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Sequential Rearrangement and Nuclear Polymerization of Actin in Baculovirus-Infected *Spodoptera frugiperda* Cells

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Proper assembly of nucleocapsids of the baculovirus *Autographa californica* nuclear polyhedrosis virus is prevented by cytochalasin D, a drug that interferes with actin microfilament function. To investigate the involvement of microfilaments in *A. californica* nuclear polyhedrosis virus replication, a fluorescence microscopy study was conducted that correlated changes in distribution of microfilaments with events in the life cycle of the virus. Tetramethylrhodamine isothiocyanate-labeled phalloidin was used to label microfilaments, and monoclonal antibody was used to label p39, the major viral capsid protein. Three microfilament arrangements were found in infected cells. During uptake of virus, thick cables were formed. These were insensitive to cycloheximide, indicating that this configuration was a rearrangement of preexisting cellular actin mediated by a component of the viral inoculum. At the time of cell rounding and before viral DNA replication, ventral aggregates of actin were observed. These were sensitive to cycloheximide but not to aphidicolin, indicating that an early viral gene mediated this actin rearrangement. Ventral aggregates did not result from the rounding process itself. Uninfected cells prerounded with colchicine did not form ventral aggregates. Cells prerounded with colchicine and then infected did form aggregates. At the time of exponential production of progeny virus, microfilaments were found in the nucleus surrounding the virogenic stroma. In this area (where nucleocapsid assembly is known to take place) microfilaments colocalized with p39. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblot analysis identified p39 among proteins retained on an f-actin affinity column. We postulate that microfilaments in the nucleus provide a scaffold to position capsids for proper assembly and filling with DNA.

Actin microfilaments are one of the major components of the cytoskeleton and are thought to be involved in such normal cellular processes as adhesion, motility, division, phagocytosis, secretion, and intracellular transport of organelles (3, 15, 20, 48, 50, 51). Until recently, it was controversial whether actin functioned in the nuclear processes of a cell. Now, actin is known to play a role in transcription (8, 41), and filamentous f-actin has been shown to be part of the nuclear matrix (35, 36, 54), the substrate upon which replication and transcription of DNA are thought to occur (18, 37, 39).

Several viruses have been shown to interact with the cytoskeleton or nuclear matrix in their replication (7, 12, 27, 39, 45). Such varied activities as genome replication, protein synthesis and transport, and assembly, transport, and release of virions have been associated with these structures. Actin microfilaments in particular have been associated with the rounding and loss of adhesion that frequently occur with viral infection or transformation (6, 16, 19, 24, 29, 43, 47, 59), with the intracellular transport of viral structural proteins and viral particles (1, 21, 49), with the budding process of many enveloped viruses (14, 33, 34, 60), and with the assembly of virions in the cytoplasm (17, 44) and in the nucleus (55).

Autographa californica nuclear polyhedrosis virus (Ac*M* NPV) is a large, double-stranded, nucleus-replicating DNA virus in the family *Baculoviridae* (28). This virus infects lepidopteran insects and has been studied extensively because of its potential for use as a biological control agent (32). It is also commonly used as an expression vector for foreign genes (26). It is an unusual virus in that two different viral forms are involved in its life cycle: an early extracellular virus (EV) that buds from the plasma membrane and a later form that is occluded in polyhedron-shaped protein matrices in the nucleus (9, 57).

Previous studies showed that f-actin formed in the nucleus of Ac*M*NPV-infected cells. Cytochalasin D, an inhibitor of actin microfilament function, prevented the proper intranuclear assembly of viral nucleocapsids and the production of infectious progeny (55, 56).

In this study, we used fluorescence microscopy to examine changes in the distribution of microfilaments during infection of Sf21 *Spodoptera frugiperda* cells with Ac*M*NPV and to correlate these rearrangements with events in the life cycle of the virus. Three microfilament configurations were found: early cables, which occurred during uptake of virions; ventral aggregates, which were seen at the time of cell rounding and DNA synthesis; and nuclear actin microfilaments, which associated with viral capsid protein during nucleocapsid morphogenesis. f-Actin affinity chromatography was used to detect two virus-induced proteins that bound specifically to microfilaments. One of these was p39, the major capsid protein of Ac*M*NPV.

MATERIALS AND METHODS

Cell culture and virus. *Spodoptera frugiperda* IPLBSf21 cells were grown at 28°C in BML-TC/10 medium (13) containing 10% fetal calf serum. The virus used was the second-passage E2 strain of Ac*M*NPV EV from culture medium 48 h postinoculation (p.i.) (46).

Antibodies. Monoclonal antibody (MAb) 39 (39P10), which is reactive with Ac*M*NPV p39 capsid antigen, was the gift of

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Sharon Roberts and Jarue Manning, University of California, Davis (61). MAb B12D5, which is reactive with the Ac*M*NPV EV major gp64 envelope antigen, was generated in this laboratory (23). Preimmune mouse serum and 14 mouse polyclonal antibodies to *Drosophila* embryo actin-binding proteins were donated by Kathy Miller and Chris Field, University of California, San Francisco (31). A sampler pack of actin-binding protein antibodies (Cyto-2) containing rabbit polyclonal antibodies to alpha-actinin, chicken spectrin, and tropomyosin as well as mouse MAb to vinculin was purchased from Sigma Chemical Co. (St. Louis, Mo.). Another MAb to vinculin (N.354) was purchased from Amersham Corp. (Arlington Heights, Ill.). A rabbit polyclonal antibody to vinculin was given to us by Joann Otto, Purdue University.

Fluorescein isothiocyanate-conjugated, affinity-purified goat anti-mouse immunoglobulin G (IgG) (gamma chain specific) was purchased from Zymed Laboratories (San Francisco, Calif.). Horseradish peroxidase-conjugated goat anti-mouse IgG (H and L chain specific) was obtained from Bio-Rad Laboratories (Richmond, Calif.).

