Isolation and Characterization of Monopinocytotic Vesicles Containing Polyomavirus from the Cytoplasm of Infected Mouse Kidney Cells†

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Monopinocytotic vesicles containing polyomavirus were isolated from the cytoplasm of mouse kidney cells infected with polyomavirus using sucrose density gradients. Nonenclosed, membrane-associated virions released by the action of neuraminidase separated from vesicle-enclosed virions in the sucrose gradient. Marker enzyme assays indicated the derivation of the vesicle membrane from the plasma membrane of the cell. The ^{125}I-labeled virus enclosed in the vesicle sedimented more slowly in the gradient and was not observed unless infection and endocytosis had occurred. Detergent treatment of virion-containing vesicles caused the release of polyomavirus with sedimentation properties similar to those of purified polyoma virions. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of virion proteins from vesicles containing virions demonstrated patterns of proteins similar to those of purified intact virions. Electron microscopy confirmed the presence of single intact virions inside vesicles. The study of these monopinocytotic virion-containing vesicles represents a further step in elucidating the early events of polyomavirus infection.

The early events of the infection of mouse kidney cells by polyomavirus have been characterized by electron microscopy (23), binding (6), and nuclear uncoating studies (23, 46). In both permissive and nonpermissive cells, the virus enters by endocytosis after attachment and penetrates into the cytoplasm contained in a monopinocytotic vesicle. These vesicles then migrate to the nucleus, where the virus is uncoated and the replication or transformation processes can begin. This mode of entry, known as receptor-mediated endocytosis, occurs for other viruses (10, 15, 19, 26, 27, 38), as well as hormones (33), toxins (16, 34, 40). However, the lysosomes are the principal destination for many of these viruses and molecules (20), whereas polyoma (23) and other papovaviruses (3, 10, 25, 28) are observed to be transported to the nucleus as well, resulting in infection.

Since the interaction of polyomavirus with the cell surface (6) and the formation of uncoating intermediates in the nucleus (46) have been studied, more information about the intermediate steps of vesicle formation, migration, and nuclear entry is needed. A thorough understanding of the early events associated with polyomavirus-host interaction requires the characterization, on a physical and chemical level, of the cytoplasmic vesicles that harbor the polyoma virions. Therefore, in this study, we have undertaken to isolate these virus-containing monopinocytotic vesicles from the cytoplasm of infected cells and to characterize their stability and enzymatic content and the nature of the enclosed virions.

MATERIALS AND METHODS

Cell and virus propagation. Primary cultures of mouse kidney cells were prepared as described previously (9, 39).

Wild-type small plaque polyomavirus was used to infect cells. Infected cultures were maintained in serum-free Dulbecco modified Eagle medium (29).

Radioactive labeling of virus. Polyomavirus was purified from infected cell lysates as previously described (7, 29). Purified virus was labeled in vitro with ^{125}I using chloramine T by the method of Frost and Bourgaux (14).

Isolation of monopinocytotic vesicles. Confluent monolayers of mouse kidney cells were washed and infected with 10^7 cpm or more of ^{125}I-labeled polyomavirus and 50 to 100 hemagglutination units of unlabeled virus per 100-mm plate. Adsorption of virus was allowed to occur for 1 h at 4°C, and penetration was allowed to occur for an appropriate time at 37°C. One hour was selected for the 37°C incubation time as the optimum time to isolate the maximum number of virion-containing vesicles from the cytoplasm. Cells were washed and treated with receptor-destroying enzyme (40 U per plate) to remove any virions remaining on the cell surface. The cells were washed again and scraped into phosphate-buffered saline and pelleted by centrifugation. Cells were then disrupted by homogenization in 0.25 M sucrose with 0.01 M Tris-chloride (pH 7.4), 1 mM MgCl$_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 0.2 U of neuraminidase per ml, and 0.1 mM phenylmethylsulfonyl fluoride, using a Plexiglas mortar and a Teflon pestle (20 strokes). The homogenate was centrifuged for 6 min at 2,000 rpm in a Sorvall HB-4 rotor, and the resulting cytoplasmic supernatant containing the vesicles was subjected to further fractionation on density gradients.

The monopinocytotic vesicles were then separated from free polyomavirus (released by the action of neuraminidase) by either 20 to 60 or 15 to 45% linear sucrose gradients. Both gradients contained 0.01 M Tris-chloride (pH 7.4), 0.15 M NaCl, and 0.01% Triton X-100. The 20 to 60% gradient was centrifuged for 16 h at 25,000 rpm in a Beckman SW41 rotor at 4°C. The 15 to 45% gradient was centrifuged for 16 h at 35,000 rpm in an SW41 rotor at 4°C and used to obtain further resolution of the vesicle peak in the gradient. The 15

to 45% gradient resulted in the pelleting of the free polyoma-virus released by neuraminidase; thus, the ^{125}I-labeled poly-omavirus peaks found in the gradient profile were only those of the virus associated with cellular material. An identical gradient corresponding to the 20 to 60% sucrose gradient described above was performed with purified ^{125}I-labeled virus to identify the sedimentation position of the nonen-closed virus. Fractions of gradients were collected by down-ward displacement with mineral oil.

Assay of marker enzymes. Alkaline phosphatase (EC 3.1.3.1), a plasma membrane marker (5, 42), was measured at pH 8.0 by the hydrolysis of p-nitrophenylphosphate (Schwarz/Mann) by the method of Malamy and Horecker (24). β-Glucuronidase (EC 3.2.1.31), a lysosomal marker (5), was determined in the presence of 0.1% Triton X-100 at pH 4.5 in 0.1 M sodium acetate buffer using p-nitrophenyl-β-glucuronide (Sigma Chemical Co.) as substrate (4). The activity of 5′-nucleotidase (EC 3.1.3.5), a plasma membrane marker (42), was measured at pH 7.6 in 0.03 M Tris-chloride buffer in the presence of 6 mM $MgCl_2$ with 1.9 mM adeno-sine-5′-monophosphate (Pabst Laboratories) as substrate. The phosphate product was measured by the method of Ames (1).

