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Golgi-Derived Membranes That Contain an Acylated Viral Polypeptide Are Used for Vaccinia Virus Envelopment

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A 37,000-dalton polypeptide (p37K) present on purified extracellular vaccinia virus but absent from intracellular virus particles of classical morphology (G. Hiller et al., J. Virol. 39:903–913, 1981; L. G. Payne, J. Virol. 27:28–37, 1978) was further characterized. The polypeptide was only expressed in infected cells after onset of viral DNA replication. Phase partition experiments showed that it is relatively hydrophobic. Although p37K apparently is not a glycoprotein, in vivo radioisotope labeling detected tightly associated palmitic acid. Antibodies to p37K were used to monitor its distribution within infected cells at the light and electron microscopic levels. After synthesis p37K first accumulated in the Golgi region due to a tight membrane association. During progressing infection p37K-carrying membranes were used to form double-walled envelopes around brick-shaped vaccinia particles. Within these specialized vesicles vaccinia particles were moved through the cytoplasm toward the cell's surface, presumably along cellular routes for certain secretory products. Finally, single enveloped viruses were released into the extracellular space by an exocytotic process.

Poxvirus DNA replication is confined to demarcated cytoplasmic regions typically situated near the cytocenter (2, 10). These specialized areas, named "viroplasms" or "factories," are also the sites for assembly of virus-specific macromolecules into immature poxvirus particles (see, for instance, references 5, 7, 22). Further maturation which is not well understood in molecular terms finally yields infectious brick-shaped particles. They still are situated close to the original factories.

An essential part of virus progeny can leave the infected host cell long before final cell lysis occurs (24, 26). A necessary prerequisite for release is that viruses can traverse the cytoplasm from their sites of assembly, i.e., factories, to the plasma membrane. Several electron microscopic studies (17, 21, 27) of infected cells indicated the presence of vaccinia particles which were surrounded by a double-walled membranous coat. These virus-carrying vesicles may be intracellular transport forms that possibly travel along some cellular routes for secretory products. Strong support for such a mechanism comes from experiments with N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine. This compound, originally shown to interfere with virus spread between neighboring cells (18), prevents envelopment of existing intracellular particles as well as virus release from infected cells (12, 27).

We have recently described an antiserum which recognizes the released extracellular vaccinia particles and not the non-enwrapped intracellular forms (12). In this communication the target antigen, a virus-coded polypeptide of molecular weight 37,000, is shown to contain tightly attached palmitic acid and to be relatively hydrophobic. We document the subcellular distribution of p37K during progressing infection at both the light and the electron microscopic levels. The results suggest a membrane localization for p37K and allow the tracing of the cellular origin of those membranes which are later used to enwrap the virus particle. Also, the intracellular route of this specialized structure could be visualized.

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MATERIALS AND METHODS

Cells and viruses. For analytical experiments either chicken embryo fibroblasts between passages 2 and 10 of an in vitro culture or an African green monkey kidney-derived cell line (BSC-1) was used. Cells were infected as described before (15) with approximately 1,000 vaccinia WR particles per chicken fibroblast or 200 virus particles per monkey cell. The virus preparation used revealed a ratio of particles (measured by optical density at 260 nm)/ PFU of 50:1 when assayed for infectivity on chicken embryo fibroblasts.

Large-scale propagation of the hemagglutinin-positive vaccinia virus strain IHD was performed on rabbit kidney cells (line RK13) grown as monolayers in roller bottles (12).

Purification of 37K viral protein. Extracellular vaccinia IHD virus obtained from culture supernatants was purified by cesium chloride gradient centrifugation; purified extracellular virus was suspended in balanced salt solution at 1 mg/ml, made 1% in Brij 58, and incubated for 10 min at room temperature with occasional shaking (12, 24). The incubation mixture was layered on a preformed discontinuous cesium chloride gradient (6-ml solution of 1.30 g/ml, 8 ml of 1.25 g/ml, and 10 ml of 1.20 g/ml) and centrifuged in a Beckman SW27 rotor (80,000 × *g* for 90 min at 20°C). Upon Brij treatment the virus band changed position to a higher buoyant density. The supernatant which had not entered the salt gradient was removed and centrifuged again (30 min at 4°C and 15,000 × *g*). Trichloroacetic acid (7% final concentration) was added to the supernatant. The precipitate formed was extracted five times with cold acetone, dried in vacuo, and dissolved in a modified Laemmli buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). After separation on a 14% polyacrylamide gel and 5-min staining with Coomassie brilliant blue, the major band showing an apparent molecular weight of 37,000 was excised and electrophoretically eluted directly into dialysis tubing, using the standard running buffer system of Laemmli. After extensive dialysis against 0.05% SDS,

samples were lyophilized and used for immunization or amino acid composition.

Amino acid analysis. Approximately 40 μg of purified SDS-denatured 37K viral protein was used. After removal of SDS by ion pair extraction (11), performic acid oxidation and subsequent acid hydrolysis were performed by conventional procedures. Amino acid analysis was done on a Biotronic analyzer.

Antisera. Antibody induction to both viral proteins (p31K and p37K) and the characterization of the two antisera have been described elsewhere (12). Reactivities with vaccinia strains IHD and WR were approximately of the same strength.

Two-dimensional analysis of virus structural proteins. Virion polypeptides were separated by isoelectric focusing in the first dimension followed by SDS-PAGE (14%) in the second dimension essentially as described by O'Farrell (23). For sensitive protein detection a silver strain method (38) was used.