Chemicals. All chemicals were reagent grade and purchased from Sigma unless otherwise indicated. Cycloheximide and colchicine were initially dissolved in BML-TC/10 medium containing 10% fetal calf serum, whereas a 100× stock solution of aphidicolin was dissolved in dimethyl sulfoxide. (When cells were treated with aphidicolin, untreated control cultures were exposed to 1% dimethyl sulfoxide.) For staining, tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin was dissolved as a 750× stock solution in methanol (0.2 mg/ml), whereas 4,6-diamidino-2-phenylindole (DAPI) was dissolved in PHEM buffer (42).

Buffers. The following buffers were used. PHEM buffer contained 60 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,N′-tetraacetic acid], and 2 mM $MgCl_2$ (pH 6.9). Actin-polymerizing buffer contained 50 mM HEPES (adjusted to pH 7.5 with KOH), 100 mM KCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 5 mM $MgCl_2$. Extraction buffer contained 5 mM HEPES (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 0.15% Nonidet P-40, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride. Wash buffer contained 50 mM HEPES (pH 7.5), 2 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, 0.005% Nonidet P-40, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 10 μg of aprotinin per ml, and 10% glycerol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer contained 62 mM Tris hydrochloride (pH 6.8), 3% SDS, 5% 2-mercaptoethanol, and 10% glycerol. Tris-buffered saline (TBS) contained 50 mM Tris (pH 7.5) and 150 mM NaCl. Blocking buffer contained 50 mM Tris (pH 7.5), 150 mM NaCl, 12% powdered milk (Carnation Co.), and 5% normal goat serum (Sigma; no. G 6767).

Immunofluorescence microscopy. Glass coverslips (22 mm^2) were soaked overnight in 70% ethanol–1% HCl, rinsed 15 times with double-distilled water, baked at 60°C for 30 min, and allowed to cool. Sterile coverslips were placed in 35-mm tissue culture dishes or 6-well tissue culture plates. A suspension of 5×10^5 cells in 500 μl of medium was applied to each coverslip, and cells were allowed to adhere for 1 h (or overnight). Cells were pretreated with either 200 μg of cycloheximide per ml for 2 h, 5 μg of aphidicolin per ml for 2 h, 10 μM colchicine for 5 h, or medium for the comparable amount of time. The cells were then infected with a multiplicity of 10 infectious particles per cell in 250 μl of medium containing the appropriate drug.

At 3, 6, 12, 20, and 44 h p.i., cells were processed for microscopy. At 3 h p.i., the inoculum was removed and replaced with 1 ml of the same medium devoid of virus. Cells were centrifuged on coverslips at 750 × *g* for 5 min at 4°C in a Beckman GPR tabletop centrifuge and then extracted for 10 min with 0.15% Triton X-100 in PHEM buffer containing 3×10^{-7} M TRITC-phalloidin. For later time points, cells were rinsed once with PHEM buffer, fixed for 10 min in 2% paraformaldehyde in PHEM buffer, and solubilized for 10 min in 0.15% Triton X-100 in PHEM containing TRITC-phalloidin. Coverslips containing cells were floated for 45 min at 27°C on 25 μl of primary antibody, rinsed twice in PHEM, and then floated for 45 min on 25 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Cells were rinsed once in PHEM, treated for 30 s with 0.1 μg of DAPI per ml in PHEM, rinsed four more times with PHEM, and mounted for viewing in a drop of nonbleach mountant (10 mg of phenylenediamine/ml of PHEM mixed 1:9 with glycerol). Excess mountant was drained from preparations. Slides were viewed and photographed with a Zeiss Axiophot photomicroscope equipped for fluorescence microscopy.

Preparation of cell extracts for chromatography. Both uninfected cells and cells at 24 h p.i. were harvested, centrifuged at 800 × *g* for 5 min, rinsed once in cold PHEM buffer, pelleted again at 800 × *g* for 5 min, and suspended in 10 volumes of cold extraction buffer. Cell suspensions were sonicated on ice eight times for 20 s each (with 1-min rests in between) with a Branson Sonifier set at 5 and tuned to 2.5 A. This disrupted about 97% of the nuclei. The resulting slurry was centrifuged at 10,000 × *g* at 4°C for 20 min, and the supernatant fluid was brought to 50 mM HEPES and 2 mM dithiothreitol. After centrifugation at 80,000 × *g* at 4°C for 1 h, samples of 10 ml of supernatant fluid containing 25 mg of protein were loaded onto appropriate affinity columns.

f-Actin affinity chromatography of cell extracts. Affinity chromatography columns were prepared with the help of Kathy Miller, University of California, San Francisco, as described previously (30). Briefly, a mixture of 1.5 ml of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) and 1.5 ml of Sepharose CL-6B (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) was packed into 6-ml sterile plastic syringes (Becton Dickinson, Rutherford, N.J.) fitted with polypropylene filter disks (Ace Glass, San Francisco, Calif.) and 18-gauge needles (Becton Dickinson). Column beds were mixed and then rinsed three times with glass-distilled water and one time with actin-polymerizing buffer. Monomeric actin purified from rabbit skeletal muscle was diluted to 2 mg/ml in actin-polymerizing buffer, and the resulting filamentous f-actin was stabilized with 10 μg of phalloidin per ml. f-Actin (1.5 ml) or bovine serum albumin (BSA) (1.5 ml at 4 mg/ml in actin-polymerizing buffer without phalloidin) was added to plugged syringes, gently mixed, and allowed to couple to resins overnight at 4°C. Coupling was terminated by the addition of 3 M redistilled ethanolamine (pH 8) to a final concentration of 50 mM for 1 h. The column was packed by circulating 7 volumes of actin-polymerizing buffer over the column in 1 h. The column was then washed first with 5 volumes of wash buffer and then with 5 volumes of extraction buffer. All washes were saved so the amount of protein retained on the column could be calculated. Extracts from uninfected cells and cells at 24 h p.i. were applied to f-actin or BSA columns containing equal amounts of protein and allowed to flow over the columns at 1 column volume per h. Columns were then washed overnight with wash

