Dissecting the Cell Entry Pathway of Baculovirus by Single-Particle Tracking and Quantitative Electron Microscopic Analysis

Fujun Qin,^{a,b} Congrui Xu,^{a,b} Jia Hu,^a Chengfeng Lei,^a Zhenhua Zheng,^a Ke Peng,^a Hanzhong Wang,^a Xiulian Sun^a

^aWuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China

^bUniversity of Chinese Academy of Sciences, Beijing, China

ABSTRACT The budded virus of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) infects insect cells through mainly clathrin-mediated endocytosis. However, the cell entry pathway of AcMNPV remains unclear. In this study, by using population-based analysis of single-virus tracking and electron microscopy, we investigated the internalization, fusion behavior, and endocytic trafficking of AcMNPV. AcMNPV internalization into host insect cells was facilitated by actin polymerization and dynamin. After incorporation into early endosomes, the AcMNPV envelope fused with the membranes of early endosome, allowing for nucleocapsid release into the cytoplasm. Microtubules were implicated in the bidirectional and long-range transport of virus-containing endosomes. In addition, microtubule depolymerization reduced the motility of virus-bearing early endosomes, impairing the progression of infection beyond enlarged early endosomes. These findings demonstrated that AcMNPV internalization was facilitated by actin polymerization in a dynamin-dependent manner, and nucleocapsid release occurred in early endosomes in a microtubule-dependent manner. This study provides mechanistic and kinetic insights into AcMNPV infection and enhance our understanding of the infection pathway of baculoviruses.

IMPORTANCE Baculoviruses are used widely as environmentally benign pesticides, protein expression systems, and potential mammalian gene delivery vectors. Despite the significant application value, little is known about the cell entry and endocytic trafficking pathways of baculoviruses. In this study, we demonstrated that the alphabaculovirus AcMNPV exhibited actin- and microtubule-dependent transport for nucleocapsid release predominantly from within early endosomes. In contrast to AcMNPV transduction in mammalian cells, its infection in host insect cells is facilitated by actin polymerization for internalization and microtubules for endocytic trafficking within early endosomes, implying that AcMNPV exhibits cell type specificity in the requirement of the cytoskeleton network. In addition, experimental depolymerization of microtubules impaired the progression of infection beyond enlarged early endosomes. This is the first study that dissects the cell entry pathway of baculoviruses in host cells at the single-particle level, which advances our understanding of the early steps of baculovirus entry.

KEYWORDS AcMNPV, actin, early endosome, microtubule, nucleocapsid release

Baculoviruses are members of the family *Baculoviridae*, which is a large family of enveloped, double-stranded DNA (dsDNA) viruses that infect insects. They are used widely for recombinant protein expression and the biological control of pests and are also being developed for mammalian gene delivery (1–5). The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), a model alphabaculovirus, has been studied

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Address correspondence to Xiulian Sun, sunxl@wh.iov.cn.

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intensely (6–10). Two diverse virion phenotypes are produced during the AcMNPV infection cycle: the occlusion-derived virus and the budded virus. The cell entry of AcMNPV budded virus is mediated by the viral fusion protein GP64, which was acquired more recently during the evolution of baculoviruses (11, 12). AcMNPV budded virus enters cells primarily through clathrin-mediated endocytosis (13–15), with a small fraction entering via direct fusion with the plasma membrane (16–18).

The cell entry pathways of viruses into host cells have raised much interest (19, 20). Many viruses infect cells by clathrin-mediated endocytosis. Initially, the virus particles bind to receptors and attachment factors at the cell surface, and they are subsequently internalized into the cytoplasm via clathrin-coated pits (CCPs) (21). Following internalization into the cytoplasm, virus particles are delivered to the endocytic system, which consists of highly dynamic vesicles and endosomes. Endosome trafficking is controlled by a large family of small Rab GTPases, which are key determinants of endosome identity. The GTPases Rab5 and Rab7 are localized primarily to early and late endosomes, respectively (22, 23). Endosomes at distinct phases of the degradation pathway exhibit significant differences in endosomal acidification (24). Viruses release the viral genome or nucleocapsids in early endosomes or late endosomes depending on the endosomal acidification required for viral membrane fusion.

During clathrin-mediated endocytosis, the actin cytoskeleton is highly dynamic and manipulated by many viruses for entry and replication (25). The actin cytoskeleton is involved in two main stages of viral entry. In the first stage, actin polymerization is required for plasma membrane deformation and viral internalization. In the later stage, actin filaments are involved in delivering the virus-bearing vesicles to early endosomes. In addition to actin-based movement, microtubule-mediated transport can also be exploited by viruses to facilitate multiple steps of infection, including endocytic trafficking of virus-bearing endosomes and transport of nucleocapsids to the site of replication (26). The dependence on actin and microtubules for infection varies for different viruses and cell types.

Actin-based motility is involved in multiple steps of AcMNPV infection in host insect cells (27–30). Immediately after nucleocapsid release into the cytosol, actin-based motility is required for movement toward the nucleus and translocation through nuclear pore complexes (27). After the expression of early genes, motility is critical for viral nucleocapsid accumulation at the cell periphery for rapid transmission to adjacent cells (28). Furthermore, actin polymerization is required for virus replication in the nucleus, and Ac34 keeps the nucleation-promoting factor Arp2/3 in the nucleus by inhibiting the CRM1-dependent nuclear export pathway (29, 30). These studies have focused mainly on the infection events downstream of nucleocapsid penetration. However, the infection events that precede nucleocapsid release remain poorly understood. Little is known about the exact manner of the endocytic trafficking route of AcMNPV, the cellular compartments where viral fusion and nucleocapsid release occur, or the cytoskeleton components implicated in endocytic trafficking in host insect cells.

Here, by tracking individual AcMNPV virus particles in living cells tagged with distinct fluorescent endocytosis-relevant markers and by performing quantitative electron microscopy to assess the effect of endocytic inhibitors on infection, we investigated the cell entry pathway of AcMNPV at the single-particle level. The results revealed that AcMNPV nucleocapsid release occurred primarily from within early endosomes in an actin- and microtubule-dependent manner. In contrast to AcMNPV internalization into mammalian cells, its internalization into insect host cells was facilitated by actin polymerization in a dynamin-dependent manner. Following internalization, endosomes containing AcMNPV exhibited long-range movement suggestive of microtubule-directed transport, and finally the AcMNPV envelope and endosomal membranes fused primarily from within early endosomes to release nucleocapsids to the cytoplasm. Microtubule depolymerization reduced the motility of virus-bearing early endosomes, impairing the progression of infection beyond enlarged Rab5-positive endosomes.

RESULTS

Construction and characterization of recombinant AcMNPV and clonal cell lines. In order to investigate the cell entry pathway of AcMNPV, a recombinant, mCherry-tagged nucleocapsid virus (vAc^{VP39-mCherry}) was generated. A second copy of the *vp39* gene fused with the *mCherry* gene was inserted under the control of the viral very late gene promoter Pp10 to generate the virus (vAc^{VP39-mCherry}). Expression of the fusion protein was confirmed by Western blotting of the purified virus (vAc^{VP39-mCherry}). An immunoreactive band of VP39-mCherry (at 66 kDa) was detected with the anti-VP39 antibody (Fig. 1A) and the anti-mCherry antibody (Fig. 1B) in vAc^{VP39-mCherry} but not in the negative-control virus. The VP39-enhanced green fluorescent protein (EGFP) fusion-expressing virus (vAc^{VP39-EGFP}) and a virus expressing only EGFP (vAc^{Pp10-EGFP}) were kindly provided by Hanzhong Wang and Xinwen Chen (Wuhan Institute of Virology, Chinese Academy of Sciences, China), respectively. Previous studies showed that fluorescent protein-labeled viruses did not compromise the viral titer or functionality (28, 31). A single EGFP gene was inserted under the control of the viral very late gene promoter Pp10 to generate the recombinant virus (vAc^{Pp10-EGFP}). Since EGFP could not be incorporated into a virus particle during the assembly of progeny virus, recombinant virus vAc^{Pp10-EGFP} could act as a control virus without any labeling. The outer envelope of the virus particle can be labeled by the spontaneous incorporation of the far-red fluorescent, lipophilic tracer DiD. Recombinant viruses (vAc^{Pp10-EGFP}, vAc^{VP39-EGFP}, and vAc^{VP39-mCherry}) were then labeled with DiD, generating DiD-labeled viruses (vAc^{Pp10-EGFP-DiD}, vAc^{VP39-EGFP-DiD}, and vAc^{VP39-mCherry-DiD}). One-step growth curves for viruses vAc^{Pp10-EGFP-DiD}, vAc^{VP39-EGFP}, and vAc^{VP39-EGFP-DiD} (Fig. 1C) and viruses vAc^{VP39-mCherry} and vAc^{VP39-mCherry-DiD} (Fig. 1D) showed growth trends similar to that of the control virus (vAc^{Pp10-EGFP}), indicating that the expression of fusion proteins or the labeling procedure did not significantly affect the replication kinetics of the viruses. Subsequently, the efficiency of DiD labeling was evaluated by calculating the percentage of dually DiD- and fluorescent protein-labeled virus particles over the total fluorescent protein-labeled virus particles (Fig. 1E and F). The efficiency of DiD labeling was about 35%, which is similar to the observation for dengue virus (32).