Routinely, a 0.1-ml sample from a gradient fraction was mixed with an equivalent volume of substrate-buffer mixture for the alkaline phosphatase and β-glucuronidase assays and with a 0.05-ml volume of a fourfold-concentrated solution reaction mixture (described above) for the 5′-nucleotidase assay. All reaction mixtures were incubated for 1 h at 37°C.

Electrophoresis. Proteins from isolated fractions were ana-lyzed using a 5 to 15% sodium dodecyl sulfate (SDS)-polyacrylamide gradient slab gel (1.5 by 14 by 31 cm in a model SE-5001 apparatus; Hoefer Scientific Instruments) by the method of Hames (17) and the discontinuous buffer system of Laemmli (22). Molecular weights of proteins were determined by using the following molecular weight stan-dards: lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase b (92,500), all pur-chased from Bio-Rad, and β-galactosidase (116,000) and myosin (205,000), purchased from Sigma Chemical Co. The running buffer was 0.1% SDS–0.38 M glycine in 0.05 M Tris buffer (pH 8.4). Gels were run for 8 h at 25 mA constant amperage, stained in 50% methanol–7.5% acetic acid with 0.1% Coomassie brilliant blue, and destained in 25% metha-nol–7.5% acetic acid. After drying, the gels were used for autoradiography with Kodak XAR-5 X-ray film with a screen. The film was developed according to the manufac-turer's directions.

Electron microscopy of vesicles. Samples for electron mi-croscopy were prepared using an adaptation of the method of Helenius (18, 19) by pooling fractions from a sucrose gradient (15 to 45%) containing the ^{125}I-labeled virus band and diluting with approximately 2 volumes of a fixation buffer containing 3% glutaraldehyde (EM grade; Poly-sciences), 0.05 M sodium cacodylate (pH 7.3), 5 mM KCl, and 2.5 mM $CaCl_2$. The mixture was immediately pelleted by centrifugation for 60 min at 35,000 rpm in an SW50.1 rotor at 4°C. The pellet was rinsed three times with 0.2 M sucrose solution in 0.05 M sodium cacodylate (pH 7.3) and postfixed for 40 min with 1% osmium tetroxide in the same buffer. After three distilled water rinses, the pellet was stained overnight in fresh aqueous 1% uranyl acetate. Then dehydra-tion with a 30 to 100% series of ethanol solutions was carried out, and the bottom of the centrifuge tube containing the pellet was cut off and dissolved in acetone. After several

acetone washes to remove the residual cellulose nitrate, the pellet was infiltrated with 2:1, 1:1, and 1:2 solutions of acetone-plastic (Mollenhauer's Epon-Araldite) (31). After embedding and hardening, thin sections were cut with a diamond knife using a Reichert OMU-2 ultramicrotome. The thin sections were stained with 5% uranyl acetate in 50% ethanol and with Reynold's lead citrate and viewed with a Philips 201 electron microscope.

RESULTS

Isolation of monopinocytotic vesicles containing polyoma-virus. Mouse kidney cells were infected with ^{125}I-labeled polyomavirus, cells were disrupted, and monopinocytotic vesicles containing ^{125}I-labeled virus were isolated as de-scribed above. The ^{125}I-labeled infecting virions were used as the radiolabeled tag to identify vesicles containing virions.

Figure 1A shows the profile of the sucrose gradient (20 to 60%) with the faster-sedimenting free virus band on the left sedimenting at the same rate as the marker virus band (arrow) from an identical gradient loaded with purified ^{125}I-labeled virus. The slower-sedimenting band on the right is the virus associated with cellular material, remaining near the top of the gradient. Application of the cytoplasmic fraction to the 15 to 45% sucrose gradient described above resulted in the resolution of the slower-sedimenting material into two peaks (I and II) of virus associated with cellular material (Fig. 1B). The free polyomavirus previously ob-served in the 20 to 60% gradient was pelleted in this gradient.

To ensure that the virus was not merely attaching to fragments of the plasma membrane and thus was not in-volved in endocytosis, purified ^{125}I-labeled virus was added to uninfected cells just before homogenization. The cells were divided into two equal portions: one portion was homogenized in the presence of neuraminidase (0.2 U/ml) and the other in the absence of neuraminidase (all other components were included, as described above). After ho-mogenization and centrifugation, the cytoplasmic superna-tant fractions were applied to sucrose gradients (20 to 60%) and centrifuged to separate the ^{125}I-labeled virions. It was found that much of the labeled virus was associated with cellular material and banded at the top of the gradient in the absence of neuraminidase treatment (Fig. 2A) but was shifted entirely to the marker virus region (indicated by the arrow) by the action of neuraminidase (Fig. 2B).

Similar findings were obtained when the purified ^{125}I-labeled virus was added to the cytoplasmic supernatant fraction obtained from uninfected cells. One portion was incubated with neuraminidase (0.2 U/ml) and the other with an equivalent volume of 0.01 M Tris-chloride (pH 7.4). After 30 min at room temperature, the reaction mixtures were applied to sucrose gradients (20 to 60%) and centrifuged as described above. Again, as shown in Fig. 2C and D, virus added to cell homogenates only remained associated with the cellular material if neuraminidase was omitted from the isolation procedure.