Phase separation of viral proteins. Bordier's procedure for phase separation (1) of viral protein was adapted in the following way: purified extracellular vaccinia WR virus corresponding to 100 μg of protein was suspended and extracted in 200 μl of ice-cold 10 mM Tris-hydrochloride buffer (pH 7.2) containing 150 mM NaCl and 0.8% Triton X-114 (Sigma Chemical, Munich, F.R.G.). Particles were sedimented (25,000 × *g*, 30 min, 2°C) and the supernatants were layered on 300 μl of a solution containing 20 mM Tris-hydrochloride (pH 7.2), 150 mM NaCl, 0.06% Triton X-114, and 6% sucrose. After warming to 37°C (3 min) samples were centrifuged (300 × *g*, 3 min, room temperature) to pellet the detergent phase as an oily droplet. Further processing followed the protocol given previously (1). Proteins recovered from either the aqueous or the detergent phase by trichloroacetic acid precipitation were analyzed on 14% polyacrylamide gels in the presence of SDS.

Radioactive labeling of cells and immunoprecipitation. To label preferentially virus-specific proteins, radioactive precursors were added to infected cultures once inhibition of host protein synthesis was established (usually between 2 and 4 h postinfection [p.i.]). Unless stated otherwise radioactivity was present until cell harvest (approximately 16 h p.i.). [^{35}S]methionine (specific activity, 1,115 Ci/mmol) was added at 10 to 15 μCi/ml in medium containing one-tenth of the usual unlabeled methionine concentration. [^{3}H]palmitic acid (specific activity, 23.5 Ci/mmol) was used at 80 μCi/ml. All labeling media contained 5% dialyzed fetal calf serum. For experiments with radioactive sugars the glucose-containing medium was replaced by a glucose-free pyruvate-enriched (3 mM) variant. [^{3}H]glucosamine (specific activity, 39.6 Ci/mmol) as well as [^{3}H]mannose (specific activity, 16 Ci/mmol) were used at 20 μCi/ml.

A total of 4×10^6 to 6×10^6 cells were homogenized in 0.5 ml of lysis buffer (20 mM Tris-hydrochloride, pH 7.2, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 200 μg of bovine serum albumin per ml, 0.1 mM phenylmethylsulfonyl fluoride) by 50 strokes with a potter-type homogenizer. After nuclei and large debris had been removed by low-speed centrifugation, the supernatants were briefly sonified on ice and then subjected to centrifugation at 10,000 × *g* (10 min, 4°C). With these clarified extracts, immunoprecipitation and subsequent SDS-PAGE were done essentially as described before (14).

Fluorescence microscopy. The indirect immunofluorescence microscopy procedure for cells grown on glass cover slips has been described (15, 36). Viral antisera were extensively preabsorbed on methanol-fixed uninfected cells and finally used at a 100-fold dilution, whereas fluorescently labeled immunoglobulins G (IgG) (Miles-Yeda, Rehovot, Israel) were used at 0.5 to 1 mg/ml.

Incubations with the DNA-binding fluorochrome Hoechst 33342 (Hoechst AG, Frankfurt, F.R.G.) were performed on prefixed cells, using 15 μM dye for 30 min in −20°C methanol. Rhodamine-labeled wheat germ agglutinin (Rh-WGA) served for the identification of the Golgi complex in fluorescence microscopy (13, 35). It was obtained from E.Y. Laboratories (San Mateo, Calif.) and used at 10 to 25 μg/ml. In triple labeling experiments where the distribution of 37K viral protein in relation to the cellular Golgi system and poxvirus factories was examined within the same cell, the fixed specimens were incubated in the following order: Hoechst 33342 in methanol, rabbit anti-vaccinia serum and simultaneously Rh-WGA in phosphate-buffered saline (PBS), and finally fluorescein-labeled goat anti-rabbit IgGs, again in the presence of Rh-WGA and in PBS. After each incubation step the samples were carefully washed with PBS. Cells were viewed and photographed with a Zeiss photomicroscope III equipped with epifluorescence optics and oil immersion objectives. The emission spectra of Hoechst 33342, fluorescein, and rhodamine were optically separated by using the standard filter combinations provided by the manufacturer.

Electron microscopy. For conventional thin sectioning, vaccinia virus WR-infected BSC-1 cells grown on cover slips were fixed and embedded in Epon as described before (12). Sample preparation for immunoperoxidase labeling experiments at the electron microscope level was performed essentially as described by Louvard et al. (20) with the following minor modifications. Vaccinia virus WR-infected BSC-1 cells (8.5 h p.i.) were only fixed for 25 min. Preabsorbed p37K antibodies were used at 0.1 mg/ml and were left overnight on saponin-permeabilized cells at room temperature. After extensive washing, a 3-h incubation with $F(ab')_2$ fragments of goat anti-rabbit IgG conjugated to peroxidase (Dianova product, diluted 1:40) followed. The diaminobenzidine reaction was allowed to proceed only for 20 min. In contrast to the original protocol (20), cells were not scraped off but remained on the cover slips during all subsequent steps. They were finally flat embedded in Epon.

For preparation of ultrathin frozen sections, we used a modification of Tokuyasu's procedure (34), which was developed at the European Molecular Biology Laboratory, Heidelberg, F.R.G. (9). Briefly, infected monolayers were detached from the supporting substratum by the combined use of 0.2 mg of protease K per ml in PBS (5 min on ice) and very gentle mechanical shearing. After successful detachment phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM and the cell sheets were centrifuged (4,500 × *g*, 1 min, 4°C). The supernatant was replaced by 0.1 M piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), pH 7.0, containing 5% sucrose and 0.5% glutaraldehyde. Fixation was at room temperature for 30 min. The fixed material was washed three times with ice-cold 0.1 M PIPES, pH 7.0, supplemented with 10% sucrose, transferred into PIPES containing 2.3 M sucrose, mounted in a drop of this solution on a copper chuck, and quickly frozen in liquid nitrogen. Sections were cut at −100°C, using glass knives in a Dupont-Sorvall ultramicrotome with an LTC-2 cryochamber attached. Ultrathin sections were placed on Formvar-carbon-coated 100-mesh grids previously ionized by a glow-discharge apparatus. Inverted grids were left for at least 10 min on agarose (0.3%)-gelatin (1%) plates covered with a film