During clathrin-mediated endocytosis, virus particles are delivered to early endosomes or late endosomes for nucleocapsid release after internalization. Actin filaments and microtubules are implicated in the endocytic trafficking of virus particles. Rab5, Rab7, mTn, and MAP4 are markers of early endosomes, late endosomes, actin filaments, and microtubules, respectively. In order to investigate the endocytic trafficking of AcMNPV, clonal cell lines that stably expressed the early endosome marker (Sf^{EGFP-Rab5}) and late endosome marker (Sf^{mCherry-Rab7}) and coexpressed the early endosome and late endosome markers (Sf^{EGFP-Rab5-mCherry-Rab7}) were generated. In addition, we also generated cell lines that stably expressed the actin filament marker (Sf^{EGFP-mTn}) and microtubule marker (Sf^{EGFP-MAP4}) to investigate the role of the cytoskeleton in AcMNPV infection. Clonal cell lines were analyzed by Western blotting to confirm expression of the fusion protein. Immunoreactive bands of EGFP-mTn (49 kDa) and EGFP-MAP4 (75 kDa) were detected with the anti-EGFP antibody in the cell lines Sf^{EGFP-mTn} and Sf^{EGFP-MAP4} (Fig. 2A). Immunoreactive bands of EGFP-Rab5 (49 kDa) were detected with the anti-EGFP antibody (Fig. 2B) or the anti-Rab5 antibody (Fig. 2C) in the cell lines Sf^{EGFP-Rab5} and Sf^{EGFP-Rab5-mCherry-Rab7}, respectively. Immunoreactive bands of mCherry-Rab7 (49 kDa) were detected with the anti-mCherry antibody (Fig. 2D) or the anti-Rab7 antibody (Fig. 2E) in the cell lines Sf^{mCherry-Rab7} and Sf^{EGFP-Rab5-mCherry-Rab7}. One-step growth curves of clonal cell lines Sf^{EGFP-Rab5}, Sf^{mCherry-Rab7}, and Sf^{EGFP-Rab5-mCherry-Rab7} (Fig. 2F) and clonal cell lines Sf^{EGFP-mTn} and Sf^{EGFP-MAP4} (Fig. 2G) showed growth trends similar to that of Sf9 cells, indicating that the expression of fusion proteins did not significantly affect the replication kinetics of the clonal lines. In the clonal cell line Sf^{EGFP-Rab5-mCherry-Rab7} coexpressing both EGFP-Rab5 and mCherry-Rab7, cells underwent a longer lag phase before cell division. This may explain why the viral replication kinetics in cells (Sf^{EGFP-Rab5-mCherry-Rab7}) were slightly delayed compared with those in the Sf9 cells. A one-step growth curve of clonal cell line Sf^{EGFP-Rab5-mCherry-Rab7} showed

FIG 1 Construction and characterization of recombinant AcMNPV. (A) Western blotting of recombinant virus vAcVP39-mCherry. A second copy of the VP39 gene fused with the mCherry gene was inserted under control of the promoter p10 to generate recombinant virus vAcVP39-mCherry. Purified budded virus of vAcPp10-EGFP and vAcVP39-mCherry was analyzed by Western blotting with an anti-VP39 antibody as the primary detection antibody. Lane 1, vAcPp10-EGFP; lane 2, vAcVP39-mCherry. (B) Western blotting of recombinant virus vAcVP39-mCherry. Purified concentrated budded virus of vAcPp10-EGFP and vAcVP39-mCherry was analyzed by Western blotting with anti-mCherry antibody as the primary detection antibody. Recombinant virus vAcVP39-mCherry was successfully constructed. Lane 1, vAcPp10-EGFP; lane 2, vAcVP39-mCherry. (C) One-step growth curves of recombinant or DiD-labeled viruses (vAcPp10-EGFP, vAcPp10-EGFP-DiD, vAcVP39-EGFP, and vAcVP39-EGFP-DiD). The envelopes of the recombinant viruses vAcVP39-EGFP and vAcVP39-mCherry were labeled with the far-red lipophilic fluorescent tracer DiD. The expression of fusion protein or labeling with DiD had no significant impact on the replication kinetics of the viruses. The experiments were performed in

(Continued on next page)

growth trends similar to those of the Sf9 cells, indicating that the expression of fusion proteins did not significantly affect the replication kinetics of the clonal lines.

AcMNPV is internalized in an actin- and dynamin-dependent manner. Actin-based motility is involved in multiple steps in the process of AcMNPV infection and replication (27–30). Previous studies investigated mainly the events downstream of nucleocapsid penetration. However, whether actin-based motility or dynamin is required for the internalization that precedes nucleocapsid release remains poorly understood.

To determine the role of actin polymerization in the uptake of virus into the cytoplasm, the capsid-labeled recombinant virus vAc^{VP39-mCherry} and the clonal cell line (SfEGFP-Rab5) stably expressing EGFP-Rab5 were employed. In our experiments, EGFP-Rab5 was expressed at appropriate levels such that enlarged early endosomes and redistribution to the perinuclear region caused by overexpression of Rab5 were not observed (Fig. 3A, control). Cells were pretreated with cytochalasin D for 10 min to inhibit actin polymerization while not disrupting the actin cytoskeleton. Cells (SfEGFP-Rab5, green) were incubated with vAc^{VP39-mCherry} (red) in the presence or absence of nocodazole, cytochalasin D, or dynasore, and then single AcMNPV particles were tracked (Fig. 3A). The virus particles that colocalized with Rab5-positive endosomes (yellow) are marked by arrows. When cells were treated with nocodazole, the majority of virus particles were internalized and colocalized with early endosomes, which was similar to the case for the untreated cells. In contrast, when cells were treated with cytochalasin D and dynasore, most virus particles were blocked at the cell surface and were not colocalized with the early endosomes.

A quantitative analysis revealed that in untreated cells, most of the vAc^{VP39-mCherry} virus particles (82%) were internalized into the cytoplasm after binding to the cell surface (Fig. 3B). Similarly, microtubule depolymerization with nocodazole did not have an obvious effect on internalization (Fig. 3B), indicating that microtubules are not required for virus internalization. In contrast, inhibition of dynamin with dynasore and inhibition of actin polymerization with cytochalasin D impaired the internalization at the cell surface (Fig. 3B). Similarly, inhibition of dynamin or actin polymerization also remarkably impaired the subsequent delivery of the viruses to Rab5-positive endosomes (Fig. 3C), further supporting the observation that actin polymerization was required for internalization. We next used flow cytometric analysis to investigate if inhibiting dynamin by treating cells with dynasore affected the viral infectivity. The results showed that dynasore inhibited the viral infectivity in a dose-dependent manner, supporting that dynamin was required for viral internalization (Fig. 3D).

To investigate the viral infection at an ultrastructural level, quantitative electron microscopy of viral infection was performed. Three different stages of viral infection in untreated and inhibitor-treated cells were analyzed: internalization (attachment to the plasma membrane and invagination) (Fig. 4A and B), trafficking in vesicles (single or multiple virus particles) (Fig. 4C to H), and nucleocapsid in the cytosol or nucleus (Fig. 4I to L). These results revealed that the percentage of virus particles at the cell surface was markedly increased from 21% in untreated cells to 82% in the cytochalasin D-treated cells (Fig. 5A). Accordingly, the percentages of virus particles for trafficking in vesicles and in the cytosol or nucleus were markedly decreased in the cytochalasin D-treated cells (Fig. 5B and C). The majority of AcMNPV particles were observed to be

FIG 1 Legend (Continued)

triplicate (error bars indicate standard deviations [SD]). (D) One-step growth curves of recombinant or DiD-labeled viruses (vAc^{Pp10-EGFP}, vAc^{VP39-mCherry}, and vAc^{VP39-mCherry-DiD}). The envelope of the recombinant virus vAc^{VP39-mCherry} was labeled with the far-red lipophilic fluorescent tracer DiD. The expression of fusion protein or labeling with DiD had no significant impact on the replication kinetics of the viruses. The experiments were performed in triplicate (error bars indicate SD). (E) Efficiency of DiD labeling. Fluorescent protein (FP)-labeled viruses vAc^{VP39-mCherry} and vAc^{VP39-EGFP} were labeled with DiD. The efficiency of DiD labeling was evaluated by calculating the percentage of dually DiD- and FP-labeled virus particles (vAc^{VP39-EGFP-DiD} and vAc^{VP39-mCherry-DiD}) over the total FP-labeled virus particles (vAc^{VP39-EGFP} and vAc^{VP39-mCherry}). DiD-labeled AcMNPV was added to a glass coverslip, and the numbers of fluorescent spots were counted in five random images in which each FP-labeled spot was taken as one virus particle as previously described (32). Three independent experiments were performed (error bars indicate SD). (F) Representative images of the colocalization of dually DiD- and FP-labeled virus particles. Fluorescent protein (FP)-labeled viruses vAc^{VP39-mCherry} and vAc^{VP39-EGFP} were labeled with DiD, resulting in dually DiD- and FP-labeled virus particles vAc^{VP39-EGFP-DiD} and vAc^{VP39-mCherry-DiD}, respectively. Dually DiD- and FP-labeled viruses were imaged by time-lapse confocal microscopy. Scale bar, 2 μ m.