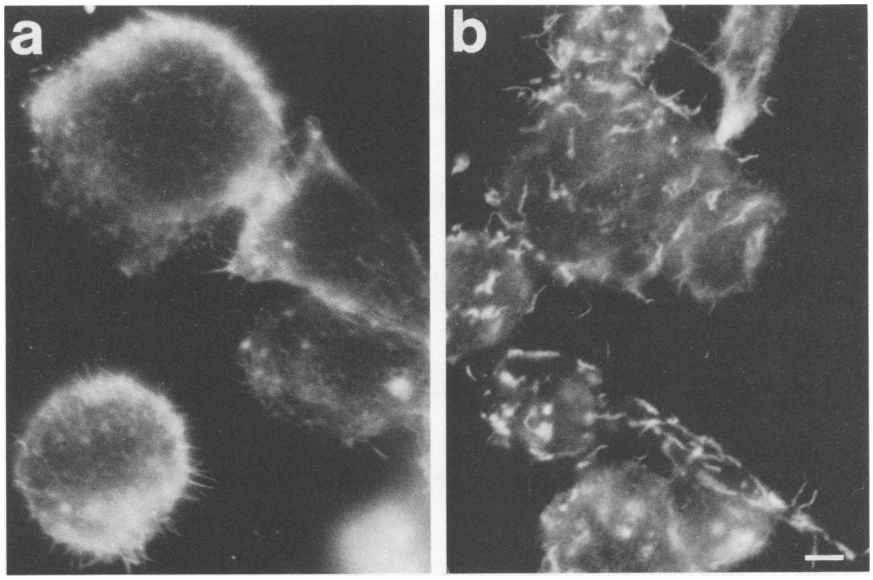

FIG. 1. Microfilaments in uninfected cells and cells at 3 h p.i. (a) Uninfected cells were incubated for 5 h in medium. (b) Cells were incubated for 2 h in medium and then infected for 3 h with Ac*M*NPV. All cells were detergent extracted, and microfilaments were labeled with TRITC-phalloidin. Bar, 2 μm.

buffer (one column volume per h). Actin- or BSA-binding proteins were then eluted from the column with wash buffer containing 1 M KCl, 2 mM ATP, and 3 mM $MgCl_2$. Fractions from column elution were collected, and protein in the fractions was determined by the Bradford assay (2). Fractions from the f-actin columns that contained protein were pooled, as were equivalent fractions from control BSA columns.

SDS-PAGE and Western immunoblotting. Proteins eluted from affinity columns were precipitated with 10% trichloroacetic acid, suspended in SDS-PAGE sample buffer, and neutralized with 2 M Tris base. Equal volumes of sample were loaded onto 10% polyacrylamide slab gels and electrophoresed in SDS (25). Samples of proteins that were applied to and that flowed through affinity columns were also electrophoresed. Proteins in gels were either visualized by Coomassie blue staining or transferred to nitrocellulose (53) for detection of specific proteins by antibody.

Immunological detection of blotted proteins. Nitrocellulose sheets were soaked in Ponceau S solution (Sigma; no. P 7767) (0.2% [wt/vol] in water) for 10 min and rinsed with TBS to visualize protein bands. Individual lanes were numbered and cut apart for labeling with different antibodies. The nitrocellulose strips were blocked for at least 90 min in blocking buffer and then rinsed three times in TBS. Strips of filter paper as large as the nitrocellulose strips were placed on Parafilm and saturated with primary antibody. Proteins on nitrocellulose strips were brought into contact with antibodies on filter paper strips and incubated for 1 h in a humid chamber at 27°C. Blotted proteins were rinsed five times for 2 min with TBS and then incubated in horseradish peroxidase-conjugated goat anti-mouse IgG for 1 h with slow rotation on an orbital shaker. Nitrocellulose strips were again rinsed five times with TBS and then stained for 5 to 10 min with a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride in TBS with 0.01% hydrogen peroxide. Stained blots were rinsed with tap water and dried away from light.

RESULTS

Early cables (1 to 3 h p.i.). TRITC-phalloidin labeling of uninfected detergent-extracted cells showed a fine homogeneous network of microfilaments with occasional small f-actin aggregates in the cytoplasm as well as microfilaments at the plasma membrane and in microspikes (Fig. 1a). No microfilaments appeared in nuclei.

Cells infected for 3 h and prepared in the same way had a dramatically different configuration of microfilaments. Coarse actin cables appeared traversing the cytoplasm, along the surface of the cell, or in microspikes (Fig. 1b). These cables were not sensitive to 200 μg of cycloheximide per ml, an amount that inhibited viral protein synthesis (data not shown). The cell shape was similar to that of uninfected cells.

Ventral aggregates (4 to 7 h p.i.). From about 4 to 7 h p.i., microfilaments formed dense aggregates on the ventral surface of the cell and early cables disappeared (Fig. 2a). In the presence of 200 μg of cycloheximide per ml, ventral aggregates did not form and early cables remained (Fig. 2b). Aphidicolin did not prevent the appearance of ventral aggregates in infected cells at 6 h p.i. (Fig. 2c), but by 20 h p.i. in the presence of aphidicolin ventral aggregates had disappeared (Fig. 2d).

One common cytopathic effect associated with Ac*M*NPV infection is that host cells round up. This is thought to be due to virus-induced rearrangement of microtubules (58). Ventral aggregates occurred at the time during infection when cells rounded up. To investigate whether formation of ventral aggregates was associated with cell rounding, cells were allowed to spread on glass coverslips overnight (Fig. 3a) and then were rounded by treatment with 10 μM colchicine for 5 h (Fig. 3b). No ventral aggregates were formed. Conversely, ventral aggregates still appeared in colchicine-prerounded, infected cells at 5 h p.i. (Fig. 3c).