of PBS, transferred on 2% gelatin in PBS (10 min), rinsed three times in PBS containing 0.02 M glycine, and finally incubated with the corresponding antibodies (40 min at room temperature). After five washes with PBS-glycine (see above), grids were incubated with colloidal gold-labeled protein A (average diameter of gold particles, approximately 5 nm; Janssen, Beerse, Belgium) for 20 min at room temperature. Unbound protein A was removed by intensive washing with PBS, followed by a short rinse with distilled water (2 to 3 min). Sections were stained with a neutral 2% uranylacetate solution containing, in addition, 0.15 M oxalic acid (5 min), briefly washed in water, and again stained with 2% aqueous uranylacetate (4 min). Finally, the grids were directly placed on the surface of large droplets of 1.5% methylcellulose solution (Tylose M 4300; Fluka AG, Buchs, Switzerland), left for 20 s, taken up with a platinum loop, and allowed to air dry after excess solution had been carefully removed with filter paper.

RESULTS

Biochemical characterization of p37K. In tissue cultures infected with vaccinia virus three variant forms of morphologically differentiated progeny particles are found: first, brick-shaped viruses apparently lying free in the cytoplasm; second, similar particles enwrapped by a double-walled vesicular structure preferentially located in peripheral cytoplasmic areas; and third, particles associated with the external face of the plasma membrane or completely free in the growth medium. These are surrounded by a single membranous coat (see, for instance, micrographs in references 17 and 27).

Double-walled particles have not yet been isolated, but the other two forms can be purified and have been compared in detail (12, 24). We have extended the polypeptide analysis of the two available vaccinia variants by a comparison of the corresponding protein patterns seen by two-dimensional separation (isoelectric focusing and subsequent SDS-PAGE). In agreement with previous reports using only one-dimensional separation according to molecular weights (12, 24), extracellular virus differs by at least one additional major polypeptide with a relative molecular weight of 37,000 (p37K) from its classical intracellular form (Fig. 1a and b). The isoelectric point of p37K is near pH 7, whereas a well-known glycoprotein (6, 16, 28) of apparent molecular weight 34,000, which is barely separated from p37K on the basis of SDS-PAGE alone, is rather acidic (pI ~5.3). Thus the two proteins can be clearly distinguished in the two-dimensional system.

For further characterization we have determined the amino acid composition of p37K (Table 1). The very low methionine content may explain why Payne (24) could not recognize the abundance of p37K after [^{35}S]methionine labeling of infected cells.

With the help of specific antibodies (12) we were able to examine by immunoprecipitation experiments possible modifications of p37K. P37K can be solubilized from particulate material by treatment with mild detergents (12, 24; see procedure for p37K purification in Materials and Methods). Therefore, Triton X-100-deoxycholate lysates from infected, adequately labeled cells were used for the experiments. The single band obtained upon analysis of a [^{35}S]methionine-labeled immunoprecipitate by SDS-PAGE and fluorography (Fig. 2, lane a) not only proves the specificity of the antibodies, but also shows that under the extraction conditions p37K is not complexed to other virally coded proteins.

Labeling of infected HeLa suspension cultures with

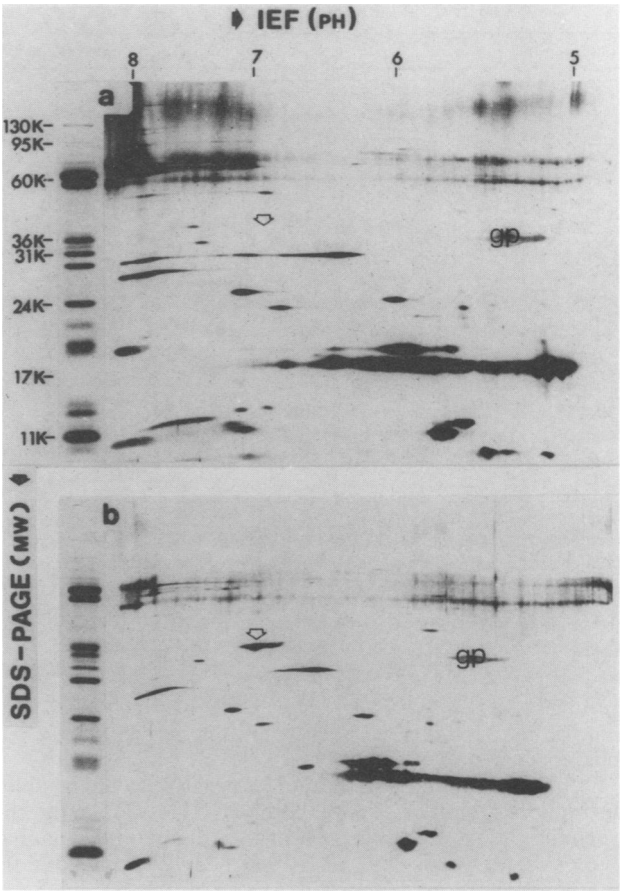

FIG. 1. Polypeptide patterns of intracellular and extracellular vaccinia IHD virus after separation by two-dimensional electrophoresis: first dimension, isoelectric focusing (IEF); second dimension, SDS-PAGE (14%). (a) Intracellular virus; (b) extracellular virus. Proteins in one-dimensional reference lanes are Coomassie blue stained, whereas those on two-dimensional gels are silver stained. A polypeptide of molecular weight 37,000 with a nearly neutral isoelectric point (position indicated by arrow) is characteristic of extracellular virus. On both two-dimensional gels a similar sized glucosamine-containing protein designated gp has a pI of about 5.3. Note the good separation of p37K and gp in two-dimensional gels of extracellular virus.

[^{3}H]palmitic acid revealed fatty acid incorporation into p37K (lane d, Fig. 2). By analyzing whole-cell lysates, we observed a change in the pattern of fatty acid-carrying polypeptides upon virus infection. In infected cells fatty acid incorporation is dramatically reduced (cf. lanes e and f) due to the inhibition of host protein synthesis and the fact that modification can only occur during biosynthesis.