FIG 2 Construction and characterization of clonal cell lines that expressed various fluorescent cellular markers. (A) Cell lines Sf^{EGFP-mTn} and Sf^{EGFP-MAP4} were analyzed by Western blotting. The cell lines were analyzed with an anti-EGFP antibody as the primary detection antibody. Lane 1, marker; lane 2, Sf^{EGFP-mTn}; lane 3, Sf^{EGFP-MAP4}. (B) Cell lines Sf^{EGFP-Rab5} and Sf^{EGFP-Rab5-mCherry-Rab7} were analyzed by Western blotting with an anti-EGFP antibody. The cell lines were analyzed with an anti-EGFP antibody as the primary detection antibody. Lane 1, Sf9; lane 2, Sf^{EGFP-Rab5}; lane 3, Sf^{EGFP-Rab5-mCherry-Rab7}. (C) Cell lines Sf^{EGFP-Rab5} and Sf^{EGFP-Rab5-mCherry-Rab7} were analyzed by Western blotting with an anti-Rab5 antibody. The cell lines were analyzed with an anti-Rab5 antibody as the primary detection antibody. Lane 1, Sf9; lane 2, Sf^{EGFP-Rab5}; lane 3, Sf^{EGFP-Rab5-mCherry-Rab7}. (D) Cell lines Sf^{mCherry-Rab7} and Sf^{EGFP-Rab5-mCherry-Rab7} were analyzed by Western blotting with an anti-mCherry antibody. The cell lines were analyzed with an anti-mCherry antibody as the primary detection antibody. Lane 1, Sf9; lane 2, Sf^{mCherry-Rab7}; lane 3, Sf^{EGFP-Rab5-mCherry-Rab7}. (E) Cell lines Sf^{mCherry-Rab7} and Sf^{EGFP-Rab5-mCherry-Rab7} were analyzed by Western blotting with an anti-Rab7 antibody. The cell lines were analyzed with an anti-Rab7 antibody as the primary detection antibody. Lane 1, Sf9; lane 2, Sf^{mCherry-Rab7}; lane 3, Sf^{EGFP-Rab5-mCherry-Rab7}. (F) One-step growth curves of Sf9 cell lines Sf^{EGFP-Rab5}, Sf^{mCherry-Rab7}, and Sf^{EGFP-Rab5-mCherry-Rab7} infected with the control virus (vAc^{Pp10}-EGFP). The expression of fluorescent cellular markers had no significant impact on the replication kinetics of the control virus (vAc^{Pp10}-EGFP). The experiments were performed in triplicate (error bars indicate SD). (G) One-step growth curves of Sf9 cell lines Sf^{EGFP-mTn} and Sf^{EGFP-MAP4} infected with the control virus (vAc^{Pp10}-EGFP). The expression of fluorescent cellular markers had no significant impact on the replication kinetics of the control virus (vAc^{Pp10}-EGFP). The experiments were performed in triplicate (error bars indicate SD).

FIG 3 AcMNPV is internalized in an actin- and dynamin-dependent manner. (A) Representative images of EGFP-Rab5 endosomes (green) and virus particles (red) in live untreated cells, nocodazole-treated cells, dynasore-treated cells, and cytochalasin D-treated cells. The cells ($S^{\text{EGFP-Rab5}}$) were pretreated with the indicated inhibitors and then incubated with vAc $_{\text{VP39-mCherry}}$ (MOI = 50) at 4°C as previously described (86). The temperature was raised to 27°C, and the cells were imaged by time-lapse confocal microscopy. Images were captured as snapshots 20 min after infection. The virus particles that colocalized with Rab5-positive endosomes (yellow) are marked by arrows. Scale bars, 5 μm. (B and C) Quantification of the percentage of AcMNPV internalization into the cytoplasm (B) or delivery into EGFP-Rab5 endosomes (C) in the absence or presence of nocodazole, cytochalasin D, or dynasore. Individual virus particles were observed for 20 min upon binding to the cell surface. Virus particles that bound to the cell surface motionlessly or moved rapidly in a diffusive manner were considered to represent invalid internalization. Virus particles that colocalized with the EGFP-Rab5 endosomes for more than 2 min were defined as having efficient delivery to EGFP-Rab5 endosomes (***, $P < 0.001$ by the Pearson χ^2 test). (D) AcMNPV infectivity in the presence of the indicated concentrations of dynasore by flow cytometry analysis. Viral infectivity was measured at 30 hpi by analyzing the percentage of cells expressing the reporter EGFP gene under the control of the viral very late gene promoter Pp10. The experiment was performed in triplicate (means with 95% confidence intervals [CI] are shown; **, $P < 0.01$; ***, $P < 0.001$ [by LSD t tests post- F test]).

trapped at the cell surface and partially invaginated (Fig. 4B), consistent with previous observations for vesicular stomatitis virus (VSV) (33). In addition, quantitative electron microscopy revealed that the percentage of virus particles located at the cytomembrane had increased to 85% in the presence of dynasore (Fig. 5A). Accordingly, the percentages of virus particles for trafficking in vesicles and in the cytosol or nucleus were markedly decreased in the dynasore-treated cells (Fig. 5B and C). The result indicated that dynamin was required for AcMNPV internalization, which was consistent with the observation that dynamin is involved in both the maturation and scission of clathrin-coated pits (34, 35).

AcMNPV fuses primarily from within early endosomes. Following their internalization into the cytoplasm, virus particles are delivered to the highly dynamic endocytic network, which is regulated by a large family of small Rab GTPases. The GTPases Rab5 and Rab7 are localized primarily to early and late endosomes, respectively (22, 23), and only a small proportion of endosomes, which are decorated by both Rab5 and Rab7, are intermediate endosomes (36, 37).

FIG 4 Electron micrographic analysis of AcMNPV entry into Sf9 cells. After prebinding to the cell surface at 4°C, AcMNPV virus particles were incubated with cells for 1 h. Cells were then fixed and processed for electron microscopy. Intact AcMNPV particles were delineated with an obvious envelope, and were distributed mainly at the cell surface or in vesicles. In contrast, unenveloped nucleocapsids were without a visible envelope and were located mainly in the cytosol or nucleus (16). Although the distribution of AcMNPV virus particles differed in untreated and inhibitor-treated cells, the images of distinct stages of AcMNPV infection were similar. Representative images of distinct stages of AcMNPV infection in untreated cells were shown. (A) An enveloped virus particle bound to the cell surface. The black arrow points to a virus particle. (B) An enveloped virus particle that was partially invaginated into the cell membrane. Boxed regions of the virus particle are magnified and shown in the inset. The empty arrowhead points to the nuclear membrane. (C) An enveloped virus particle that was completely invaginated into the cell membrane. The black arrow points to the virus particle. (D) A nascent virus-bearing vesicle that was formed in the extreme periphery of the cell. (E) Adjacent virus-bearing vesicles that presumably would fuse with each other. (F) An enlarged vesicle containing multiple virus particles near the nucleus, indicating that virus-bearing endosomes had undergone fusion events. (G) An enveloped virus particle that was partially fused with a vesicle, resulting in the formation of a hemifusion intermediate. (H) Complete fusion between the viral envelope with endosomal membranes, allowing the onset of nucleocapsid release into the cytoplasm. The boxed region is shown in the inset. (I) A nucleocapsid located in the vicinity of a vesicle, with one apical end of the nucleocapsid still connected with the endosomes, indicating that the release of the nucleocapsid has just been achieved. (J) Nucleocapsid alignment along filament-like structures. Stars mark the filaments. (K) A nucleocapsid associated with the extreme periphery of the nucleus, facilitating the subsequent viral nuclear entry. (L) A nucleocapsid located in the nucleus, indicating that nucleocapsid translocation to the nucleus has been completed. Scale bars, 0.5 μ m.

FIG 5 Quantitative electron microscopic analysis of AcMNPV entry into Sf9 cells. (A) Quantitative electron microscopic analysis of the fraction of virus particles for internalization. Sf9 cells were infected with AcMNPV in the presence or absence of different inhibitors, including chlorpromazine, cytochalasin D, dynasore, nocodazole, and bafilomycin A1. Each value represents a quantitative analysis of about 200 cell sections for each treatment. All the virus particles observed, including enveloped viruses and nucleocapsids, were recorded for analysis. The fraction of virus particles for internalization over the total virus particles was plotted. The number of the virus particles indicated in the figure represents pooled virus particles from 200 cell sections (***, $P < 0.001$ by a Pearson χ^2 test). (B) Quantitative electron microscopic analysis of the fraction of virus particles in vesicles. The fraction of virus particles in vesicles over the total virus particles was plotted (***, $P < 0.001$ by a Pearson χ^2 test). (C) Quantitative electron microscopic analysis of the fraction of virus particles in the cytosol and nucleus. The fraction of nucleocapsids in the cytosol and nucleus over the total virus particles was plotted (***, $P < 0.001$ by a Pearson χ^2 test).