Ac*M*NPV-induced ventral microfilament aggregates had a similar appearance to the ventral adhesion plaques of f-actin

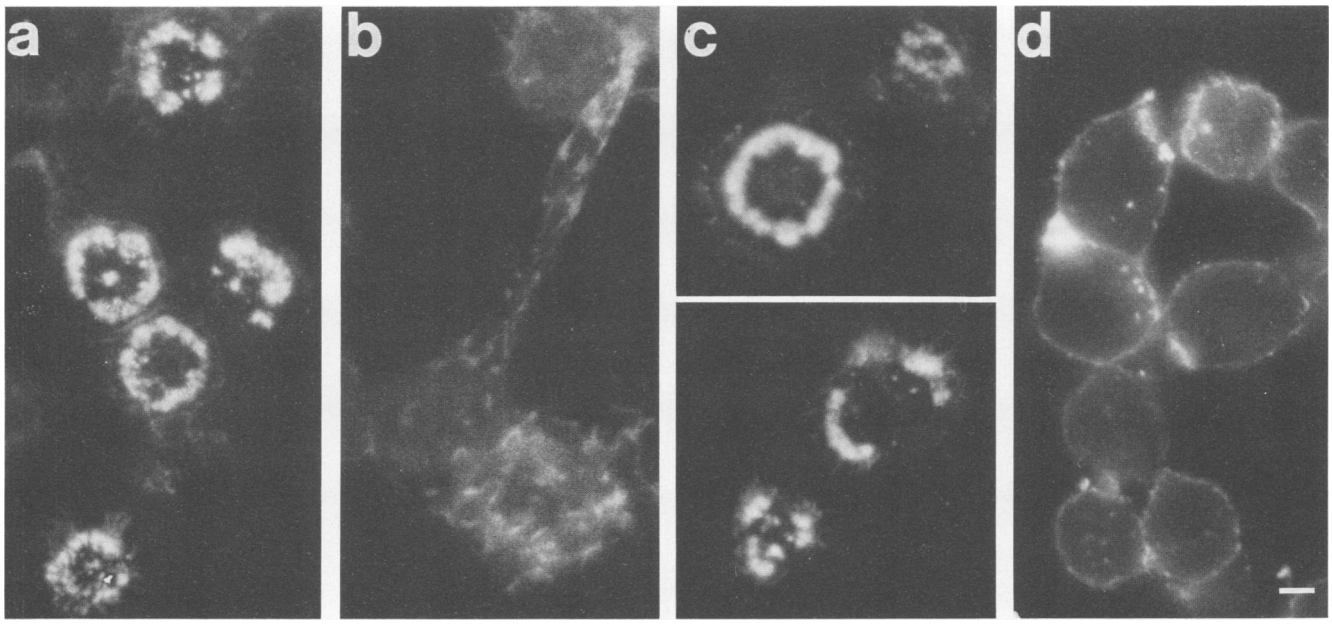

FIG. 2. Effect of protein synthesis and DNA replication inhibitors on formation of ventral microfilament aggregates. (a) Cells were pretreated for 2 h with medium alone and then infected for 6 h with Ac*M*NPV. (b) Cells were pretreated for 2 h with medium containing 200 μg of cycloheximide per ml and then infected for 6 h in the presence of 200 μg of cycloheximide per ml. (c) Cells were pretreated for 2 h with medium containing 5 μg of aphidicolin per ml and then infected for 6 h in the presence of 5 μg of aphidicolin per ml. (d) Cells were pretreated for 2 h with 5 μg of aphidicolin per ml and then infected for 20 h in the presence of 5 μg of aphidicolin per ml. All cells were fixed and detergent extracted, and microfilaments were labeled with TRITC-phalloidin. Bar, 2 μm.

found in Rous sarcoma virus-transformed cells (6). Since Rous sarcoma virus-induced adhesion plaques are known to associate with various actin-binding proteins (5), we tried labeling ventral aggregates with antibodies to vinculin, spectrin, alpha-actinin, tropomyosin, and 14 polyclonal antibodies made to *Drosophila* embryo actin-binding proteins (31). Although most of these antibodies gave positive staining patterns in infected and uninfected cells, none of them