In the converse experiment using cells infected with ^{125}I-labeled virus, the absence and presence of neuraminidase (0.2 U/ml) in the homogenization mixture resulted in the gradient profiles of Fig. 3A and B, respectively. A large portion of the virus label was shifted to the marker virus region by neuraminidase, but a significant portion was not. This ratio of neuraminidase-sensitive and neuraminidase-insensitive labeled virus varied with different multiplicities of infection (Fig. 1A). This is consistent with the conclusion that only actual infection of mouse kidney cells with poly-

omavirus can produce an association of the virus with cellular material that was unaffected by, or inaccessible to, the action of neuraminidase.

Similar experiments (data not shown) using receptor-destroying enzyme (100 U/ml) or a mixture of trypsin and chymotrypsin (10 μg/ml) in the homogenization mixture of infected cells showed that receptor-destroying enzyme was able to remove some but not all of the virus that neuraminidase could and that the proteases could not release virus from the cellular material at the top of the sucrose gradient. Thus, neuraminidase was routinely included in all homogenization mixtures, such that the membrane-associated virus
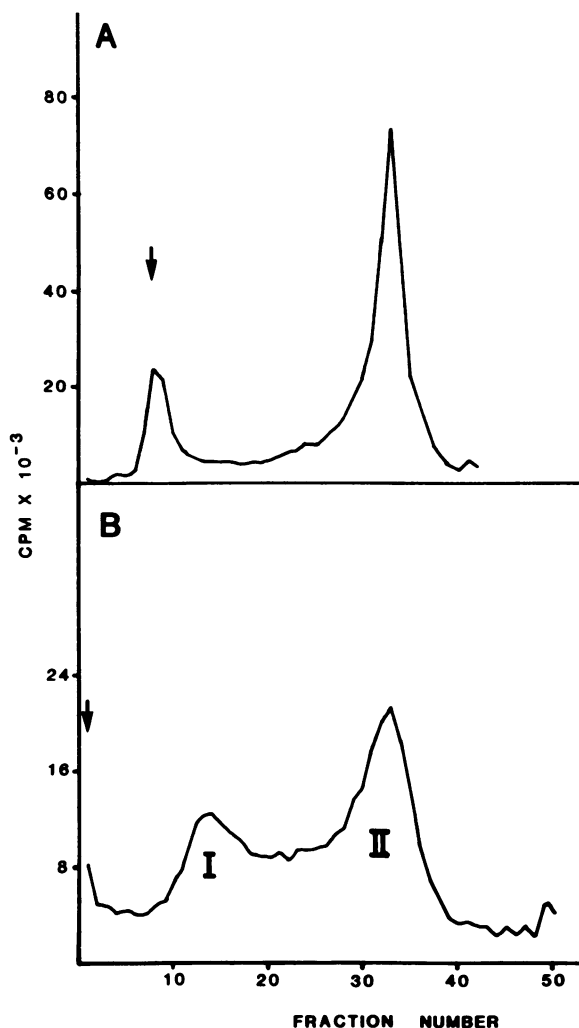

FIG. 1. Sedimentation pattern of vesicles containing ^{125}I-labeled polyomavirus. (A) A 20 to 60% sucrose gradient. The cytoplasmic fraction, containing 7.44×10^5 cpm of ^{125}I-labeled virus, was applied to a sucrose gradient (20 to 60%) containing 0.01 M Tris-chloride (pH 7.4), 0.15 M NaCl, and 0.01% Triton X-100 and centrifuged for 16 h at 25,000 rpm in an SW41 rotor at 4°C. (B) A 15 to 45% sucrose gradient. The cytoplasmic fraction, containing 6.94×10^5 cpm of ^{125}I-labeled virus, was applied to a sucrose gradient (15 to 45%) containing 0.01 M Tris-chloride (pH 7.4), 0.15 M NaCl, and 0.01% Triton X-100 and centrifuged for 16 h at 35,000 rpm in an SW41 rotor at 4°C. See the text for the preparation of the cytoplasmic fraction. The arrow indicates the sedimentation position of purified ^{125}I-labeled polyoma virions.
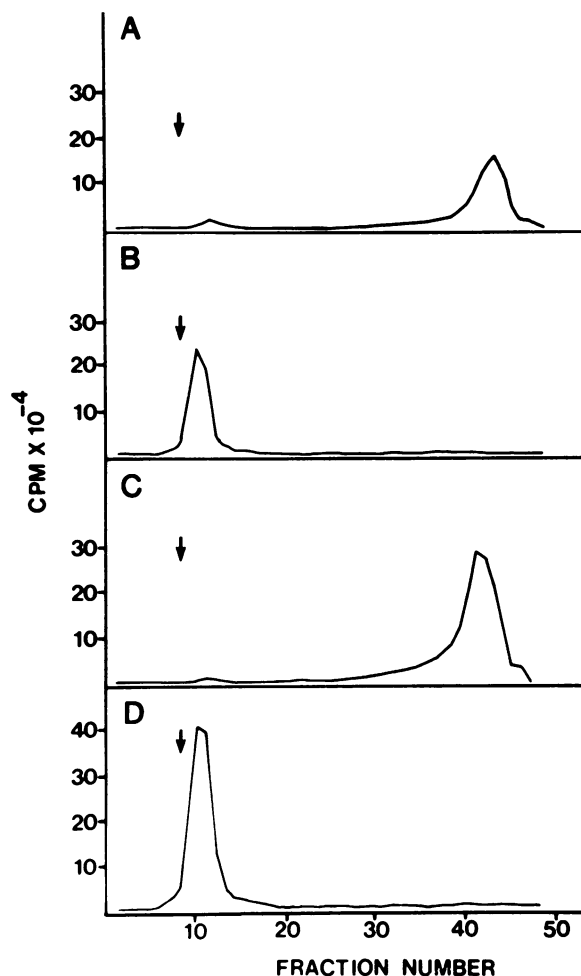