To identify the role of Rab5 and Rab7 in AcMNPV entry, we performed single-particle tracking in the cell line Sf^{EGFP-Rab5-mCherry-Rab7}, stably coexpressing EGFP-Rab5 and mCherry-Rab7. The fusion of the virus particles with the endosomes was detected by a sudden increase of DiD fluorescence due to the dilution of DiD from the viral envelope into the membranes of the endosome (38). Here, we used the DiD-labeled virus vAc^{Pp10}-EGFP-DiD and Sf^{EGFP-Rab5-mCherry-Rab7} cells for live-cell imaging (Fig. 6A). Both Rab5- and Rab7-positive endosomes were visible as discrete punctate structures of fluorescence signals, and the Rab7-positive endosomes were usually larger than the Rab5-positive endosomes. A representative example of viral fusion in an early endosome is depicted in Fig. 6B to D and in Movies S1 and S2 in the supplemental material. Selected frames from boxed regions of a virus particle in a cell are magnified and shown on the right (Fig. 6B). The viral membrane fusion is marked by arrows. A sudden increase in the amount of DiD fluorescence was detected in an early endosome which is EGFP-Rab5 positive but lacks mCherry-Rab7 signal (Fig. 6C and D).

A quantitative analysis of viral fusion revealed that the majority (78%) of the viral particles induced membrane fusion from within Rab5-positive early endosomes devoid of Rab7 signal, while 8% of virus particles fused from within intermediate endosomes, which were identified by the colocalization of Rab5 and Rab7. Only 14% of the viral fusions occurred in Rab7-positive late endosomes without Rab5 signal (Fig. 6E). The results suggest that the AcMNPV envelope fuses with the membranes of primarily early endosomes.

We next employed dominant negative (DN) mutants to investigate which endosomes were required for AcMNPV infection. The DN mutants Rab5(S43N) and Rab7(T22N) were generated by mutating the same conserved amino acids as reported previously (39, 40). Compared with the wild type, both EGFP-Rab5(S43N) and mCherry-Rab7(T22N) endosomes displayed a more dispersed localization pattern and fewer punctate structures (see Fig. S1 in the supplemental material), which was consistent with previously observed DN mutants of Rab5 and Rab7 (41). Rab5 dominant negative overexpression remarkably reduced the infectivity, while Rab7 mutant overexpression did not impair infection as drastically as that of Rab5 (Fig. 6F). The result was consistent with the observation by the single-particle tracking that AcMNPV fused predominantly from within early endosomes, with a small fraction fusing from within intermediate endosomes and late endosomes. Collectively, these data indicate that AcMNPV nucleocapsid release into the cytosol occurred predominantly within early endosomes.

FIG 6 AcMNPV fuses primarily from within early endosomes. (A) Selected frames from a representative fluorescence image of cells coexpressing EGFP-Rab5 (green) and mCherry-Rab7 (red) infected with envelope-labeled (DiD, blue) virus particles. Rab5-positive (Continued on next page)

AcMNPV nucleocapsid is released primarily from within early endosomes. Fusion of the viral envelope with endosomal membranes and nucleocapsid release are typically two sequential, rather than concomitant, steps of the endocytic pathway (42). Although the quantitative analysis of the membrane fusion events showed that AcMNPV fused primarily from within Rab5-positive early endosomes, the identity of endosomes in which nucleocapsid release occurred remained uncertain. To address this issue, the cells expressing early endosomes (SfEGFP-Rab5) and late endosomes (SfmCherry-Rab7) were infected with the capsid-labeled recombinant viruses vAc^{VP39-mCherry} and vAc^{VP39-EGFP}, respectively. As shown in images of cells (SfEGFP-Rab5) infected with capsid-labeled vAc^{VP39-mCherry} and cells (SfmCherry-Rab7) infected with capsid-labeled vAc^{VP39-EGFP} at a series of different time postinfection in Fig. 7A, while most AcMNPV particles colocalized with the Rab5-positive endosomes, few virus particles were found to colocalize with Rab7-positive endosomes. A quantitative analysis revealed that 86% of the vAc^{VP39-mCherry} virus particles were delivered to the Rab5-positive endosomes in the clonal cell line SfEGFP-Rab5 (Fig. 7B). In contrast, only 16% of the vAc^{VP39-EGFP} virus particles were transported to the Rab7-positive endosomes in the clonal cell line SfmCherry-Rab7 (Fig. 7B), indicating that most virus particles had released their nucleocapsids into the cytosol from within early endosomes. The results suggest that AcMNPV particles are trafficked primarily into Rab5-positive endosomes rather than Rab7-positive endosomes.

To directly examine the nucleocapsid release of individual viruses within endosomes, dually envelope- and capsid-labeled virus (vAc^{VP39-EGFP-DiD} and vAc^{VP39-mCherry-DiD}) particles were employed. The dually labeled virus allowed the detection of nucleocapsid release when the DiD-labeled envelope and fluorescent protein-labeled capsid were observed to separate from each other, leaving the DiD-labeled envelope retained within the endosome. A quantitative analysis revealed that 83% of the vAc^{VP39-mCherry-DiD} virus particles were observed to undergo a separation of envelope and capsid in Rab5-positive endosomes. In contrast, only 27% of the vAc^{VP39-EGFP-DiD} virus particles were observed to undergo a separation of envelope and capsid in Rab7-positive endosomes (Fig. 7C). A representative example of the separation of viral envelope and nucleocapsid from within EGFP-Rab5 endosomes is shown in Fig. 7D and in Movie S3 in the supplemental material. These data indicate that AcMNPV infects cells mainly via Rab5-positive endosomes rather than Rab7-positive endosomes, which is consistent with the results of membrane fusion assays.

Microtubules are required for the transport of virus-bearing early endosomes. In host insect cells, the role of microtubule networks in AcMNPV transport remains unclear. A range of nocodazole concentrations were previously employed without obvious toxic effects on Sf9 cells (43). Here, compared with the untreated control, inhibition assays revealed that nocodazole inhibited the viral infectivity significantly, indicating that microtubule networks are required for virus transport to the nucleus

FIG 6 Legend (Continued)

endosomes devoid of any detectable Rab7 signal are early endosomes, whereas Rab7-positive endosomes that lack Rab7 are late endosomes. Rab5/Rab7-copositive endosomes are intermediate endosomes. Scale bar, 10 μ m. (B) Selected frames from a single AcMNPV particle (blue) during its fusion within an early endosome. Boxed regions of a virus particle in a cell are magnified and shown on the right. A sudden increase in the amount of DiD fluorescence was detected, indicating the occurrence of fusion between the virus and endosome membranes. The viral membrane fusion is marked by arrows. Scale bar, 5 μ m. (C) Time trajectories of the DiD fluorescence intensity of the virus shown. The fusion of viruses with endosomes was indicated by a sudden increase in the amount of DiD fluorescence to the dilution of the DiD from the viral envelope into the endosomal membranes. The viral membrane fusion is marked by an arrow. (D) Time trajectories of the EGFP-Rab5 and mCherry-Rab7 fluorescence intensity of the virus shown. While the intensity of the EGFP-Rab5 fluorescence signal was very high, the intensity of the mCherry-Rab7 fluorescence signal was relative low, approximately that of background. The viral membrane fusion is marked by arrows. (E) Quantitative analysis of the proportion of membrane fusion events in the different endocytic endosomes. The “n” represents the number of independent pooled membrane fusion events from 12 experiments. (F) Effects of DN mutants of Rab5 (Rab5S43N) or Rab7 (Rab7T22N) on the infectivity of AcMNPV in Sf9 cell lines. At 30 hpi, vAc^{VP39-EGFP} and vAc^{VP39-mCherry} were used to separately infect Sf9 cells that had been transfected with Rab7-DN or Rab5-DN mutants (MOI = 5). Viral infectivity was measured at 30 hpi by analyzing the percentage of cells expressing the EGFP reporter gene under the control of the promoter Pp10. The results are expressed as the percentage of infectivity in transfected cells. The experiment was performed in triplicate (means with 95% CI are shown; *, $P < 0.05$; ***, $P < 0.001$ [by a two-tailed Student t test]).

FIG 7 Nucleocapsid release occurs primarily from within Rab5-Positive endosomes. (A) Selected frames from cells (Sf^{EGFP-Rab5}) infected with capsid-labeled vAc^{VP39-mCherry} and cells (Sf^{mCherry-Rab7}) infected with capsid-labeled vAc^{VP39-EGFP} at 5, 15, 30, 45, and 60 min after infection. The virus (Continued on next page)

(Fig. 8A). Notably, this was not due to microtubules playing a role in the transport of cytosolic nucleocapsids, because numerous studies have shown that actin polymerization is responsible for delivering nucleocapsids toward the nuclear periphery and transit through nuclear pore complexes (27–29).

Although the inhibition assay suggested that microtubules are involved in viral infection, the exact stage of infection in which the microtubules play a role remained unclear. Given that the internalization and the transport of nucleocapsids toward or within the nucleus were all previously shown to be dependent on actin-based motility, the transport of virus-bearing endosomes prior to nucleocapsid release was assumed to depend on microtubules.