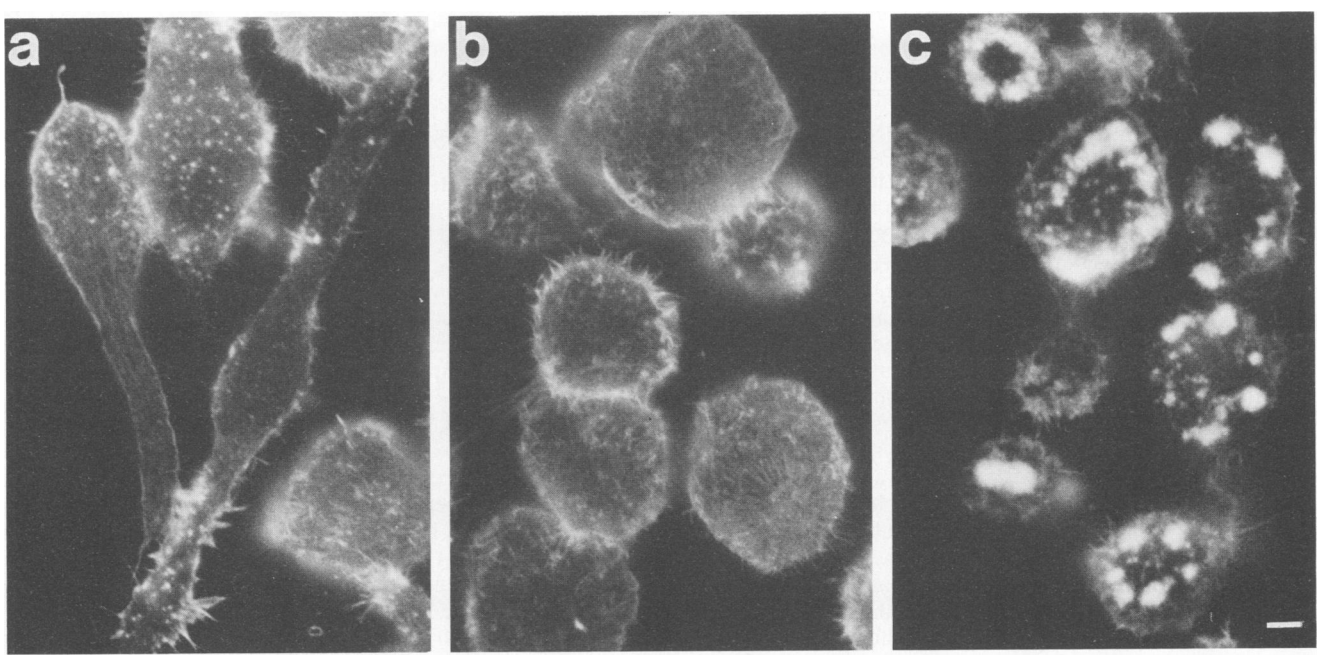

FIG. 3. Effect of cell rounding on formation of ventral microfilament aggregates. (a) Uninfected cells were plated overnight. (b) Uninfected cells were plated overnight and then rounded by incubation for 5 h in medium containing 10 μM colchicine. (c) Cells were treated as in (b) and then infected for 5 h with Ac*M*NPV in medium containing 10 μM colchicine. All cells were fixed and detergent extracted, and microfilaments were labeled with TRITC-phalloidin. Bar, 2 μm.

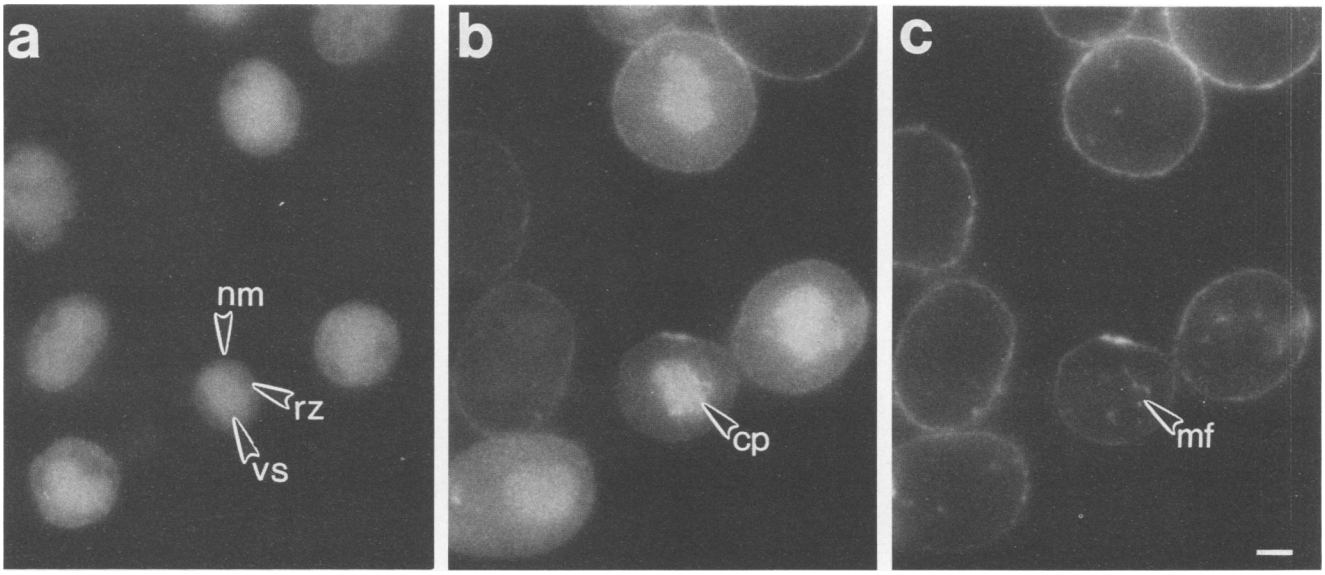

FIG. 4. Microfilaments in the nucleus, peripheral to the virogenic stroma. Cells were fixed at 12 h p.i., detergent extracted, and colabeled with (a) DAPI, (b) MAb p39 and fluorescein isothiocyanate-conjugated goat anti-mouse IgG, and (c) TRITC-phalloidin. Photographs were all taken in the same plane of focus. vs, Virogenic stroma; rz, ring zone; nm, nuclear membrane; cp, p39, the major viral capsid protein; mf, microfilament. Bar, 2 μm.

localized in Ac*M*NPV-induced ventral microfilament aggregates (data not shown).

Nuclear f-actin (10 to 30 h p.i.). At 12 h p.i., DAPI labeling of DNA showed a bright nuclear virogenic stroma surrounded by a lighter ring zone within the nuclear membrane (Fig. 4a). These features are characteristic of baculovirus infections (10). In these same cells, antibody to p39, the major viral capsid protein, localized in the stroma but also reacted strongly with areas in the ring zone (Fig. 4b). TRITC-phalloidin labeling of microfilaments in these cells showed that f-actin had begun to polymerize at the periphery of the stroma and that microfilament labeling colocalized with bright p39 labeling in the ring zone (Fig. 4).

By 20 h p.i., considerably more f-actin was evident within the ring zone, where it colocalized with highly concentrated p39 (Fig. 5). At this time, p39 could also be detected in bright spots at the plasma membrane of most cells, consistent with virus budding. The appearance of nuclear f-actin was prevented by treatment with aphidicolin (Fig. 2d). At 20 h p.i., polyhedra were not yet evident (data not shown).