FIG. 2. Control experiments for the isolation of monopinocytotic vesicles. Sedimentation patterns on sucrose gradients (20 to 60%) containing 0.01 M Tris-chloride (pH 7.4), 0.15 M NaCl, and 0.01% Triton X-100 and centrifuged for 16 h at 25,000 rpm in an SW41 rotor at 4°C. (A) ^{125}I-labeled virus (2.17×10^6 cpm) was added to the homogenization mixture of uninfected cells in the absence of neuraminidase, and the resulting cytoplasmic fraction was applied to the gradient. (B) An identical amount of labeled virus was added to the homogenization mixture of uninfected cells in the presence of neuraminidase (0.2 U/ml). (C) ^{125}I-labeled virus (2.17×10^6 cpm) was added to the cytoplasmic fraction from uninfected cells just before application to the gradient. No neuraminidase was present in the homogenization mixture (and thus not in the cytoplasmic fraction). (D) An identical amount of labeled virus was added to the cytoplasmic fraction from uninfected cells just before application to the gradient. Neuraminidase (0.2 U/ml) was present in the homogenization mixture (and thus the cytoplasmic fraction). The arrow indicates the sedimentation position of purified ^{125}I-labeled polyoma virions.

that was subsequently followed in the isolation was only that which was neuraminidase insensitive.

In another control experiment (data not shown), omission of the 37°C incubation period required for virion penetration resulted in only a very small percentage of neuraminidase-insensitive ^{125}I-labeled virus associated with the host cell material in the top of the gradient. Therefore, not only adsorption but penetration of the virus was essential for the
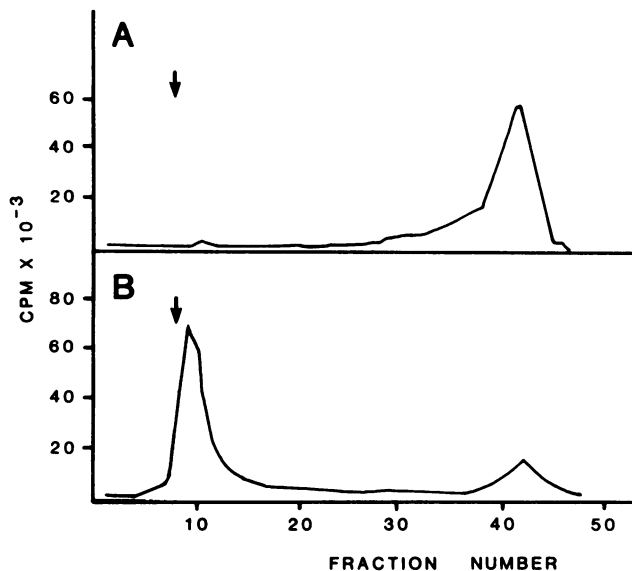
FIG. 3. Sedimentation pattern of the cytoplasmic fraction from cells infected with ^{125}I-labeled virus in the absence and presence of neuraminidase. The gradient and centrifugation conditions were identical to those described in the legend to Fig. 2. (A) No neuraminidase was included in the homogenization mixture. (B) Neuraminidase (0.2 U/ml) was included to the homogenization mixture. The arrow indicates the sedimentation position of purified ^{125}I-labeled polyoma virions.

formation of the peak of vesicle-enclosed virions that were not affected by neuraminidase.

Detergent treatment of isolated vesicles. Samples of the vesicle material from pooled fractions of a sucrose gradient (20 to 60%) were treated with deoxycholate (0.2%) and Nonidet P-40 (0.02%) to determine whether the virions of the isolated monopinocytotic vesicles could be released by detergent solubilization of the vesicles. These samples were then applied to a second sucrose gradient (20 to 60%). In Fig. 4A, the profile of the untreated sample shows that a small amount (6%) of free virus was released from the vesicles, most likely by mechanical or osmotic damage during handling or dilution, but the vesicles containing virions retained their sedimentation properties on this second gradient.

Treatment with deoxycholate caused a release of 76% of the labeled virus from the vesicles (Fig. 4B), whereas Nonidet P-40 treatment released 68% of the virus from the vesicles (Fig. 4C). The detergent-released virus was found to sediment in the marker virus region (arrow). The extent of this release could be controlled by the concentration of detergent. Additional experiments demonstrated that the detergents and concentrations used had no adverse structural effect on purified polyoma virions and did not change their sedimentation properties in this gradient.

In a separate experiment (data not shown), treatment of the vesicle-enclosed polyomavirus preparations with neuraminidase (0.2 U/ml) did not free the virions from their membrane association. These treatments confirmed the membrane-like nature of the cellular material associated with polyomavirus and the inaccessibility of the structure to neuraminidase and further suggested its enclosure within a membrane. The sedimentation properties of the freed virions indicated that the structural integrity of these internalized virions was unchanged during endocytosis and the isolation

procedure, which is in agreement with the results of Mackay and Consigli (23).