Early endosomes usually undergo fusion and fission events, resulting in fewer and larger early endosomes before maturation into late endosomes (44). When cells expressing early endosomes (SfEGFP-Rab5) were infected with capsid-labeled virus vAc^{VP39-mCherry}, virus-carrying Rab5-positive endosomes were observed to undergo fusions (Fig. 8B and C). At first, several viruses were separately transported within the Rab5-positive endosomes, and virus-bearing Rab5-positive endosomes were observed to move in a bidirectional and intermittent manner. Later, two viruses bearing Rab5-positive endosomes were fused, resulting in a larger Rab5-positive endosome (see Movie S4 in the supplemental material). The virus particles typically exhibited two types of movements, kinetically characteristic of microtubule- and actin-directed transport. For human immunodeficiency virus type 1 (HIV-1), microtubule- and actin-dependent movements are represented by gating maximal velocity either at 0 to 0.05 $\mu\text{m/s}$ for actin-directed movements or at 0.05 to 1 $\mu\text{m/s}$ for microtubule-like movements (45). Analysis of the trajectory indicated that the velocity of virus-bearing endosomes approached 0.5 $\mu\text{m/s}$, obviously suggestive of microtubule-dependent movements (Fig. 8B). These results indicate that virus-bearing endosomes undergo fusion in a microtubule-dependent manner.

The implication of microtubule-based viral transport, deduced on the basis of a kinetic analysis of fusions between virus-bearing endosomes, was further supported by single-virus tracking along microtubules. To visualize virus transport along microtubules in host cells, the cell lines expressing actin filaments (SfEGFP-mTn) and microtubules (SfEGFP-MAP4) were infected with AcMNPV (vAc^{VP39-mCherry}). The virus particle alignment along actin filaments was observed to be present in the cell periphery. In contrast, the virus particle alignment along microtubules was present in the center of cell, indicating that the virus particles have probably been delivered into the endosomes (Fig. 8D).

In addition, mCherry-labeled virus particles (vAc^{VP39-mCherry}) were observed to co-localize with and move along microtubules for an extended period (Fig. 8E; see Movie S5 in the supplemental material). The maximum velocity of the virus was roughly 0.8 $\mu\text{m/s}$ (Fig. 8F, left panel), which was consistent with the previously reported kinetics of microtubule-dependent transport of influenza virus (38, 45). To further reveal the mode of transport along the microtubules, the mean square displacement (MSD) of virus particles was plotted as a function of time. The superlinear relationship between MSD and time (t) in the low- Δt range indicated that the movement during this period

FIG 7 Legend (Continued)

particles that colocalized with endosomes are marked by arrows. Scale bar, 5 μm . (B) Quantitative analyses of the percentage of vAc^{VP39-mCherry} virus particles delivered to the Rab5-positive endosomes in the cell line SfEGFP-Rab5 and of vAc^{VP39-EGFP} virus particles delivered to Rab7-positive endosomes in the cell line Sf^{mCherry-Rab7}. The number of the virus particles indicated in the figure represents pooled virus particles from 5 experiments (***, $P < 0.001$ by a Pearson χ^2 test). (C) Quantitative analysis of the percentage of vAc^{VP39-mCherry-DiD} or vAc^{VP39-EGFP-DiD} virus nucleocapsid release in cell line SfEGFP-Rab5 or Sf^{mCherry-Rab7}, respectively. The dually labeled virus allowed the detection of nucleocapsid release when a DiD-labeled envelope and fluorescent protein-labeled capsid were observed to separate from each other, leaving the DiD-labeled envelope retained within the endosome. The number of virus particles indicated in the figure represents pooled virus particles from 5 experiments (***, $P < 0.001$ by a Pearson χ^2 test). (D) A representative example of the separation of viral envelope and nucleocapsid from within EGFP-Rab5 endosomes. Left panel, snapshot of a cell (SfEGFP-Rab5) infected with dually labeled vAc^{VP39-mCherry-DiD}. Boxed regions of virus particle are magnified and shown on the right. Scale bar, 5 μm . Right panel, Selected frames from a dually labeled virus particle nucleocapsid release from EGFP-Rab5 endosomes. The envelope was labeled with DiD, and the nucleocapsid was labeled with mCherry. When the dually labeled virus vAc^{VP39-mCherry-DiD} released nucleocapsids from within EGFP-positive early endosomes, the mCherry-labeled nucleocapsids separated from the DiD-labeled envelope, leaving the envelope retained within the EGFP-positive early endosomes.

FIG 8 Virus-bearing early endosomes move along the microtubule in a bidirectional and intermittent manner. (A) Flow cytometry analysis of AcMNPV infectivity in the presence of different concentrations of
(Continued on next page)

is directed, while the fluctuant relationship between MSD and time in the high- Δt range suggested that the movement during this period is directional and intermittent (Fig. 8F, right panel). In this phase of infection, the viruses moved along the microtubules in a bidirectional and intermittent manner and were observed to be transported backwards and forwards along the same microtubule, clearly suggesting that the movement was directed. The results indicate that the transport of virus-bearing vesicles is bidirectional and intermittent on the microtubules.

To investigate the role of microtubules at an ultrastructural level, quantitative electron microscopic analyses of AcMNPV infection were performed. Microtubule depolymerization with nocodazole significantly increased the proportion of viruses within vesicles (Fig. 5B), but it also clearly reduced the percentage of nucleocapsids within the cytosol and nucleus (Fig. 5C). Microtubule depolymerization resulted in an accumulation of virus particles in vesicles (Fig. 4F), indicating that nucleocapsid release was blocked. Similarly, inhibition of endosomal acidification with bafilomycin A1 also blocked the transport of nucleocapsids out of enlarged vesicles (Fig. 5B and C). These results suggest that microtubules may be involved in the trafficking of virus-bearing endosomes.

Microtubule depolymerization impairs the progression of infection beyond enlarged early endosomes by reducing motility. Next, the mechanism by which microtubules facilitate AcMNPV infection was investigated. Data from randomly selected cells were pooled to quantitatively assess the motility of virus-bearing Rab5-positive endosomes. Figure 9A showed examples of virus-bearing Rab5-positive endosomes in live control, nocodazole-treated, or cytochalasin-D-treated cells, corresponding to Movies S6 to S9 in the supplemental material. Compared with untreated cells, several distinct parameters were affected in the inhibitor-treated cells. First, the average velocity of virus-bearing endosomes in nocodazole-treated cells was markedly decreased to $0.06 \mu\text{m/s}$ (Fig. 9B). Second, in comparison with the average track length of $1.01 \mu\text{m}$ in control cells, it decreased to $0.63 \mu\text{m}$ in nocodazole-treated cells (Fig. 9C). Third, microtubule depolymerization also decreased the number of motile virus-bearing endosomes by 57% (Fig. 9D). Since nocodazole is a reversible drug causing microtubule depolymerization, removing the nocodazole results in the reconstruction of microtubules. When nocodazole was washed out, both the velocity and motility of virus-bearing early endosomes were restored to those of the control group to some degree (Fig. 9B to D). In contrast, when actin was disrupted with cytochalasin D, the velocity and motility of virus-bearing early endosomes were not impaired but rather were stimulated (Fig. 9B and C), consistent with previous observations for early endosomes devoid of virus particles (46, 47). Together, these results suggest that microtubules facilitate the virus transport within early endosomes.

To determine the role of microtubules in nucleocapsid release from within early endosomes, we investigated whether microtubule depolymerization affected the virus escape from Rab5-positive endosomes. In contrast with depolymerization of actin filaments, which was implicated in AcMNPV internalization, microtubule depolymeriza-

FIG 8 Legend (Continued)

nocodazole. Cells were pretreated with the indicated concentrations of nocodazole for 1 h and then infected with vAcPp10-EGFP (MOI = 5). Viral infectivity was measured at 30 hpi by analyzing the percentage of cells expressing the EGFP reporter gene under the control of the promoter Pp10. The experiment was performed in triplicate (means with 95% CI are shown; ***, $P < 0.001$ [by LSD t tests post- F test]). (B) Time trajectories of the velocity of the virus shown in panel C. (C) Selected frames from virus-carrying Rab5-positive endosomes undergoing fusions in the cell line SfEGFP-Rab5 (green). Cells expressing early endosomes (SfEGFP-Rab5) were infected with capsid-labeled virus vAcVP39-mCherry at an MOI of 50. (D) Representative images of virus particle alignment along actin and tubulin filaments. Cell lines expressing actin filaments (SfEGFP-mTn) and microtubules (SfEGFP-MAP4) were infected with AcMNPV (vAcVP39-mCherry, red). The virus particles that colocalized with actin filaments or microtubules are marked by arrows. Scale bar, 5 μm . (E) Selected frames from a single AcMNPV particle (vAcVP39-mCherry, red) moving along microtubules in the cell line SfEGFP-MAP4 (green). The virus particle is indicated with an arrow. Scale bar, 5 μm . (F) Time trajectories of the velocity (left) and MSD (right) of the virus shown in panel E.

