3**:367–381.
- Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the golgi complex. *Science* **234**:438–443.
- Griffiths, G., G. Warren, I. Stulfauth, and B. Jockusch. 1981. The role of clathrin-coated vesicles in acrosome formation. *Eur. J. Cell Biol.* **26**:52–60.
- Gruenberg, J., G. Griffiths, and K. E. Howell. 1989. Characterization of the early endosome and putative endocytic carrier vesicles *in vivo* and with an assay of vesicle fusion *in vitro*. *J. Cell Biol.* **108**:1301–1316.
- Hauri, H. P., and A. Schweizer. 1992. The endoplasmic reticulum-Golgi intermediate compartment. *Curr. Opin. Cell Biol.* **4**:600–608.

28. Herzog, H., and H. Reggio. 1980. Pathways of endocytosis from luminal plasma membrane in rat exocrine pancreas. *Eur. J. Cell Biol.* **21**:141–150.
29. Herzog, V., and M. G. Farquhar. 1977. Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. *Proc. Natl. Acad. Sci. USA* **74**:5073–5077.
30. Hiller, G., and K. Weber. 1985. Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *J. Virol.* **55**:651–659.
31. Ichihashi, Y., and S. Dales. 1971. Biogenesis of poxviruses: Interrelationship between hemagglutinin production and polykaryocytosis. *Virology* **46**:533–543.
32. Isaacs, S. N., E. J. Wolffe, L. G. Payne, and B. Moss. 1992. Characterization of a vaccinia virus-encoded 42-kilodalton class I membrane glycoprotein component of the extracellular virus envelope. *J. Virol.* **66**:7217–7224.
33. Killisch, I., P. Steinlein, K. Römisch, R. Hollinshead, H. Beug, and G. Griffiths. 1992. Characterization of early and late endocytic compartments of the transferrin cycle. *J. Cell Sci.* **103**:211–232.
34. Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**:483–525.
35. Krijnse-Locker, J., G. Griffiths, M. C. Horzinek, and P. J. M. Rottier. 1992. O-glycosylation of the coronavirus M protein—differential localization of sialyltransferases in N- and O-linked glycosylation. *J. Biol. Chem.* **267**:14094–14101.
36. Krijnse-Locker, J., J. K. Rose, M. C. Horzinek, and P. J. M. Rottier. 1992. Membrane assembly of the triple-spanning coronavirus M protein—individual transmembrane domains show preferred orientation. *J. Biol. Chem.* **267**:21911–21918.
37. Lewis, V., S. A. Green, M. Marsh, P. Vihko, A. Helenius, and I. Mellman. 1985. Glycoproteins of the lysosomal membrane. *J. Cell Biol.* **100**:1839–1847.
38. Matsuoka, Y., T. Ihara, D. H. L. Bishop, and R. W. Compans. 1988. Intracellular accumulation of Punta Toro virus glycoproteins expressed from cloned cDNA. *Virology* **167**:251–260.
39. Morgan, C. 1976. Vaccinia virus reexamined: development and release. *Virology* **73**:43–58.
40. Moss, B. 1990. Poxviridae and their replication, p. 2079–2111. In B. N. Fields and D. M. Knipe (ed.), *Virology*. Raven Press, Ltd., New York.
41. Moss, B. 1990. Regulation of vaccinia virus transcription. *Annu. Rev. Biochem.* **59**:661–688.
42. Neeffes, J. J., J. M. H. Verkerk, H. J. G. Broxterman, G. A. van der Marel, J. H. van Boom, and H. L. Ploegh. 1988. Recycling glycoproteins do not return to the cis-Golgi. *J. Cell Biol.* **107**:79–87.
43. Orci, L., M. Ravazzola, M. Amherdt, D. Louvard, and A. Perrelet. 1985. Clathrin-immunoreactive sites in the Golgi apparatus are concentrated at the trans pole in polypeptide hormone-secreting cells. *Proc. Natl. Acad. Sci. USA* **82**:5385–5389.
44. Palade, G. E. 1983. Membrane biogenesis: an overview. *Methods Enzymol.* **96**:XXIX–LV.
45. Patzak, A., and H. Winkler. 1986. Exocytotic exposure and recycling of membrane antigens of chromaffin granules: ultrastructural evaluation after immunolabeling. *J. Cell Biol.* **102**:510–515.
46. Payne, L. G. 1978. Polypeptide composition of extracellular enveloped vaccinia virus. *J. Virol.* **27**:28–37.
47. Payne, L. G. 1979. The biology of extracellular enveloped vaccinia virus. Ph.D thesis. Department of Virology, Karolinska Institute School of Medicine, Stockholm, Sweden.
48. Payne, L. G. 1979. Identification of the vaccinia hemagglutinin polypeptide from a cell system yielding large amounts of extracellular enveloped virus. *J. Virol.* **31**:147–155.
49. Payne, L. G. 1992. Characterization of vaccinia virus glycoproteins by monoclonal antibody precipitation. *Virology* **187**:251–260.
50. Payne, L. G., and K. Kristenson. 1979. Mechanism of vaccinia virus release and its specific inhibition by  $N_1$ -isonicotinoyl- $N_2$ -3-methyl-4-chlorobenzoylhydrazine. *J. Virol.* **32**:614–622.
51. Pifat, D. Y., M. C. Osterling, and J. F. Smith. 1988. Antigenic analysis of Punta Toro virus and identification of protective determinants with monoclonal antibodies. *Virology* **167**:442–450.
52. Rabinowitz, S., H. Horstmann, S. Gordon, and G. Griffiths. 1992. Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. *J. Cell Biol.* **116**:95–112.
53. Schmutz, C., L. G. Payne, J. Gubser, and R. Wittek. 1991. A mutation in the gene encoding the vaccinia virus 37,000- $M_r$  protein confers resistance to an inhibitor of virus envelopment and release. *J. Virol.* **65**:3435–3442.
54. Shida, H. 1986. Nucleotide sequence of the vaccinia virus hemagglutinin gene. *Virology* **150**:451–462.
55. Shida, H., and S. Matsumoto. 1983. Analysis of the hemagglutinin glycoprotein from mutants of vaccinia virus that accumulate on the nuclear envelope. *Cell* **33**:423–434.
56. Slot, J. W., and H. J. Geuze. 1985. A novel method to make gold probes for multiple labelling cytochemistry. *Eur. J. Cell Biol.* **38**:87–93.
57. Sodeik, B., R. W. Doms, M. Ericsson, G. Hiller, C. E. Machamer, W. van't Hof, G. van Meer, and G. Griffiths. 1993. Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. *J. Cell Biol.* **121**:521–541.
58. Tooze, J., M. Hollinshead, B. Reis, K. Radsak, and H. Kern. 1993. Progeny vaccinia viruses and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *Eur. J. Cell Biol.* **60**:163–178.
59. Trowbridge, J. S., R. A. Newman, D. L. Dominges, and C. Sauvage. 1984. Transferrin receptors: structure and function. *Biochem. Pharmacol.* **33**:925–932.
60. van Deurs, B., K. Sandvig, O. W. Petersen, S. Olsnes, K. Simons, and G. Griffiths. 1988. Estimation of the amount of internalized ricin that reaches the trans-Golgi network. *J. Cell Biol.* **106**:253–267.
61. Wolffe, E. J., S. N. Isaacs, and B. Moss. 1993. Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. *J. Virol.* **67**:4732–4741.
62. Wolffe, E. J., and B. Moss. Unpublished data.