Marker enzymes associated with isolated vesicles. Fractions of a sucrose gradient (15 to 45%) were assayed for the activities of the marker enzymes alkaline phosphatase and β-glucuronidase to determine the original cellular source of the vesicles containing the virions. Samples of each gradient fraction were incubated with the appropriate substrate-buffer mixture to obtain a relative measure of the distribution of these activities through the gradient. As Fig. 5 demonstrates, the plasma membrane activity alkaline phosphatase was principally associated with peak I of the gradient. The lysosomal marker β-glucuronidase, however, showed most of its activity on the shoulder of peak II, toward the top of the gradient, with relatively little activity in peak I. Additional experiments (data not shown) showed that the plasma membrane marker 5'-nucleotidase was associated
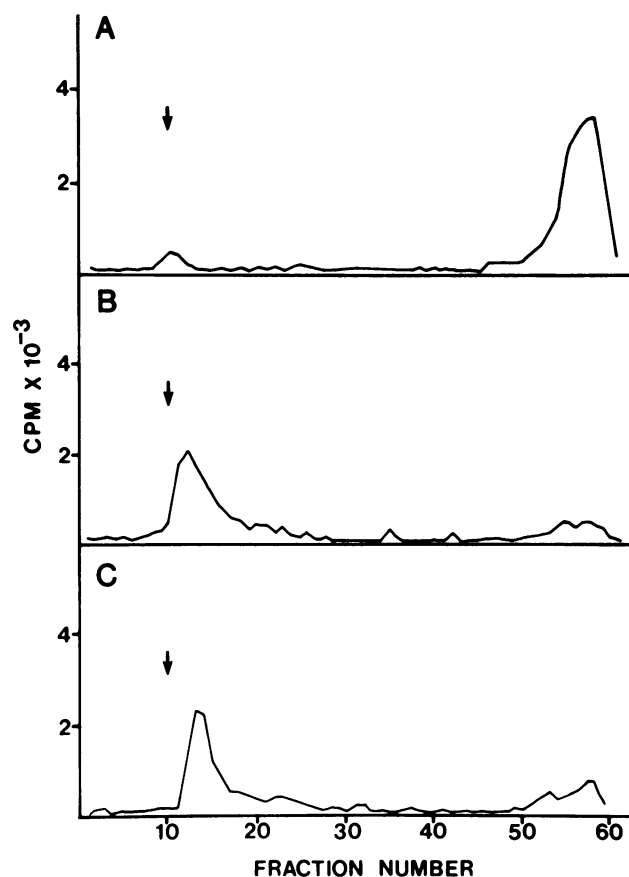

FIG. 4. Sedimentation pattern of detergent-treated vesicles. For each sample, 2.7 × 10⁴ cpm of pooled material from the slower-sedimenting peak of the 20 to 60% sucrose gradient (as in Fig. 1A) was incubated for 30 min at room temperature with the detergent and diluted with an equal volume of 0.01 M Tris-chloride (pH 7.4) before application to a second sucrose gradient (20 to 60%) identical to that described in (A) of the legend to Fig. 1 and centrifuged under the same conditions. (A) Untreated ^{125}I-labeled polyomavirus contained in monopinocytotic vesicles. An equivalent volume of 0.01 M Tris-chloride buffer (pH 7.4) was substituted in the incubation. (B) ^{125}I-labeled polyomavirus contained in monopinocytotic vesicles, treated with deoxycholate (0.2%). (C) ^{125}I-labeled polyomavirus contained in monopinocytotic vesicles, treated with Nonidet P-40 (0.02%). The arrow indicates the sedimentation position of purified ^{125}I-labeled polyoma virions.
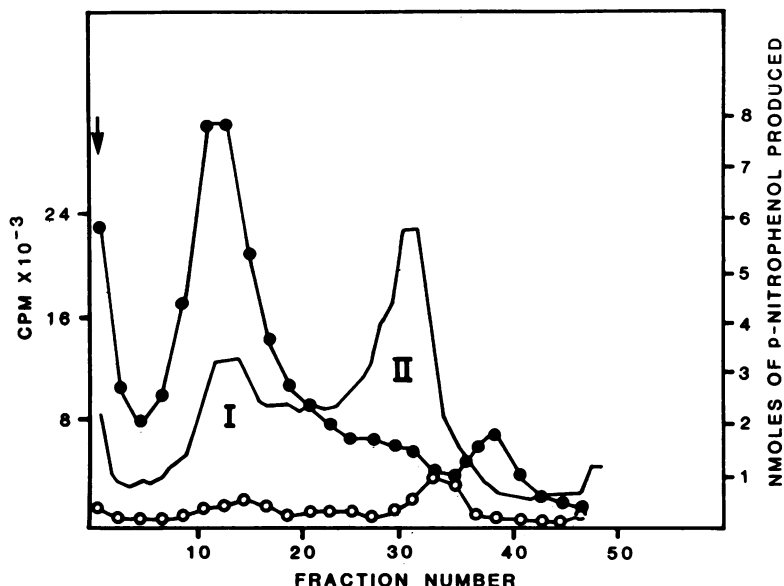
FIG. 5. Sedimentation pattern of vesicles and their associated marker enzyme activities. The cytoplasmic fraction, prepared as described in the text, containing 6.94×10^5 cpm, was applied to a sucrose gradient (15 to 45%) identical to that described in (B) of the legend to Fig. 1 and centrifuged under the same conditions. Samples of 0.1 ml were taken from each gradient fraction and assayed for alkaline phosphatase and β-glucuronidase as described in the text. Activities were plotted as nanomoles of product formed during the incubation. Symbols: solid line, ^{125}I-labeled virus; ●, alkaline phosphatase; ○, β-glucuronidase. The arrow indicates the sedimentation position of purified ^{125}I-labeled polyoma virions.
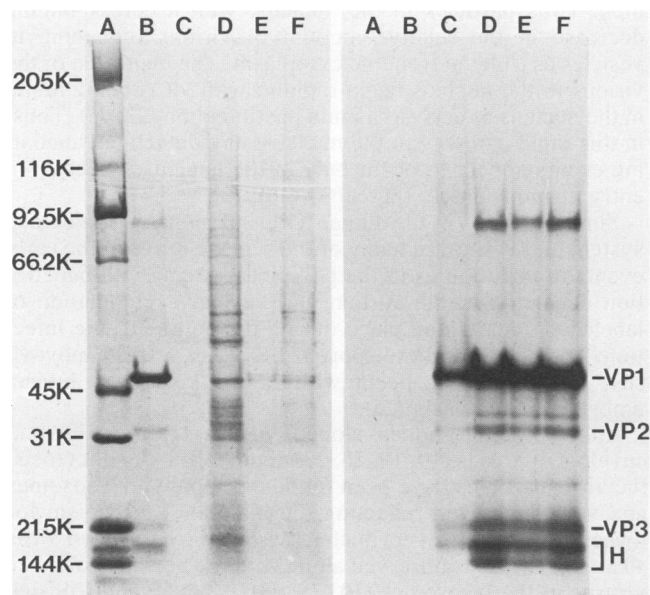