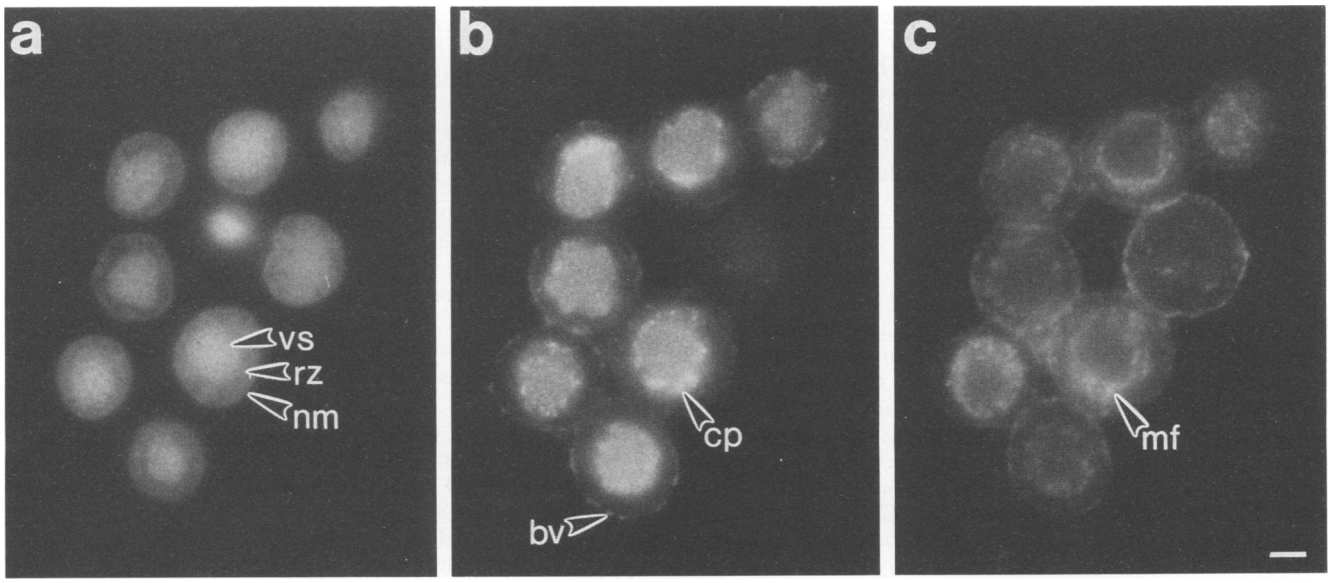

FIG. 5. Colocalization of viral capsid protein with nuclear microfilaments. Cells were fixed at 20 h p.i., detergent extracted, and colabeled with (a) DAPI, (b) MAb p39 and fluorescein isothiocyanate-conjugated goat anti-mouse IgG, and (c) TRITC-phalloidin. Photographs were all taken in the same plane of focus. vs, Virogenic stroma; rz, ring zone; nm, nuclear membrane; cp, p39, the major viral capsid protein; bv, budding virus; mf, microfilament. Bar, 2 μm.

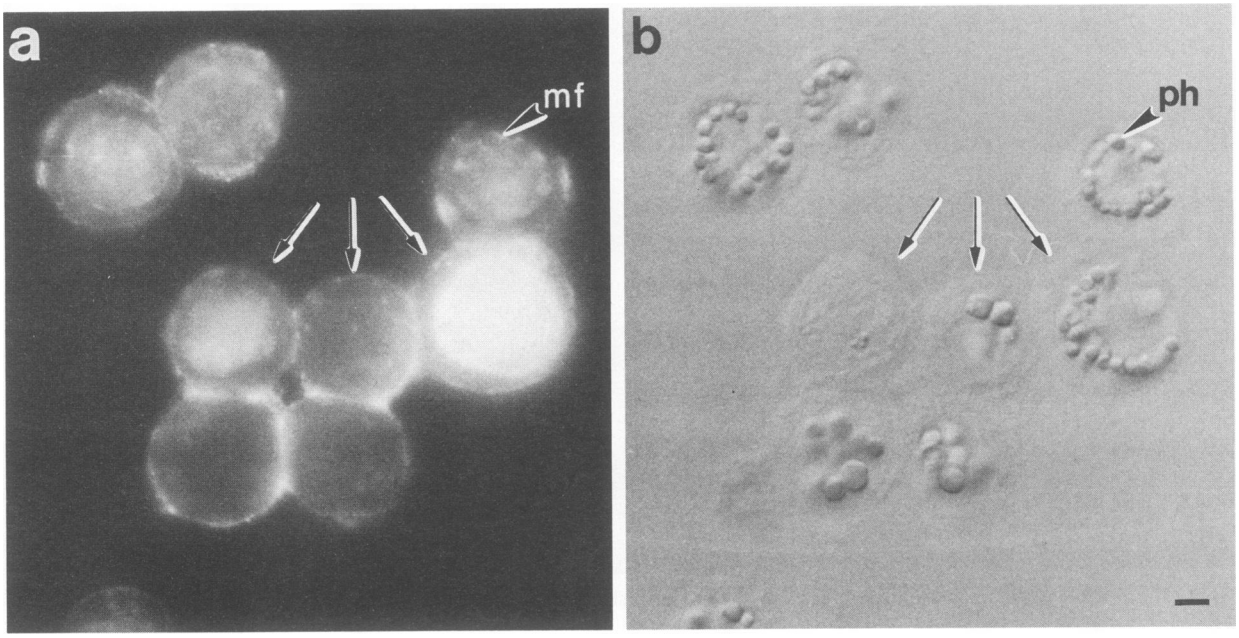

FIG. 6. Microfilaments during polyhedron formation. (a) Microfilaments (mf) were labeled with TRITC-phalloidin. (b) Polyhedra (ph) in these same cells were visualized with differential interference contrast optics. Bar, 2 μm.