FIG 9 Microtubule depolymerization impairs the progression of infection beyond early endosomes by reducing motility of endosomes. (A) Representative images of virus-bearing EGFP-Rab5 endosome tracks and displacement (white arrows) in live untreated control cells, cytochalasin-D-treated cells, nocodazole-treated cells, and nocodazole-washed-out cells infected with vAcVP39-mCherry (red). To avoid the effect of cytochalasin D on AcMNPV internalization, virus particles were incubated with cells for 10 min to allow internalization before addition of cytochalasin D. The drug was present throughout the experiment. The color of the trajectory corresponds to the time-color scale with a time axis from 0 s (blue) to 30 s (red). Movement of the Rab5-positive endosomes was analyzed using Imaris. The tracking images correspond to Movies S6 to S9 in the supplemental material, respectively. Scale bars, 5 μm . (B and C) Box plots of the average track velocity (B) and track displacement length (C) of virus-bearing EGFP-Rab5 endosomes in live cells treated as described above. The “n” represents the number of independent pooled movies. The box plot shows the median (line) and first and 99th (crosses) and 25th and 75th (boxes) percentiles of vesicle velocity or track displacement length. The average track velocity and track displacement length of moving virus-bearing endosomes were calculated using Imaris (**, $P < 0.01$; ***, $P < 0.001$ [by LSD t tests post- F test]). (D) Histogram representing the percentage of motile virus-bearing endosomes out of the total endosomes. Virus-bearing endosomes whose track length exceeded 3 μm within 30 s were defined as motile endosomes. The number of virus particles indicated in the figure represents pooled virus trajectories from 10 experiments (***, $P < 0.001$ by a Pearson χ^2 test). (E) Quantitative analysis of the percentage of individual virus particles trapped in Rab5-positive endosomes within 10 min in the cell line SfEGFP-Rab5 in the absence or presence of nocodazole. The number of virus particles indicated in the figure represents pooled virus particles from 10 experiments (***, $P < 0.001$ by a Pearson χ^2 test).

tion by treatment with nocodazole did not affect either the internalization or delivery to early endosomes (Fig. 3B and C). However, microtubule depolymerization resulted in the retention of virus particles (55%), impairing the progression of infection beyond the EGFP-Rab5 endosomes (Fig. 9E, nocodazole). Accordingly, washing out nocodazole restored AcMNPV infection to control levels, suggesting that the infection by AcMNPV trapped in the EGFP-Rab5 endosomes resumed after the reconstruction of microtubules (Fig. 9E). In untreated cells, a minority of virus particles (32%) were trapped in EGFP-Rab5-positive endosomes after their entry into endosomes. Consistently, in contrast with untreated cells, nucleocapsid release was markedly reduced from 30% to 9% by nocodazole based on observations from quantitative electron microscopy of viral infection, supporting the view that microtubules are involved in the nucleocapsid release of AcMNPV (Fig. 5C). In addition, the majority of virus particles were trapped in enlarged vesicles (Fig. 4E and F), indicating that virus-bearing endosomes had undergone fusions, consistent with the observations from single-particle tracking described above. Together, these experiments show that microtubule depolymerization not only reduced the velocity and track length of virus-bearing endosomes but also decreased the number of mobile endosomes, which impaired the progression of infection beyond Rab5-positive endosomes.

DISCUSSION

Despite its significance, the cell entry pathway of AcMNPV remains largely uncharacterized. In this study, using time-resolved tracking and population-based analysis of individual virus particles, we demonstrated that, following actin-dependent internalization, AcMNPV virus particles release nucleocapsids primarily from within early endosomes in a microtubule-dependent manner. Microtubule depolymerization not only reduced the velocity and track length of virus-bearing early endosomes but also decreased the proportion of motile endosomes, blocking infection by trapping viruses in early endosomes.

Actin polymerization is required for AcMNPV internalization. Internalization is the initial stage of viral entry, which allows the delivery of virus particles into the cytoplasm. The requirement for actin polymerization in virus internalization varies for different viruses and cell types. Many viruses rely on actin polymerization for internalization, including vesicular stomatitis virus, simian virus 40, and West Nile virus (34, 48, 49). In contrast, several other viruses are internalized into the cytoplasm independently of actin assembly, such as Borna disease virus and avian reovirus (50, 51). When AcMNPV transduces mammalian cells, actin-based motility is dispensable for both its internalization and nucleocapsid release (52). Here, we demonstrated that, in contrast with its dispensability in mammalian cells, actin polymerization was required for AcMNPV internalization in host insect cells. These results indicate that the source of the cells (mammalian versus insect) plays a key role in determining whether actin-based motility is required during virus entry, consistent with a previous observation influenza virus. While influenza virus relies on both actin polymerization and myosin-based motility to infect polarized epithelial cells, these are both dispensable for the entry of influenza virus into nonpolarized cells (53).

Since actin-based motility is involved in multiple steps during AcMNPV infection (27–30), it is not possible to merely inhibit actin-dependent internalization at the cell surface without affecting multiple steps downstream of viral internalization. Therefore, it is difficult to use flow cytometric analysis to investigate whether merely inhibiting actin polymerization affects viral infectivity, when infectivity is involved in multiple steps of viral infection. This is the reason why we used flow cytometric analysis to investigate the role of dynamin but did not investigate whether inhibiting actin polymerization affects viral infectivity by flow cytometric analysis. In this study, by using population-based analysis of single-virus tracking and electron microscopy, we demonstrated that inhibiting actin polymerization trapped AcMNPV virus particles in partially invaginated vesicles at the cytomembrane. This finding is consistent with the model that the isometric versus rod-shaped geometry of virus particles dictates the

need for actin assembly. The elongated shape of AcMNPV particles, which is similar to that of vesicular stomatitis virus particles, may cause clathrin assembly to halt prematurely and trigger actin polymerization to achieve particle invagination (33, 49).

AcMNPV nucleocapsid is released from within primarily early endosomes in a microtubule-dependent manner. Following internalization into the cytoplasm, virus particles are delivered to endosomal compartments for nucleocapsid release. Most enveloped viruses, which infect cells via clathrin-mediated endocytosis, have been reported to release nucleocapsids in late endosomes, such as influenza virus, dengue virus, Ebola virus, and HIV-1 (37, 54–57). Enveloped viruses that release nucleocapsids from within late endosomes are thought to rely on microtubules for endocytic trafficking (58). However, the dependence on microtubules for the transport of virus-bearing early endosomes varies for different virus types. Several viruses that enter cells via early endosomes do not require an intact microtubule network, such as foot-and-mouth disease virus and Semliki Forest virus (59, 60). Some other viruses that penetrate into the cytoplasm from within early endosomes require microtubules for infection, such as Japanese encephalitis virus and Borna disease virus (50, 61). In this study, we demonstrated that AcMNPV released nucleocapsid from within early endosomes in a microtubule-dependent manner.

A kinetic analysis of viral transport allows the distinction between microtubule-like and actin-like directed movements. Virus particles can exhibit two representative types of movements, kinetically characteristic of microtubule- and actin-directed transport (38). For human immunodeficiency virus type 1 (HIV-1), microtubule- and actin-dependent movements are represented by gating maximal velocity either at 0 to 0.05 $\mu\text{m/s}$ for actin-directed movements or at 0.05 to 1 $\mu\text{m/s}$ for microtubule-like movements (45). In this study, our analysis of the AcMNPV trajectory indicated that the velocity of virus-bearing endosomes is highly suggestive of microtubule-dependent movements (Fig. 8A to F). Virus-bearing endosomes underwent rapid movements to fuse in a microtubule-dependent manner. In addition, individual virus particles were observed to move along the microtubules in a bidirectional and intermittent manner. Since the nucleocapsid of AcMNPV underwent intracellular motility driven by actin polymerization, the virus particles moving along the microtubule were presumably intact enveloped viruses within the endosomes.

It is generally thought that the pH of early endosomes in most mammalian cells is about 6.0 to 6.5 (62, 63). However, other studies have shown that the pH of early endosome exhibits cell type specificity. In some mammalian cells, the pH of early endosomes is 5.5 to 6.0 (64–70), while other reports state that the pH of early endosomes in certain mammalian cells is 5.0 to 6.0 (71, 72). Because the pH threshold for GP64-mediated membrane fusion was measured at approximately pH 5.5 (73), nucleocapsid release was previously thought to occur in late endosomes (12). However, this hypothesis was based on the assumption that the pHs of early endosomes and late endosomes are 6.0 to 6.5 and 5.5 to 6.0, respectively. However, since the pH of media is 6 and the early endosomes become gradually acidic through the activity of V-ATPase (74), the pH of early endosomes in Sf9 insect cells is postulated to be lower than 6.0, presumably 5.5 to 6.0. Hence, the assumption that the pH of early endosomes is 6.0 to 6.5 is probably not applicable for endosomes in insect host Sf9 cells. Our study is further confirmed by the findings that AcMNPV nucleocapsid release in mammalian cells occurs also primarily in early endosomes but not late endosomes (31, 75, 76).

The mechanism underlying the role of microtubules in AcMNPV infection. The molecular mechanism underlying the role of microtubules in the endocytic trafficking of AcMNPV was investigated. Here, microtubule depolymerization with nocodazole not only slowed the velocity of virus-bearing early endosomes but also impaired the motility of endosome movement, indicating that the movement of early endosomes was dependent on microtubules. Unexpectedly, microtubule depolymerization blocked virus particle transport out of enlarged endosomes rather than out of nascent endosomes, indicating that the fusion between virus-bearing

endosomes was not affected. The discrepancy may be due to the transport of early endosomes through a switch from microtubules to actin filaments after the disruption of endosomal vesicle-microtubule interactions. The movement of early endosomes has been reported to switch between microtubules and actin filaments, which is regulated by a series of complexes, such as RhoD-hDia2C and HAP40-Htt (47, 77). Under physiological conditions, the transport of virus-bearing endosomes was preferentially dependent on microtubules. However, following microtubule depolymerization, the movement of virus-bearing endosomes may switch from microtubules to actin filaments. This hypothesis is consistent with the observation that the depolymerization of microtubules caused a drastic reduction in the velocity and movement of virus-bearing early endosomes.