FIG. 6. SDS-PAGE of isolated monopinocytotic vesicles containing ^{125}I-labeled polyoma virions. Samples from pooled fractions of a sucrose gradient (15 to 45%) were prepared, and equivalent counts per minute (8×10^4 cpm) of ^{125}I-labeled virus enclosed in vesicles or purified ^{125}I-labeled virus were applied to a 5 to 15% gradient gel and run as described in the text. The Coomassie-stained proteins are on the left, and the accompanying autoradiogram is on the right. Lane A, molecular weight markers: lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase b (92,500), β-galactosidase (116,000), and myosin (205,000); lane B, purified, unlabeled polyoma virions (9 μg); lane C, purified ^{125}I-labeled polyoma virions; lane D, cytoplasmic fraction; lane E, monopinocytotic vesicles containing polyoma virions from peak I

directly with peaks I and II in the same gradient (15 to 45%). These results suggested that the plasma membrane, rather than the lysosomes, was the principal organelle associated with the virus as it was isolated in vesicles on this sucrose gradient.

SDS-PAGE of viral proteins from isolated vesicles. The pooled fractions from the vesicle peaks (I and II) on a sucrose gradient (15 to 45%) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) to identify the virion structural proteins and the mouse cell vesicle proteins. Figure 6 shows the resulting patterns: the Coomassie-stained gel on the left indicates the molecular weight markers (lane A), purified, unlabeled polyomavirus (lane B), and the virion and membrane proteins of the vesicles (lanes D, E, and F). The autoradiogram on the right indicates the proteins of purified ^{125}I-labeled polyoma virions (lane C) and the proteins from the ^{125}I-labeled virions as isolated in vesicles (lanes D, E, and F). Comparing lanes D, E, and F with the marker virus proteins in lane C on the autoradiogram, all the polyoma proteins (VP1, VP2, VP3, and histones) were found to be present in the vesicle-associated virions, with no major degradation having occurred at this stage of infection.

It also may be observed that, in the Coomassie-stained proteins on the left, with equivalent amounts of ^{125}I-labeled virus enclosed in vesicles applied, fewer host protein bands were observed from the gradient-purified monopinocytotic vesicles (lanes E and F), especially for peak I (lane E), than from the cytoplasmic fraction (lane D). Apparently, some separation of host proteins from the virion-containing vesi-

(see Fig. 1B); lane F, monopinocytotic vesicles containing polyoma virions from peak II (see Fig. 1B).

cles was accomplished in the sucrose gradient. The factors causing the occurrence of two peaks with different sedimentation properties (I and II) in the sucrose gradient (Fig. 1B) are not well understood, but both peaks contained monopinocytotic vesicles that enclose polyomavirus. Peak I appeared to be a purer preparation of these vesicles, as observed in lane E, possibly being more free of association with other cellular components such as the endoplasmic reticulum. It is also worthy of mention that no significant protein bands in the molecular weight range of clathrin monomers (175,000) were observed in any of the preparations applied to the gel in lanes D, E, or F. Since numerous host proteins as well as virion proteins were visible by Coomassie staining, a clathrin band would have been expected if coats were present in these vesicle preparations.

Electron microscopy of isolated vesicles. Mouse kidney cells were infected with a higher multiplicity of virus consisting of 46,000 hemagglutination units of unlabeled polyomavirus as well as 1.72×10^8 cpm of ^{125}I-labeled virus per 100-mm plate. The cytoplasmic fraction was applied to a sucrose gradient (15 to 45%), and the ^{125}I-labeled virions contained in vesicles were pooled from the gradient fractions and fixed, pelleted, stained, and sectioned to visualize them by electron microscopy.

In the field of vesicles in Fig. 7A, numerous vesicles containing virions can be seen, some of which are indicated by the arrows. The virions found in the isolated vesicles appeared structurally intact and similar to purified virions (Fig. 7C), having diameters of 40 to 42 nm in both specimens. The diameter of the virions found in the vesicles (Fig. 7A) depended on the position at which the section was cut; thus, some virions were smaller than others and measured less than 40 nm in diameter. The monopinocytotic vesicles containing polyoma virions had an average diameter of 50 to 60 nm; occasionally some vesicles were observed in the 70-nm range. Figure 7B is a higher magnification of the outlined section of Fig. 7A, showing several virion-containing vesicles. It is worthy of mention that some virions appeared to remain attached to the inside of the vesicle membrane (previously the outside of the plasma membrane). This may reflect the state of attachment of the virion to its cellular receptor.

The field of vesicles presented in Fig. 7A indicates that polyoma virions can indeed be isolated in significant numbers enclosed in vesicles. The actual percentage of vesicles that contained polyoma virions was dependent on the multiplicity of infection. A low multiplicity of infection resulted in fewer virion-containing vesicles found in the thin sections than a relatively high multiplicity of infection, as shown here. The existence of vesicles not containing virions, or "empty vesicles," in these preparations may have occurred because the sectioning process excluded the portion of the vesicle that actually contained the virion, thus leaving the empty portion to be viewed in the electron micrograph. Also, the formation of endocytic vesicles is a continuous, ongoing process in the cell, and vesicles containing other ligands or no ligands at all are being formed during polyoma infection.