The apparent molecular weight of the major labeled polypeptide characteristic of lysates derived from infected cells is indistinguishable from that of immunoprecipitated authentic p37K. All previously characterized acylated proteins of animal virus origin (for review, see reference 31) represent integral components of the virion envelope. Another common property seems to be their glycosylation. However, for vaccinia p37K neither mannose nor glucosamine incorporation could be detected (Fig. 2, lanes b and c), although in the same samples another viral polypeptide of apparent molecular weight 42,000 clearly had both sugars incorporated (unpublished data). This agrees with Payne's observation

TABLE 1. Amino acid composition of p37K[a]

Amino acid	mol/mol of protein
Asp	33.5
Thr	17.2
Ser	30.6
Glu	20.9
Pro	11.7
Gly	39.5
Ala	27.3
Cys	6.4
Val	16.8
Met	1.9
Ile	13.2
Leu	23.0
Tyr	6.3
Phe	8.8
Lys	14.7
His	5.2
Arg	7.8
Trp	ND

[a] The value for glycine may be too high due to possible contamination arising from the running buffer used in preparative SDS-PAGE and subsequent electrophoretic elution. The molecular weight calculated on the basis of this amino acid composition is approximately 34,400. As in SDS-PAGE the viral protein moves slightly slower than glyceraldehyde dehydrogenase from rabbit skeletal muscle (molecular weight, 36,500); p37K seems an appropriate designation. Note the low value of methionine. ND, Not determined.

using radioactive fucose for glycoprotein detection on extracellular vaccinia viruses (24).

For further characterization p37K was subjected to phase partition experiments, using Triton X-114 (1). With this procedure a fast determination of the relative hydrophobicity of proteins is possible. P37K was heavily enriched in the detergent phase as might be expected for a membrane protein.

Temporary p37K association with Golgi structures. To learn more about the intracellular p37K distribution, we used p37K-specific antibodies in indirect immunofluorescence microscopy on vaccinia virus (strain WR)-infected BSC-1 cells. P37K must be a virus-specified protein because no cross-reactive material is found in uninfected cells. Even 4 to 6 h after infection when cells already show pronounced viral DNA replication in the cytoplasmic poxvirus factories, p37K could not be detected (Fig. 3b). Therefore, by definition it belongs to the class of late viral proteins. Between 6 and 8 h p.i. p37K appears concentrated near the cytocenter in discrete cytoplasmic areas (Fig. 3e) which by size and location could represent poxvirus factories at later stages. Examination of viral DNA distribution within the same cell (Fig. 3f) proves, however, that p37K is not factory associated (cf. Fig. 3e and f). Instead, the same area labeled by p37K antibodies is also strongly decorated by rhodamine-coupled WGA (Fig. 3d). This lectin has a sugar-binding specificity for *N*-acetylglucosamine (8), and we have shown previously that it can be used for Golgi detection in uninfected BSC-1 cells (13). Thus, shortly after synthesis p37K appears to be associated with the Golgi system.

In Fig. 3d clear fluorescence of the cell surface is not evident, although some plasma membrane labeling by WGA could be expected due to the reported lectin-binding activity of the viral hemagglutinin (32). Since hemagglutinin is a viral late-late function the corresponding cells might not yet have reached the stage of hemagglutinin expression on the cell surface.

Virus-modified Golgi membranes and virus envelopment. During the course of progressing infection p37K finally appears distributed through the whole cytoplasm in the form of distinct particulate structures (Fig. 4a). Simultaneously, the staining intensity of the original Golgi area often gradually decreases. In Fig. 4b the intracellular distribution of another viral antigen representing a structural protein (molecular weight, 31,000) of classical vaccinia particles is documented for the same cell (see also reference 12). This 31K protein is believed to participate in virus assembly soon after its synthesis since no soluble pool is detectable. The smallest fluorescently labeled units in Fig. 4b do represent vaccinia particles (see also reference 15). Judged by the partial overlap of labeling in Fig. 4a and b, especially recognizable in small punctuate structures distributed within the cytoplasm or accumulated in a pseudopod region, p37K becomes virus associated after it has departed from the Golgi. To confirm this interpretation, immunolabeling experiments at the electron microscopic level were performed. At 9 h p.i. monolayer cultures were fixed in situ and processed according to a preembedding immunocytochemical method in which the intracellular antigen localization is revealed by the electron-dense reaction product of antibody-conjugated peroxidase (for details and further references see Materials and Methods). Thin sections of such immunolabeled, peroxidase-reacted and Epon-embedded samples are shown in Fig. 5. The infected cell in the first micrograph

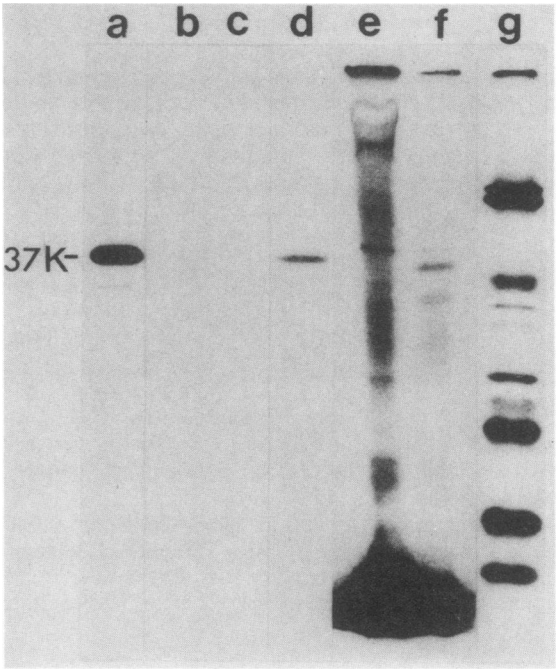

FIG. 2. Post-translational modification of p37K. SDS-polyacrylamide (14%) gel: (a) anti-p37K immunoprecipitate from extracts of infected, [^{35}S]methionine labeled cells; (b and c) similar immunoprecipitates after labeling with [^{3}H]mannose or [^{3}H]glucosamine; (d) p37K immunoprecipitated from extracts of infected, [^{3}H]palmitate-labeled cells; (e and f) pattern of [^{3}H]palmitate incorporation into total SDS lysates of either uninfected (e) or vaccinia IHD-infected (f) HeLa cells. Upon coelectrophoresis the major band in (f) migrates exactly with authentic p37K. For comparison and molecular weight calibration the protein pattern of [^{35}S]methionine-labeled purified classical IHD virus is shown in lane g. ^{3}H lanes have been exposed for 15 days; ^{35}S exposure was for only 2 days.