Polyhedron formation (44 h p.i.). Cells in various stages of polyhedron formation could be seen at 44 h p.i. (Fig. 6b). There was no correlation between the amount or location of microfilaments within the nucleus and the amount or location of polyhedra (Fig. 6; compare cells indicated by arrows).

Actin-binding proteins. The close association (colocalization) of p39 and nuclear f-actin suggested that p39 might be an f-actin-binding protein. To test this possibility, 80,000 × *g* supernatant fractions of infected (20 h p.i.) and uninfected cells were passed over f-actin or BSA affinity columns. Two proteins, 67K and 39K, were greatly concentrated on f-actin columns from infected cell extracts but not on those from uninfected cell extracts as determined by SDS-PAGE (Fig. 7a). Western blot analysis of these gels indicated that the 39K protein was p39, the major viral capsid protein. Gp64, the major Ac*M*NPV EV envelope protein, was not significantly retained on f-actin affinity columns (Fig. 7b). The 67K protein that was retained on the f-actin column from infected cell supernatants was not identified.

DISCUSSION

It has been estimated that the Ac*M*NPV genome codes for 70 to 100 protein products (11). The synthesis of these products is regulated in a cascade fashion in four separate classes; the expression of each successive class is dependent on the expression of products of the previous class. Products of the first class, or immediate-early genes, require only host cell factors for their expression. The delayed-early genes precede and are necessary for replication of the viral genome, which begins at about 6 h p.i. Expression of late genes, many of which code for viral structural proteins of both forms of Ac*M*NPV, commences only after viral DNA replication has begun. The very late genes are primarily involved in occlusion of the late form of the virus in protective polyhedral matrices of protein (11).

Several inhibitors have been used to study the synthesis of viral proteins and to assign these proteins to different temporal classes (40). Cycloheximide at a concentration of 200 μg/ml is known to inhibit both viral and host protein synthesis. Aphidicolin at a concentration of 5 μg/ml inhibits the function of viral and host DNA polymerases, thus preventing both genome replication and the replication-dependent expression of late gene products. As one means of correlating changes in actin microfilaments with events in the life cycle of Ac*M*NPV, we used cycloheximide and aphidicolin to determine which specific classes of virus genes mediated particular actin rearrangements.

Early cables (1 to 3 h p.i.). The thick microfilament cables observed in infected cells from 1 to 3 h p.i. formed without protein synthesis, indicating that a component of the viral inoculum induced this first rearrangement of microfilaments from preexisting cellular actin. In a normal infection, these cables disappeared from infected cells by 6 h p.i. If, however, the infection was carried out in the presence of cycloheximide, the cables remained at 6 h p.i., indicating that protein synthesis was necessary for their depolymerization and subsequent rearrangement. Although the actin cables seen early in Ac*M*NPV-infected cells sometimes appeared in microspikes, careful focusing up and down showed that most cables penetrated well into the body of the cell. These actin cables were different both in timing and in location from the long f-actin-containing microvilli that have been reported to occur in association with budding of progeny of several different viruses (1, 17, 22, 33, 34).

Ventral aggregates (4 to 7 h p.i.). About 6 h p.i., cells begin to round up, and viral DNA synthesis begins (52). At approximately this same time, in addition, ventral aggregates of f-actin appeared. These microfilament aggregates were sensitive to cycloheximide and insensitive to aphidicolin, indicating that an early viral gene was responsible for inducing this arrangement of actin.

The ventral aggregates appeared to be rather transient structures. They were evident in only 10 to 20% of infected