DISCUSSION

As first studied by Mackay and Consigli (23) and confirmed in this work, polyomavirus, in the early stage of infection, is enclosed in monopinocytotic vesicles that form as the virions pass through the cell membrane. Vesicles observed in thin sections of infected whole cells (23) and in

thin sections of material isolated from the cytoplasm as presented here are identical in appearance, structure, and dimensions of the virions as well as the plasma membrane surrounding the virions. Mackay and Consigli also reported that the virus particles do not change in morphology, density, antigenicity, or ability to cause hemagglutination during enclosure in the monopinocytotic vesicles. Our present results concur with these earlier observations in that the virion proteins were essentially unchanged and the virions retain their original sedimentation properties when released from the vesicles by detergent treatment and their structural integrity as judged by electron microscopy. In preliminary experiments not described here, the virion-containing vesicles demonstrated an increase in titer in the hemagglutination assay and in the number of PFU after detergent treatment. This suggests that the biological activities of vesicle-enclosed virions also remain unchanged.

Although not shown by our present studies, the processes of receptor binding and enclosure in vesicles or nonspecific binding to neuraminidase-sensitive sites may create subtle alterations in the surface of polyoma virions, resulting in slightly slower sedimentation rates (225 to 230 s) compared with purified ^{125}I-labeled virions (240 s) (Fig. 2B and D, 3B, and 4B and C). This might possibly reflect the presence of cellular receptor moieties bound to virions or a very early stage of virion uncoating preceding nuclear delivery.

Time course infection studies following the amount of ^{125}I-labeled virus that is found in isolated monopinocytotic vesicles and in nuclei after 15 min and 1, 4, and 12 h (unpublished data) have demonstrated the accumulation of input virus particles in the nucleus, with a corresponding decrease in the relative amount of virions remaining in vesicles as isolated from the cytoplasm. The migration of the virions to the nucleus begin rapidly, with virus being found in the nucleus as early as 15 min postinfection (23, 46). Thus, in this rapid pathway to the nucleus, any detectable uncoating events appear to occur only in the nucleus, either upon entry or immediately thereafter (46).

Similar studies in the simian virus 40 monkey kidney cell system (3, 21) showed many of the same features of the early events of infection as in the polyoma system, with penetration occurring within 10 min and nuclear accumulation of labeled virions taking place during the course of the infection. The uptake and transport of BK virus, also a papovavirus, appears also to occur with similar kinetics in human embryonic fibroblasts (25).

Although other nonenveloped viruses (10, 15, 38) and enveloped viruses (10, 19, 26, 27) enter cells by endocytosis, the virus particles have been found principally in lysosomes and vacuoles, often reflecting the infection mechanism for these viruses. However, nuclear association of simian virus 40 and BK virus is observed along with the presence of these viruses in the lysosomes (10, 25, 28). Mackay and Consigli (23) reported the accumulation of polyoma capsids in larger vesicles, but intact virions were observed only as single particles in monopinocytotic vesicles. Bolen and Consigli (6) described two types of binding at the surface of mouse kidney cells: one that is specific, involving intact virions, and one that is nonspecific, involving capsids. All these results suggest the existence of two possible fates for the infecting particle: a productive pathway of monopinocytotic vesicles eventually transported to the nucleus, and a degradative, nonproductive pathway terminating in the lysosomes. The point of segregation of these two pathways, however, is not clear. It may occur at the cell surface, mediated by different cellular receptors (6), or it may take
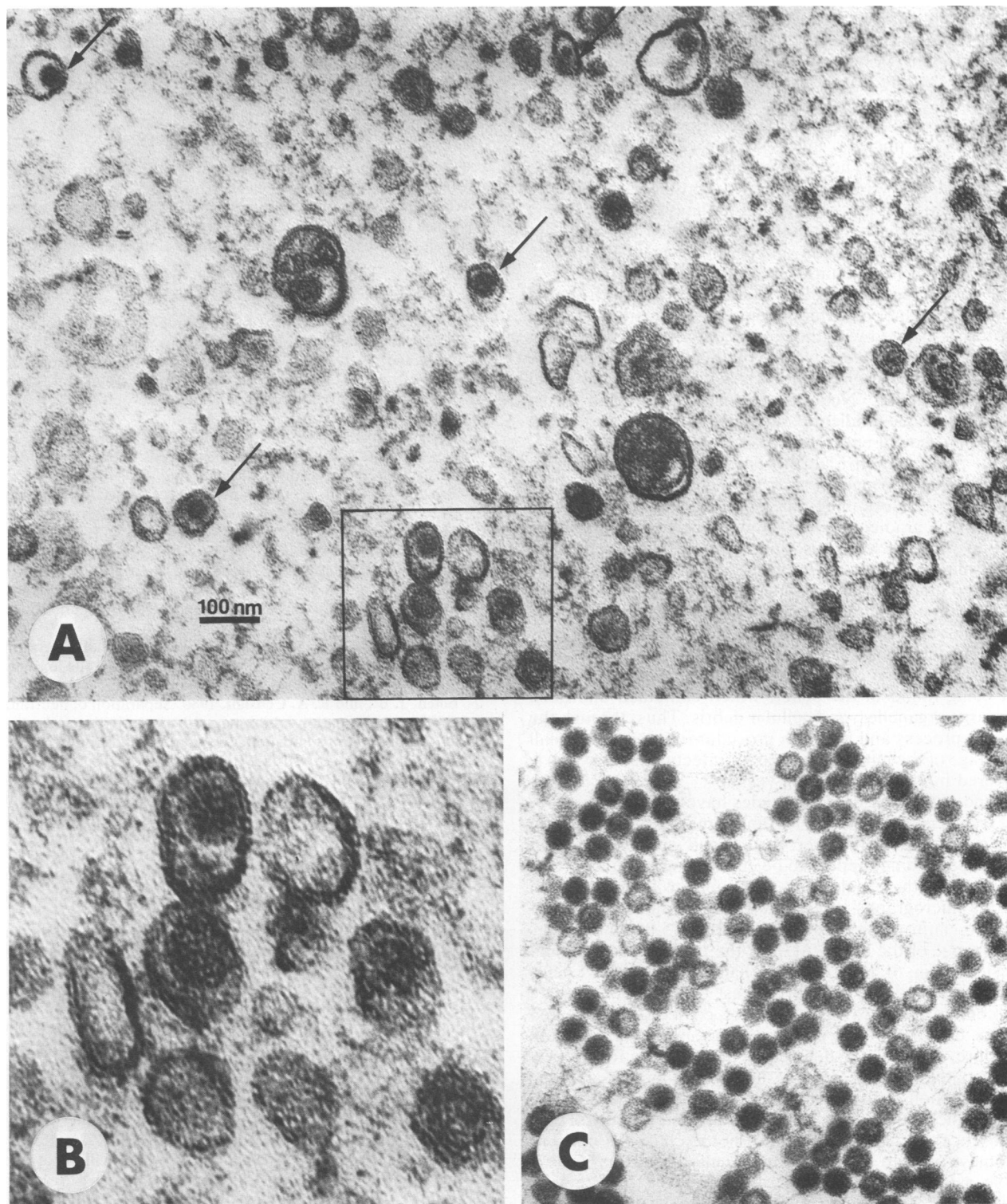
FIG. 7. Electron microscopy of isolated monopinocytotic vesicles containing polyomavirus. Pooled fractions from peak I of a sucrose gradient (15 to 45%) were obtained as described for the gradient in Fig. 1B and fixed, pelleted, and stained for thin sectioning as described in the text. (A) Field of vesicles. Selected monopinocytotic vesicles containing polyoma virions are indicated by arrows. Bar, 100 nm. ×97,200. (B) Higher magnification of outlined portion of (A) of monopinocytotic vesicles. ×345,600. (C) Purified polyomavirus prepared for sectioning in a manner identical to that for monopinocytotic vesicles in (A). ×97,200.