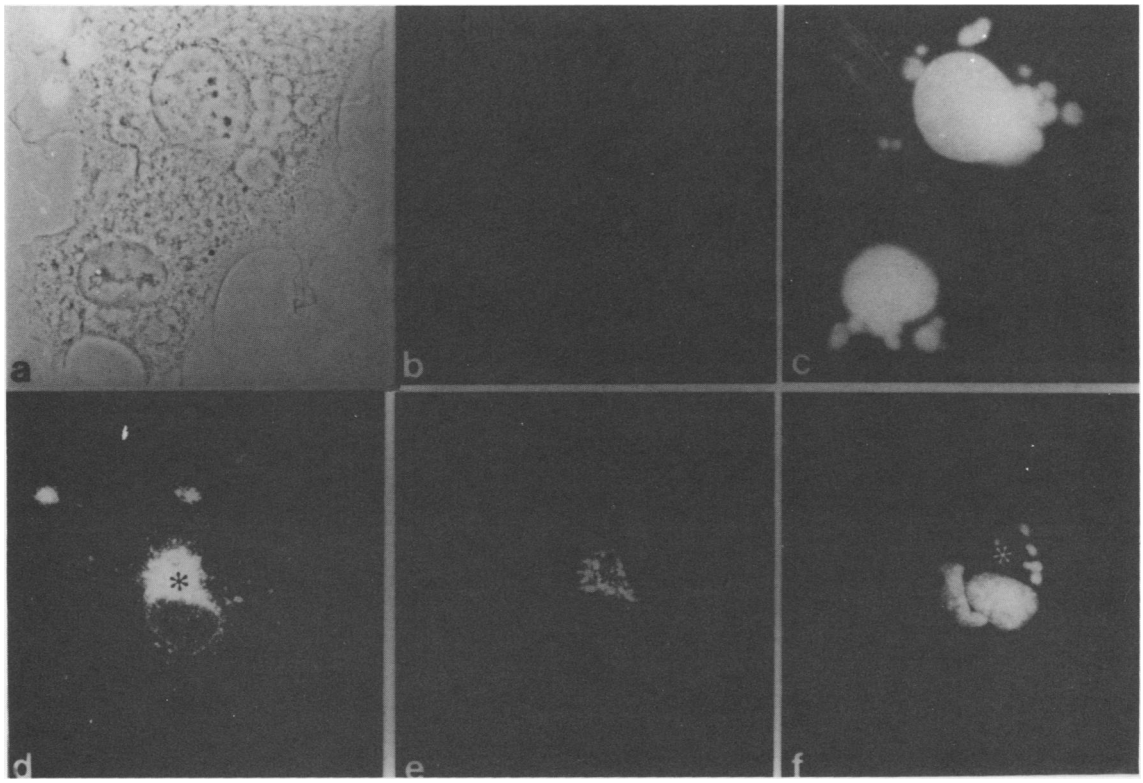

FIG. 3. Time of p37K appearance and subcellular location shortly after synthesis. (a), (b), and (c) show the same fields of view, as do (d), (e), and (f). (b) and (e) document the intracellular p37K distribution in indirect immunofluorescence microscopy (fluorescein isothiocyanate label). (c) and (f) detect DNA stained by Hoechst 33342. In (d) the cell is decorated with Rh-WGA. Although both cells shown in the phase-contrast micrograph (a) contain several small viral factories in their cytoplasm (c), detectable p37K synthesis has not yet started (b) at 5 h p.i. Two hours later (7 h p.i.) cells contain newly synthesized p37K restricted to a juxtanuclear position (e) which by comparison with the WGA label (d) is identified as a Golgi area (position on all three micrographs indicated by asterisk). Poxvirus factories (f) are lined up around the Golgi organelle. They do not contain p37K. Magnification: (a–c) ×900; (d–f) ×750.

(Fig. 5a) was strongly extracted during the saponin permeabilization. This and the heavy precipitate of the peroxidase reaction product at the sites of p37K accumulation obscure an underlying finer structure. Nevertheless, at favorite locations a system of membranous structures can be detected (arrows). Form and intracellular distribution sug-