In summary, we propose the following model for the host cell entry of AcMNPV. First, virus particles are internalized into host cells with the help of actin polymerization and dynamin. When actin polymerization was inhibited with cytochalasin D, virus particles were observed to be trapped in the partially invaginated vesicle at the cytomembrane. Subsequently, dynamin mediates the scission of the virus-bearing pit, resulting a virus-bearing endocytic vesicle. That vesicle then fuses with an early endosome to form an AcMNPV-containing endosome. Immediately after virus delivery to early endosomes, virus-containing endosomes are preferentially moving along the microtubules. Virus-bearing early endosomes undergo long-range bidirectional and intermittent movements and then mature via fusions into larger early endosomes. While some AcMNPV-bearing endosomes may fuse to form larger endosomes, some other AcMNPV-bearing endosome may simply fuse with any other endosome, containing other extracellular material. Finally, the events of AcMNPV envelope fusion with the membranes of endosomes occur predominantly from within early endosomes to release nucleocapsids. Additionally, microtubule depolymerization with nocodazole impaired the motility of virus-bearing endosomes, blocking infection by trapping viruses in enlarged early endosomes. Collectively, AcMNPV infects cells via actin polymerization-dependent internalization and microtubule-dependent nucleocapsid release primarily from within early endosomes (Fig. 10).

This is the first systematic study that reveals the host cell endocytic trafficking of baculovirus budded viruses at the single-particle level. Single-particle tracking in live cells provides mechanistic and kinetic insights into the internalization route, endocytic transport, and fusion events for individual viruses with endosomes. In contrast to AcMNPV transduction in mammalian cells, its infection in host insect cells is facilitated by both actin polymerization and microtubules, implying that AcMNPV exhibits cell type specificity in the requirement of the cytoskeleton network. The mechanistic and kinetic insights presented in this study further enhance our understanding of the infection pathway of baculoviruses.

MATERIALS AND METHODS

Cells and viruses. Sf9 cells (Thermo Fisher Scientific) were cultured at 27°C in Grace's insect medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) (78). A second copy of the *vp39* gene fused with the mCherry gene was inserted under control of the p10 promoter to generate recombinant plasmid pFastBac^{VP39-mCherry}. The primers (vp39-mCherry For-1, Rev-1, For-1, and Rev-1) used for overlap extension PCR (OE-PCR) amplification are shown in Table 1. The recombinant virus vAc^{VP39-mCherry} was generated by transforming *Escherichia coli* DH10Bac (Thermo Fisher Scientific) and transfecting the indicated bacmids into Sf9 cells according to the manufacturer's instructions (Bac-to-Bac baculovirus expression system; Thermo Fisher Scientific) (79). Purified virus vAc^{VP39-mCherry} was analyzed by Western blotting with an anti-VP39 antibody (a gift from Zhihong Hu, Wuhan Institute of Virology, Chinese Academy of Sciences, China) or anti-mCherry antibody (TaKaRa) as the primary detection antibody.

Clonal cell lines expressing endocytic markers. The coding sequence (CDS) of full-length *Rab7* was amplified from Sf9 cells, while the CDS of full-length *Rab5* was amplified from *Bombyx mori*. EGFP with a linker (GCTGCCGCCGCTGCCGCCGCC) at its C terminus was inserted into the pIB/V5-His vector (Invitrogen) using primers EGFP For and Rev, resulting in the pIB/V5^{EGFP} vector. *Rab5* was inserted into the pIB/V5^{EGFP} vector using primers Rab5 For and Rev, resulting in pIB/V5^{EGFP-Rab5}. Similarly, *Rab7* fused with the mCherry gene linked with the above-mentioned linker was inserted into the pIZ/V5-His vector using primers EGFP For and Rev and Rab7 For and Rev, resulting in pIZ/V5^{mCherry-Rab7}. *MTn* and *MAP4* were separately amplified from pcDNA3.1GFP-mTn and pcDNA3.1GFP-MAP4 (gifts from Gengfu Xiao,

FIG 10 Proposed model for the host cell entry of AcMNPV. AcMNPV undergoes an actin- and microtubule-dependent movement for nucleocapsid release predominantly from within early endosomes. At first, virus particles are internalized into host cells in an actin- and dynamin-dependent manner. When actin polymerization was inhibited with cytochalasin D, virus particles were observed to be trapped in the partially invaginated vesicle at the cytomembrane. Subsequently, dynamin mediates the scission of virus-bearing pits, resulting a virus-bearing endocytic vesicle. That vesicle then fuses with an early endosome to form an AcMNPV-containing endosome. Virus-containing endosomes are preferentially moving along the microtubules. Virus-bearing early endosomes undergo long-range bidirectional and intermittent movements and then mature via fusions into larger Rab5-positive endosomes. While some AcMNPV-bearing endosomes may fuse to form larger endosomes, some other AcMNPV-bearing endosome may simply fuse with any other endosome, containing other extracellular material. Finally, the events for fusion of virus particles with endosomes occur predominantly from within Rab5-positive early endosomes to release nucleocapsids. Inhibition of endosomal acidification with bafilomycin A1 blocked nucleocapsid release from early endosomes. Additionally, microtubule depolymerization with nocodazole impaired the motility of virus-bearing endosomes, impairing infection by trapping viruses in enlarged early endosomes. Collectively, AcMNPV infects cells via actin polymerization-dependent internalization and microtubule-dependent nucleocapsid release primarily from within early endosomes. Since motor proteins are much smaller than the virus particles and vesicles, the motors and virus particles in the diagram are drawn to scale as described before (20, 36, 58).

Wuhan Institute of Virology, Chinese Academy of Sciences, China) using primers EGFP-MAP4 For and Rev and EGFP-mTn For and Rev and then inserted into pIB/V5^{EGFP}, resulting in pIB/V5^{EGFP-mTn} and pIB/V5^{EGFP-MAP4}, respectively. The primers used for PCR amplification are shown in Table 1.

Stable polyclonal cell lines were selected after the transfection of Sf9 cells with the above-described constructs in accordance with the manufacturer's specifications (pIB/V5-His vector kit; Thermo Fisher Scientific). The cell lines coexpressing EGFP-Rab5 and mCherry-Rab7 were constructed by cotransfecting the plasmids pIB/V5^{EGFP-Rab5} and pIZ/V5^{mCherry-Rab7} and were then coselected with the antibiotics blasticidin (Thermo Fisher Scientific) and zeocin (Thermo Fisher Scientific). Millicell hanging cell culture inserts (Merck Millipore) were used to isolate clonal stable cell lines as described previously (80). In total, five different clonal cell lines (SfEGFP-Rab5, Sf^{mCherry-Rab7}, SfEGFP-Rab5-mCherry-Rab7, SfEGFP-mTn, and SfEGFP-MAP4) were constructed. The clonal cell lines were examined by confocal imaging, and the cell lines that had the appropriate fluorescently labeled markers were selected for Western blotting and live-cell imaging. The cell lines SfEGFP-mTn, SfEGFP-MAP4, SfEGFP-Rab5, and SfEGFP-Rab5-mCherry-Rab7 were analyzed using an anti-

TABLE 1 Primers used for construction of recombinant AcMNPV, clonal cell lines, and DN mutants

Primer	Sequence (5'→3')
vp39-mCherry For-1	ATATACTCGAGATGGCGCTAGTGCCCGTGG
vp39-mCherry Rev-1	ATGTTCTCCTTAATCAGCTCGCTCATGACGGTATTCTCCACCTGCTT
vp39-mCherry For-2	AAGCAGGTGGAGGAATAGCCGTCATGAGCGAGCTGATTAAGGAGAACAT
vp39-mCherry Rev-2	ATATAGGTACCGCTTTAGTGCCCCAGTTTGCTAGGGAG
EGFP-MAP4 For	CGCGGATCCAACCTCTAAAAAACGCCACCATGGGTAAAGGAGAAGAACT
EGFP-MAP4 Rev	GCTCTAGATTAACTCTGCAGGAAA
EGFP-mTn For	CGCGGATCCAACCTCTAAAAAACGCCACCATGGGTAAAGGAGAAGAACT
EGFP-mTn Rev	GCTCTAGATTAGTGTCTGCTCTCG
EGFP For	CGCGGATCCAACCTCTAAAAAACGCCACCATGGGTAGCAAGGGCGAGGAG
EGFP Rev	ATAGAATTCTGCTGCCGCCGCTGCCGCCCTTGACAGCTCGTCCA TGCCG
Rab5 For	ATAGAATTCATGGCGACAAACAGGACCGG
Rab5 Rev	ATACTCGAGTCACTTGCGAGCACTGCGAGGG
Rab7 For	ATAGAATTCATGTCGTCGAGAAAAAGGCACTACT
Rab7 Rev	ATACTCGAGCTAGCAAGCACAGTTGTCAACATCC
Rab5-DN For	ATAGAATTCATGGCAACCACTCCACGCAG
Rab5-DN Rev	ATACTCGAGTCACTTGCGAGCACTGTTCTCGT
Rab7-DN For-1	ATTCGCTTATGAATCAGTTTGTCAC
Rab7-DN Rev-1	CCTCGCCCTTGCTCAACAT
Rab7-DN For-2	GTGAGCAAGGGCGAGGAGG
Rab7-DN Rev-2	TGATTCAATAGCGAATTTTGCCTAC

EGFP antibody (ProteinTech). The cell lines SfEGFP-Rab5 and SfEGFP-Rab5-mCherry-Rab7 were analyzed using an anti-Rab5 antibody (Cell Signaling Technology). The cell lines Sf9, Sf^{mCherry}-Rab7, and SfEGFP-Rab5-mCherry-Rab7 were analyzed using an anti-mCherry antibody (TaKaRa). The cell lines Sf9, Sf^{mCherry}-Rab7, and SfEGFP-Rab5-mCherry-Rab7 were analyzed using an anti-Rab7 antibody (Abcam).