place after endocytosis has occurred, in the cytoplasm. In the latter possibility, reports of the formation of intermediate vesicles before fusion with the lysosomes for other ligands (2, 30, 41) indicate a potential segregation point for polyomavirus-containing vesicles if such intermediate vesicles are forming in the infected mouse cells. This would necessitate some sort of control for those vesicles that become acidified (41) and become part of the lysosomes and those that go directly to the nucleus, unless the process of selection is completely random. The involvement of the Golgi system in lysosomal delivery (43, 44) suggests a possible cellular site for the initiation of this segregation to the lysosomes. In any case, the mechanism(s) directing polyoma virions and capsids in their internalization and/or infection in the mouse kidney cell system remain to be identified.

The vesicles isolated in this study were derived from the plasma membrane, as demonstrated by the marker enzyme activities associated with the virion-containing vesicles (Fig. 5). It was our purpose to exclude by this isolation procedure any cellular associations of the virus with the lysosomes, and this was achieved. None of the isolated labeled virus-containing vesicle gradient bands showed strong association with the lysosomal enzyme marker β-glucuronidase at 1, 4, or 12 h postinfection (data not shown). Also, preliminary experiments involving cell surface labeling of mouse kidney cells with ^{125}I before infection with unlabeled polyomavirus resulted in a gradient profile similar to that obtained with infection with labeled virus, suggesting the involvement of the plasma membrane in the formation of this vesicle fraction of the gradient (unpublished data).

The virion-containing vesicles that were isolated from the cytoplasm represented 10% of the labeled virus that was present in the homogenate. At 1 h postinfection, another 10% was located in the nucleus, with the remainder of the label being found in the pellet, which contained unbroken cells, large organelles, and cellular debris. Thus, through the infection process and isolation procedure, only a very small percentage of the input virus used for infection (2% or less) is recovered in virion-containing vesicles.

Ligand-containing coated vesicles have been observed (8, 12, 13) and isolated (36, 37) from animal tissues and from tissue culture cells. However, the isolated vesicles described in this report do not appear to be enclosed in a clathrin coat, but are smooth vesicles, as they appear in thin-section electron micrographs after gradient purification. Furthermore, no clathrin can be detected in the 175,000-molecular-weight range of the SDS-PAGE of the pooled gradient band of vesicles (Fig. 6). Observations that newly formed vesicles soon lose their coats (35, 45) suggest that our homogenate may contain a mixed population of coated and uncoated vesicles containing polyomavirus. Possibly, our isolation procedure, either by the differences in sedimentation properties of coated and uncoated vesicles or by factors affecting the dissociation of the coats (32, 47), causes the loss of any coated vesicles that might have been present initially. Thus, the absence of clathrin coats does not exclude the entry of polyomavirus into mouse kidney cells from the coated vesicle pathway observed for other ligands (15). Previous studies (6, 23) do suggest that polyomavirus enters by receptor-mediated endocytosis.

This procedure for the isolation of polyomavirus contained in vesicles will facilitate our further studies of the early events of infection of mouse kidney cells by polyomavirus, particularly the segregation of infecting particles into the lysosomes or the nucleus and the event of nuclear entry that results from the interaction of the monopinocytotic

vesicle and the nucleus before the actual uncoating events (6, 23, 46). The purification of such virus-containing vesicles derived from the plasma membrane also provides an enriched source of the cellular receptor(s) for polyomavirus, as reported for other internalized ligands (11, 34), thus aiding our future efforts to identify, characterize, and isolate these receptors.

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