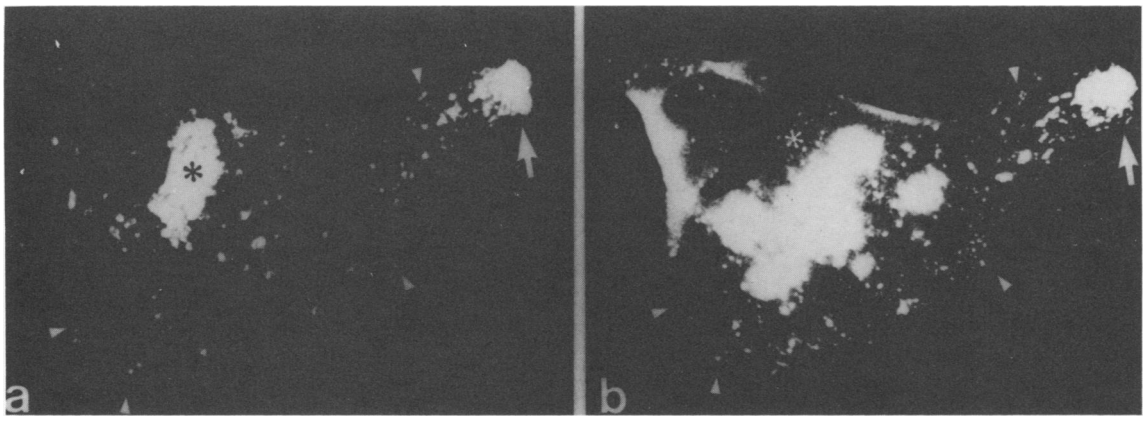

FIG. 4. Later stage of p37K distribution correlated to intracellular virus dissemination. At 12 h after infection p37K is no longer restricted to the Golgi complex (marked by asterisk) but is distributed in a particulate manner throughout the whole cytoplasm (a). A pseudopod region is particularly strongly labeled, blurring structural details (large arrow). In (b) the distribution of assembled vaccinia particles within the same cell is revealed by antibodies directed against a 31K virus structural protein which apparently does not exist in a detectable soluble pool. The heavily labeled structures (b) in the center of the cell correspond to heaps of assembled particles having not yet left factory areas. Only structures accumulating in the pseudopod as well as some small units distributed throughout the cytoplasm (representatives indicated by small arrowheads) are simultaneously detected by both antibodies. Magnification, ×930.

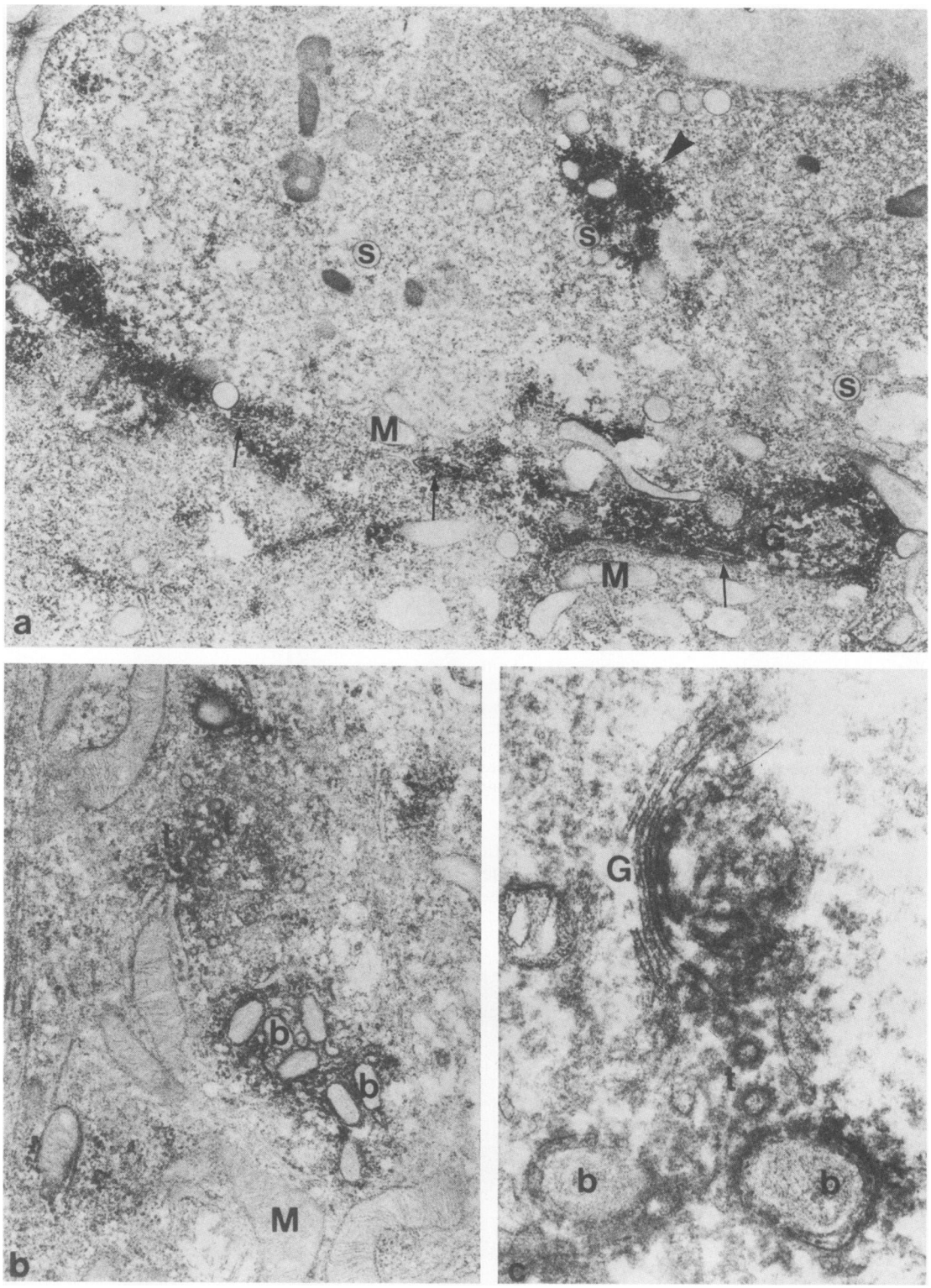

FIG. 5. Ultrastructural p37K localization by a preembedding immunoperoxidase technique. BSC-1 cells (9 h after start of infection) were fixed and subsequently permeabilized by saponin. The electron-dense peroxidase reaction product detects the intracellular p37K distribution. (a) The main structure immunolabeled consists of an elaborated extended membranous system. The only intracellular structure of comparable appearance and spatial arrangement is the Golgi apparatus. Separated from the extended organelle a second small area is strongly immunostained. Here a brick-shaped vaccinia particle is seen. Magnification, ×16,000. (b) In this section from a different cell which appears to have been less extracted and stronger contrasted by osmium tetroxide, several brick-shaped viruses with faintly visible characteristic morphology have accumulated. They again are surrounded by p37K-containing membranous structures of various sizes. Transitional elements characterized by a round appearance also seem to carry p37K on their coats. Magnification, ×32,000. (c) This micrograph shows