DNA transfection of cells with DN mutants. The plasmid encoding the mCherry-tagged dominant negative (DN) Rab7(T22N) mutant was constructed by mutation at the indicated sites of Rab7 using primers (Rab7-DN For-1, Rev-1, For-2, and Rev-2) on the base of the plasmid pLZ/V5^{mCherry}-Rab7. The Rab5(S43N) mutant was amplified from pUAS^{YFP}-Rab5-DN (Addgene) using primers Rab5-DN For and Rev and was inserted into the pLb/V5-EGFP vector, resulting in pLb/V5^{EGFP}-Rab5(S43N). Similarly, Rab5(WT) was amplified from pUAS^{YFP}-Rab5-WT (Addgene) using primers Rab5-DN For and Rev and was inserted into the pLb/V5-EGFP vector, resulting in pLb/V5^{EGFP}-Rab5(WT). The primers used for PCR amplification are shown in Table 1. To investigate the roles of Rab5 and Rab7 in the route of AcMNPV cell entry, viral infectivity was analyzed in Sf9 cells expressing dominant negative Rab5 or Rab7 mutants. These plasmids were transfected into Sf9 cells using the transfection reagent Cellfectin II (Thermo Fisher Scientific), according to the manufacturer's protocol. At 30 h postinfection (hpi), vAc^{VP39-EGFP} and vAc^{VP39-mCherry} were used to infect the Sf9 cells that had been transfected with wild-type Rab7 and Rab7-DN mutant or wild-type Rab5 and Rab5-DN mutant at a multiplicity of infection (MOI) of 1. At 24 hpi, the cells were washed with phosphate-buffered saline (PBS) and collected for flow cytometry analysis. The infectivity was calculated as the percentage of infectivity in the transfected cells. Three independent experiments were performed.

DiD labeling and one-step growth curve assay. Virus infection, labeling, and purification were performed as described previously (81–83). Briefly, to amplify the viral stock, Sf9 cells were infected at an MOI of 0.1. The supernatant was collected at 72 hpi and centrifuged at 5,000 × g for 10 min to remove large cell debris. The supernatant was filtered through a 0.45-μm filter (Millipore) to further remove unwanted cell debris and centrifuged at 100,000 × g at 4°C for 2 h on 5 ml of a 30% (wt/vol) sucrose cushion in PBS (pH 7.4) buffer. The virion pellet was resuspended in PBS. Twenty nanomoles of DiD (Molecular Probes, Life Technologies) dissolved in dimethyl sulfoxide (DMSO) was mixed with approximately 1 × 10¹⁰ virus particles. DiD-labeled particles were purified by discontinuous sucrose density gradient ultracentrifugation. The mixture was filtered through a 0.22-μm filter after incubation for 2 h at 27°C and was carefully laid onto a series of sucrose solutions (densities [wt/vol], 65%, 55%, 45%, 35%, 25%, and 15%; the volume of each layer was 1.5 ml). Purified viruses were resuspended in PBS containing 1% bovine serum albumin (BSA) and stored at 4°C. Viruses were filtered through a 0.22-μm-pore-size filter (Millipore) immediately before confocal imaging. To measure the effect of DiD (Thermo Fisher Scientific) labeling on virus infectivity, identical amounts of viruses with the same titers were labeled as described above along with additional control groups in which equivalent amounts of DMSO (Sigma) were added. To investigate the influence of recombinant protein expression on virus production, Sf9 cells were infected with recombinant AcMNPV (vAc^{Pp10-EGFP}, vAc^{VP39-EGFP}, vAc^{VP39-mCherry}, vAc^{Pp10-EGFP-DiD}, vAc^{VP39-EGFP-DiD}, or vAc^{VP39-mCherry-DiD}) at an MOI of 5. Similarly, to test the influence of recombinant protein expression on baculovirus production by the clonal cell lines, vAc^{Pp10-GFP} was used to infect the clonal cell lines (SfEGFP-Rab5, Sf^{mCherry}-Rab7, SfEGFP-Rab5-mCherry-Rab7, SfEGFP-mTn, or SfEGFP-MAP4) at an MOI of 5. Culture medium was harvested at 12, 24, 36, 48, 60, 72, and 96 hpi. All samples were stored at 80°C before titers were determined by the endpoint dilution assay (EPDA) in Sf9 cells. These experiments were performed in triplicate.

Inhibitor treatment of Sf9 cells. Cells were pretreated with a series of inhibitors. Cells without drug treatment were set up as a control group. The concentrations of inhibitors used to treat cells for the flow cytometric analysis as previously described (15, 18, 43, 84) were as follows: 20 $\mu\text{g/ml}$ chlorpromazine, 20 to 100 μM nocodazole, 10 μM cytochalasin D, 40 to 120 nM dynasore, and 10 nM bafilomycin A1. The six-well tissue culture plates were seeded prior to infection at a density of 5×10^5 cells per well and were pretreated with Grace's medium containing inhibitors at 27°C for 1 h. Subsequently, cells were incubated with vAc^{CP10-EGFP} at an MOI of 5 in the inhibitor-containing medium at 27°C for 1 h. The nocodazole was further maintained in Grace's medium for 8 h after the virus particles were washed out. For other inhibitors, after being washed three times with fresh Grace's medium, the cells were cultured with normal Grace's insect medium supplemented with 10% FBS for 30 h before being harvested for flow cytometric analysis. Three independent experiments were performed.

Live-cell imaging. The clonal stable cell lines were seeded on glass-bottom culture dishes and grown to 50% confluence prior to infection for confocal imaging. The emission signal was collected using a 60 $\times$ oil immersion objective with a PerkinElmer UltraView VOX system. EGFP, mCherry, and DiD were excited at 488 nm, 561 nm, and 640 nm, respectively. For the emission of EGFP, mCherry, and DiD, bandpass filters of 527/W55 nm, 615/W70 nm, and 707/W90 nm were used, respectively. Single-particle tracking in live cells was performed as described previously (85). The velocity, trajectory, and MSD of fluorescent signals were analyzed with Volocity (PerkinElmer) and Imaris (Bitplane) software. The virus stock was diluted in Grace's medium and incubated with the cells at an MOI of 50 as previously described (86, 87). Dishes were incubated in a heated chamber at 27°C during live-cell imaging. For drug treatment in live-cell imaging, cells were pretreated with Grace's medium containing inhibitors at 27°C for 30 min. The concentrations of inhibitors used to treat cells were as follows: chlorpromazine, 20 $\mu\text{g/ml}$; nocodazole, 80 μM ; cytochalasin D, 10 μM ; and dynasore, 80 nM. The inhibitors were maintained in the medium throughout the live-cell imaging.

Transmission electron microscopy. Virus stock was concentrated after ultracentrifugation, followed by being filtered through a 0.22- μm filter (Millipore) immediately before infection to remove viral aggregates. Since it was difficult to detect virus particles at low doses by electron microscopy, cells were infected at a high MOI as described before, such as 5,000 for VSV (42, 49, 52). Sf9 cells were incubated with the wild-type AcMNPV at an MOI of 1,000 for 30 min at 4°C, allowing the virus to attach to the cell surface. The cells were treated with drugs as described above, after which they were fixed in 2.5% glutaraldehyde in PBS at 4°C for 1 h. Cells were processed as described before (79, 88). Ultrathin sections of these cells were examined by FEI Tecnai G² 20 TWIN transmission electron microscopy.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.00033-19>.

SUPPLEMENTAL FILE 1, AVI file, 5.1 MB.

SUPPLEMENTAL FILE 2, AVI file, 1.1 MB.

SUPPLEMENTAL FILE 3, AVI file, 0.7 MB.

SUPPLEMENTAL FILE 4, AVI file, 8.6 MB.

SUPPLEMENTAL FILE 5, AVI file, 1.5 MB.

SUPPLEMENTAL FILE 6, AVI file, 0.9 MB.

SUPPLEMENTAL FILE 7, AVI file, 1.1 MB.

SUPPLEMENTAL FILE 8, AVI file, 0.7 MB.

SUPPLEMENTAL FILE 9, AVI file, 1.2 MB.

SUPPLEMENTAL FILE 10, PDF file, 0.2 MB.

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