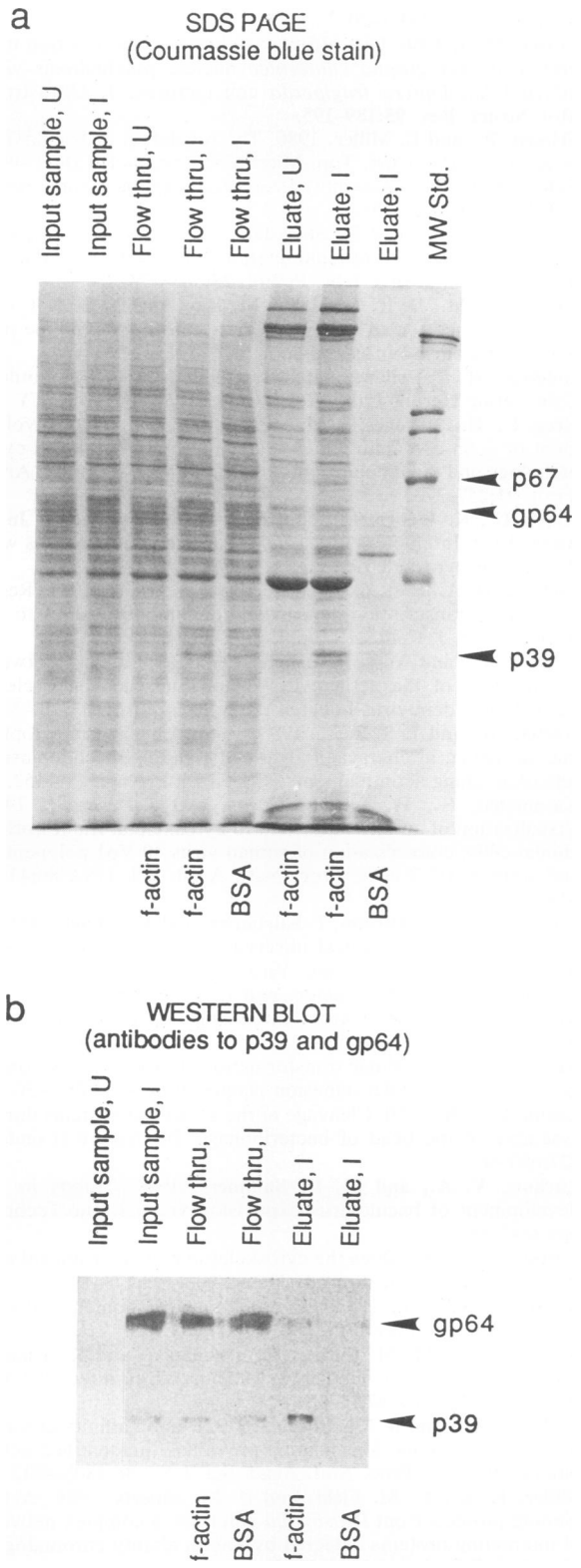

FIG. 7. Proteins from uninfected and infected (20 h p.i.) cell extracts that bound to f-actin affinity columns. (a) SDS-PAGE of proteins applied to and eluted from columns. (b) Immunological detection of proteins applied to and eluted from columns using Western blot and labeling with MAbs to p39, the major viral capsid protein and to gp64, the major envelope protein of Ac*M*NPV EV. U, Uninfected cell extract; I, infected cell extract; the type of column (f-actin or control BSA) is indicated under each lane; Molecular mass standards (MW Std.) were as follows: 200 (myosin), 116, 97.4, 66.2, and 45 kDa.

cells at any given time between 4 and 7 h p.i., and they disappeared before detectable amounts of p39 (a late protein) were synthesized (data not shown). There was no accumulation of aggregates in cells infected in the presence of aphidicolin for 20 h, indicating that viral DNA replication or late gene expression was not necessary for their depolymerization.

Rounding is a common cytopathic effect of viral infection. Rounding of Ac*M*NPV-infected Sf21 cells appears to be the result of virus-induced microtubule reorganization (58). Uninfected cells can be similarly rounded with the microtubule-depolymerizing drug colchicine. Since rounding occurred in Ac*M*NPV-infected cells at about the time that ventral microfilament aggregates were seen, we conducted a study to determine whether the appearance of aggregates was related to cell rounding. We found that uninfected, colchicine-rounded cells contained no ventral microfilament aggregates, whereas infected cells prerounded with colchicine contained ventral aggregates 4 to 7 h p.i. Apparently, microfilament aggregates were not the result of cell rounding per se but occurred as a result of infection.

Transformation of cells by many agents, including viruses, disrupts ventral adhesion plaques which contain microfilaments and many actin-binding proteins. This causes cells to lose association with the substrate and to round up. Microfilaments typically aggregate in patches (5, 6, 24). Because of the similarity in appearance of Ac*M*NPV ventral aggregates to the adhesion plaques of Rous sarcoma virus-transformed cells and because Ac*M*NPV-infected cells also underwent cell rounding and loss of adhesion at about the time of appearance of ventral aggregates, we probed cells with antibodies to vinculin, spectrin, alpha-actinin, tropomyosin, and 14 antibodies to *Drosophila* embryo actin-binding proteins (including myosin). Although many antibodies gave positive staining patterns, none localized in ventral microfilament aggregates, indicating that, at least within the limits of detection by antibody reaction, Ac*M*NPV ventral microfilament aggregates did not contain any of these actin-binding proteins. These results suggested that Ac*M*NPV-induced ventral aggregates were not analogous to adhesion plaques.

Nuclear f-actin and the scaffolding hypothesis. After ventral aggregates of f-actin disappeared, cells were relatively free of polymerized microfilaments (except at the plasma membrane) until the time of virus assembly in the nucleus several hours later. Polymerization of microfilaments in the nucleus around the virogenic stroma began at about 12 h p.i. Viral DNA replication or a late gene product was necessary for this polymerization to occur, since the process was sensitive to aphidicolin.

At 12 h p.i., when assembly of nucleocapsids was beginning, p39, the major capsid protein, localized primarily in the virogenic stroma. Only small patches of p39 colocalized with microfilaments in the nuclear ring zone at this time. The colocalization of microfilaments with p39 increased in the ring zone with increased production of EV.

A previous study showed that cytochalasin D, a fungal metabolite that interferes with the function of f-actin (4), prevents the formation of f-actin in the nucleus of Ac*M*NPV-infected cells (55). Treatment of infected cells with cytochalasin D also inhibits the production of infectious progeny

virus by interfering with nucleocapsid morphogenesis. Aberrantly long and improperly oriented tubules containing p39 fill the ring zone and are largely devoid of viral DNA. Taken together with our study, these results suggest that microfilaments may be involved in nucleocapsid morphogenesis.

Electron microscopy studies indicate that Ac*M*NPV nucleocapsids are composed of a basal structure, a cylindrical capsid sheath with nucleoprotein core, and an apical cap (10). The basal structure apparently associates with the nuclear matrix. Capsid sheaths are seen within the ring zone surrounding the virogenic stroma, where they appear to assemble on the basal structures. The apex of the capsid is oriented toward the virogenic stroma. While positioned in this manner, nucleoprotein enters the sheath through the apical cap and fills the capsid from base to apex. Biochemical and electron microscopy evidence also indicates that Ac*M*NPV capsids associate with the nuclear matrix in infected cells at 24 h p.i. (62).

In the present study, we found that p39, the major capsid protein of Ac*M*NPV, may be an f-actin-binding protein, because it was specifically retained by an f-actin affinity column. These same results were obtained when f-actin binding was assayed by direct pelleting of microfilaments (data not shown). Because of the appearance of microfilaments in the ring zone at the time of nucleocapsid assembly, their colocalization with and apparent binding of the major capsid protein, and the previously reported association of capsids with the nuclear matrix, we postulate that f-actin attaches to the nuclear matrix in infected cells, where it provides a scaffold for assembly and filling of capsids.

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