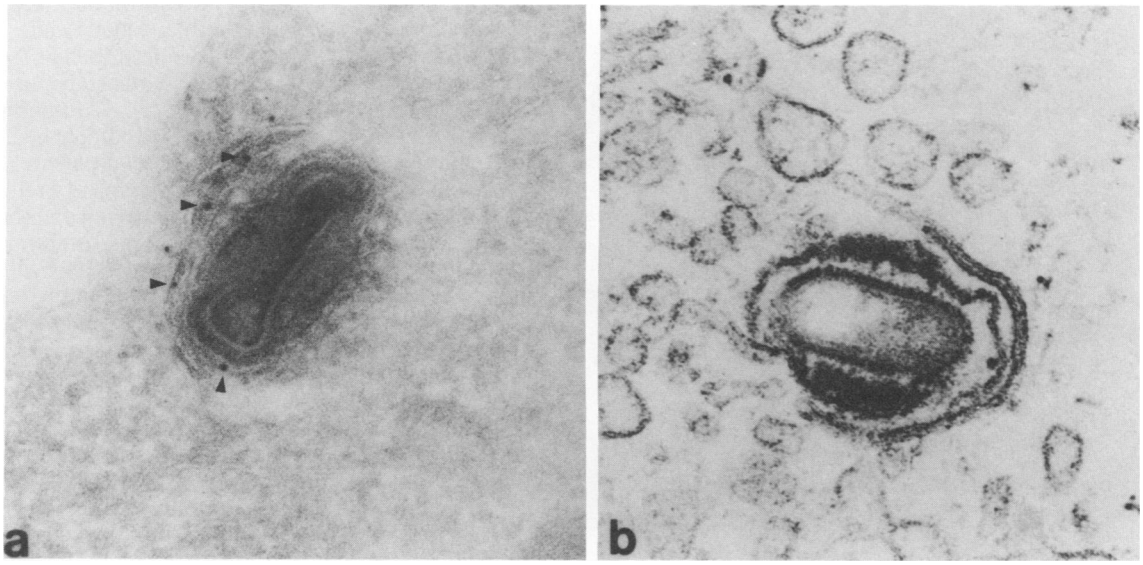

FIG. 6. Immunochemical localization of virus-associated p37K at the ultrastructural level. Micrographs show intermediate stages in virus envelopment present in BSC-1 cells 12 h after infection. On an ultrathin frozen section (a) electron-dense gold particles (arrowheads) locate p37K on virus-enwrapping membranes. Particles themselves appear unlabeled. To facilitate identification of structures, similar but not immunolabeled arrangements seen by conventional processing for standard electron microscopic examination (b) are given. Antibody staining in (a) was with purified rabbit anti-p37K IgGs followed by gold-labeled protein A.

gest that the heavy label is on the cellular Golgi apparatus. Completely separated from the immunolabeled long and extended Golgi system is a second area of patchy label (arrowhead) where a brick-shaped virus particle can be seen. More peripheral to this small labeled area are some immature virus particles identified by their still roundish appearance. More of these immature virus forms are scattered through the cytoplasm but none seem to be associated with p37K-positive structural elements.

The section in Fig. 5b shows seven brick-shaped virus particles accumulated in a p37K-labeled area. Due to a longer exposure to osmium tetroxide in this specimen the fine structure of mitochondria and viruses is somewhat better recognized, allowing an unambiguous identification of virus particles. In addition, the interior of this cell appears less extracted than in Fig. 5a and the membranous nature of the immunolabeled structures is clearly revealed. Interestingly, some vesicles of the size of transitional elements also carry p37K label in their coats. Figure 5c summarizes the proposed sequence of events: virus-specified p37K is recognized as an integral component of the membranes that form the Golgi cisternae, it is found in transitional elements of a vesicular nature directly adjacent to the Golgi stack, and, finally, it is identified in both membranes of the double-walled structure which have been constructed around two separate brick-shaped vaccinia particles situated near the Golgi system.

On ultrathin frozen sections from infected cells p37K association with viruses can also be detected by gold-labeled protein A. Again only the double-walled structure surrounding pox particles of classical morphology carries gold particles (Fig. 6a). In the selected example the double-walled envelope seems to be just under construction. For comparison, a similar stage of uncompleted envelopment is also shown in a conventionally processed non-immunolabeled Epon section (Fig. 6b).

DISCUSSION

From the supernatant growth medium of infected tissue culture cells a vaccinia virus variant can be purified. It differs in several aspects from the "classical" vaccinia virus isolated from infected cells after experimentally induced lysis (12, 24, 25). When the polypeptide compositions of the two virus populations are examined by two-dimensional gel electrophoresis, it becomes evident that extracellular vaccinia virus is characterized by one additional major polypeptide which has an apparent molecular weight of 37,000. Earlier reports on this protein (12, 24) were solely based on one-dimensional SDS-PAGE which did not allow a clear and reproducible separation of p37K from a well-known vaccinia structural protein containing glucosamine (6, 16, 30). Figure 1a and b, however, clearly documents the quite distinct nature of the two proteins in two-dimensional gels. Other but minor polypeptide constituents thought to be specific for extracellular virus (23; G. Hiller, unpublished data) are not evident in our two-dimensional analysis. This is probably due to a decreased sensitivity (silver stain versus fluorography) or the lack of isoelectric focusing in the first dimension.

Metabolic labeling of infected cells by various precursors and subsequent immunoprecipitation of p37K detected a fatty acid incorporation. All hitherto identified acylated

a well preserved stack of Golgi cisternae where the membranes are slightly but noticeably stained. This also holds for the membranes of transitional elements which are situated between the Golgi and of two virus particles. Around the latter a double-walled vesicular structure is locally resolved. At favorable sites both vesicle walls appear slightly stained and thus contain p37K. Magnification, ×76,000. G, Golgi; s, spherical immature viruses; b, brick-shaped viruses; t, transitional elements; M, mitochondria.

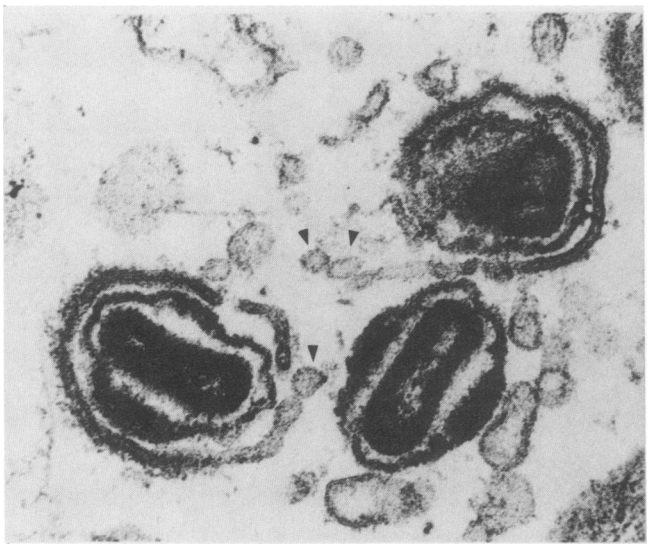

FIG. 7. Various stages in vaccinia virus envelopment. An Epon section of an infected, non-immunolabeled BSC-1 cell (12 h p.i.) is shown. The vaccinia particle in the middle seems contacted by at least three different-sized vesicles which might initiate the process of envelopment. On the two other viruses envelope formation is advanced. Single entities of small vesicles (indicated by arrowheads) appear associated with the "ends" of nascent envelope structures. Magnification, ×90,000.

proteins of animal virus origin (for review, see reference 31) are integral membrane components that also carry carbohydrate side chains. Although in phase partition experiments (see Materials and Methods) p37K proved to be relatively hydrophobic, all attempts to characterize the protein as a glycoprotein have failed (Fig. 2; unpublished data). Thus it might belong to a novel category of unglycosylated but acylated viral membrane proteins. P37K is clearly absent from uninfected cells and is coded by the virus genome (R. Wittek and G. Hiller, unpublished data). In indirect immunofluorescence microscopy p37K first becomes detectable in the Golgi area where it appears to accumulate. The rough endoplasmic reticulum, presumably the site of p37K synthesis, does not contain enough antigen to become clearly stained. Interestingly, the poxvirus factories also remain unstained (cf. Fig. 3e and f).

The restriction of p37K to the Golgi area seems to be caused by an incorporation or tight association of the protein with both Golgi cisternal membranes and transitional elements presumably derived from the ends of Golgi stacks (Fig. 5a to c). These p37K-positive vesicles of varying size probably become immobilized after contact with the surface of morphologically mature brick-shaped vaccinia particles. A putative recognition reaction could be based on a virus-specified vesicle component such as p37K itself or on other, not yet identified minor polypeptides of viral origin. Several such vesicles appear to fuse to allow the formation of a single large double-walled structure around the corresponding virus (Fig. 7). These distinct morphological units have already been described in several reports (17, 21, 27). Some of them (17, 21) have even tentatively suggested a Golgi origin solely by morphological criteria (rarely observable direct budding into Golgi cisternae). P37K is now clearly identified on the membranous envelope around vaccinia particles. It is found there during construction and remains after the structure has been completed. Immunoelectron micrographs such as Fig. 5c indicate that p37K may be present on both the inner and the outer membranes of the double-walled shell.

The large virus-carrying vesicles described above seem to serve as a vehicle for intracellular virus transport probably along the routes of secretory products. We think that virus release from the still unlysed cell is accomplished by fusion of the outer vesicle layer with the cellular plasma membrane (see, for instance, some of the micrographs in references 17 and 27). In consequence, a virus particle within a now single-walled vesicle is released. These structural units are harvested as "extracellular" virus from the growth medium. Upon fusion, that part of p37K contained in the outer of the two vesicle walls should become a constitutive component of the plasma membrane. As we have never seen general plasma membrane staining by p37K antibodies we tentatively assume that the amount of p37K, when evenly spread over the whole surface, is too small to be detected by immunofluorescence microscopy.

Our data on the construction of the double-walled envelope suggest that the inner and outer coats might be identical. Thus the inner membrane remaining around the released extracellular vaccinia particles should represent an ideal structure for fusing such viruses into uninfected cells. All fusion events mentioned could be brought about through the presence of acylated p37K, as one common feature of fatty acid-containing viral glycoproteins is their ability to induce membrane fusion (31).

The results obtained here combined with earlier ultrastructural studies (17, 21, 27) suggest that pox particles with classical morphology do not represent fully matured virions, although they are undoubtedly infectious. Similar to other enveloped viruses, which receive their surrounding lipid-containing shells from various preexisting cellular membranes modified by the incorporation of virus-specific proteins (for review see references 3, 33), poxviruses apparently obtain their final envelopes also from modified cellular membranes which in this case are of Golgi origin. That only a fraction of assembled virus particles become enveloped and that very late under one-step growth conditions most intracellular particles accumulate in the free form (see reference 27) could be due to an extreme loss of Golgi-derived membranes. The virus release process discussed most likely exhausts the capacity of the host cell to provide enough newly synthesized membranous material. Indeed, Golgi morphology and distribution studied at the light microscopic level with Rh-WGA changes during the course of infection. Late in infection the cellular organelle appears fragmented and widely scattered throughout the cytoplasm (not shown), whereas for uninfected or briefly infected cells the juxtanuclear position of a unit structure is very characteristic (Fig. 3d).

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