Microtubule-Mediated and Microtubule-Independent Transport of Adenovirus Type 5 in HEK293 Cells

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Adenovirus serotypes 2 and 5 are taken into cells by receptor-mediated endocytosis, and following release from endosomes, destabilized virions travel along microtubules to accumulate around the nucleus. The entry process culminates in delivery of the viral genome through nuclear pores. This model is based on studies with conventional cell lines, such as HeLa and HEp-2, but in HEK293 cells, which are routinely used in this laboratory because they are permissive for replication of multiple adenovirus serotypes, a different trafficking pattern has been observed. Nuclei of 293 cells have an irregular shape, with an indented region, and virions directly labeled with carboxyfluorescein accumulate in a cluster within that indented region. The clusters, which form in close proximity to the microtubule organizing center (MTOC) and to the Golgi apparatus, are remarkably stable; a fluorescent signal can be seen in the MTOC region up to 16 h postinfection. Furthermore, if cells are infected and then undergo mitosis after the cluster is formed, the signal is found at each spindle pole. Despite the sequestration of virions near the MTOC, 293 cells are no less sensitive than other cells to productive infection with adenovirus. Even though cluster formation depends on intact microtubules, infectivity is not compromised by disruption of microtubules with either nocodazole or colchicine, as determined by expression of an enhanced green fluorescent protein reporter gene inserted in the viral genome. These results indicate that virion clusters do not represent the infectious pathway and suggest an alternative route to the nucleus that does not depend on nocodazole-sensitive microtubules.

Fifty-one serotypes of human adenoviruses have been identified to date and classified into six species (A to F) based on the degree of DNA relatedness (42). In the natural host, adenoviruses infect predominantly epithelial cells of the respiratory tract (species B, C, and E), urinary tract (species B), small intestine (species A and F) and the conjunctiva (species B, D, and E) (20, 43). Species C serotypes (adenovirus serotype 2 [Ad2] and Ad5) can be propagated readily in conventional cell lines (HeLa, HEp-2, and A549) and have been well characterized in many respects, including virus entry. According to the current model (29), the process of adenovirus entry begins with attachment of the fiber knob (on the virion) to the host cell receptor. Interaction of the viral penton base with integrins on the cell surface then triggers endocytosis, and following release from endosomes, destabilized virions travel along microtubules to accumulate around the nucleus. The entry process culminates in delivery of the viral genome through nuclear pores. However, not all adenoviruses utilize the same pathway. Most species B serotypes recognize CD46 (14, 34, 39) rather than the Coxsackie and adenovirus receptor (CAR). Two species B serotypes (Ad3 and Ad7) bind to other receptors (33), identified as CD80 and CD86, in the case of Ad3 (38). Unlike Ad2 and Ad5, which rapidly escape endosomes soon after uptake, virions of species B serotypes are transported within vesicles towards the nucleus, being released from late endosomes or lysosomes (32, 36). Species D serotypes also appear to use a receptor other than CAR. In different studies, Ad37 (species D) has been shown to utilize CD46 (45), like species B serotypes, and sialic acid (1, 2, 3, 9).

Direct labeling of virions with fluorescent dyes, like carboxyfluorescein, has made it possible to follow virions by microscopy during transport to the host cell nucleus. In experiments initiated to study the transport of different adenovirus serotypes, we observed an atypical trafficking pattern in 293 cells, even with Ad5. Further investigation focused on Ad5 and addressed microtubule-mediated transport in relation to the pattern observed. The juxtanuclear virion clusters in 293 cells revealed a pathway which is microtubule dependent but is not required for successful genome delivery.

MATERIALS AND METHODS

Cells and viruses. HeLa (cervical carcinoma) and A549 (lung carcinoma) cells were obtained from the ATCC and used between passages 33 and 47 following receipt. 293 cells, a line of human embryonic kidney cells which express the E1 sequences of the Ad5 genome (18, 35), were obtained from F. Graham, McMaster University, Hamilton, Ontario, Canada, at passage 24 and were used in these experiments between passages 58 and 80. HEp-2 (laryngeal carcinoma) cells were obtained from S. Gray-Owen, University of Toronto, and used within 30 passages of receipt. Cell lines were maintained in minimal essential medium (auto claveable; Gibco) supplemented with 10% fetal calf serum (FCS) as well as penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were subcultured once or twice weekly at a split ratio of 1:2 to 1:5 (293 cells) or 1:5 to 1:10 (HEp-2, HeLa, and A549 cells). Ad5 was initially obtained from ATCC. Ad5.CMV-LacZ and Ad5EGFP are both ΔE1ΔE3 viruses, expressing β-galactosidase and enhanced green fluorescent protein (EGFP), respectively, from a cassette (cytomegalovirus immediate-early promoter, LacZ, or EGFP coding sequence, simian...
phosphate buffer, pH 7.4, for 30 min and then washed twice (5 min each time) in 0.1 M phosphate buffer, pH 7.4, followed by two washes (5 min each) in distilled water. Cells were stained for 30 min in 1% aqueous uranyl acetate, followed by three washes (5 min each) in distilled water. The cells were dehydrated through a graded series of ethanol and then infiltrated and embedded in Epon. Sections were stained with 2% uranyl acetate and 0.2% lead citrate. The samples were examined with a FEI Tecnai 20 electron microscope at 80 kV. Processing of the samples from the osmium tetroxide fixation step onwards and assistance with viewing the stained sections were provided by Robert Temkin, Advanced Bioimaging Centre, Mount Sinai Hospital, Toronto.

**Microtubule disruption experiments.** Nocodazole (Sigma-Aldrich) was prepared as a 10 mM stock solution in dimethyl sulfoxide, stored in aliquots at −20°C, and diluted to a concentration of 30 μM in culture medium or in binding buffer immediately prior to use. Colchicine (Sigma-Aldrich) was prepared as a 20 mM stock solution in distilled deionized water, stored in aliquots at −20°C, and diluted to a concentration of 100 μM in culture medium immediately prior to use. 293 and A549 cells were seeded on 12-mm coverslips in 24-well plates (1.5 × 10^5 cells/well) for microscopy or in 6-well plates (6 × 10^5 cells/well) for analysis by flow cytometry and used the following day.

For analysis of virion trafficking by microscopy, cells were treated for 60 min at 37°C with 30 μM nocodazole in binding buffer prior to infection with carboxyfluorescein-labeled Ad5 diluted in binding buffer containing 30 μM nocodazole. Following adsorption at 4°C for 60 min, the inoculum was removed; cells were washed with binding buffer containing 30 μM nocodazole, and fresh binding buffer containing 30 μM nocodazole was added. Cells were incubated at 37°C for 90 min and then fixed with 4% paraformaldehyde and stained for β-tubulin to confirm the absence of microtubules.

For experiments to monitor the effect of microtubule disruption on genome delivery, nocodazole pretreatment was extended to 2 h to minimize the number of surviving microtubules, and adsorption took place at 37°C to avoid any alterations to the cells induced by exposure to low temperature. The input MOI was based on results of preliminary dose-response experiments and was chosen to give a detectable number of GFP-positive cells, but less than 50% of the population, at 12 h postinfection (p.i.). Accordingly, cells were pretreated with 30 μM nocodazole, diluted in complete medium containing 10% FCS, for 2 h at 37°C prior to infection with 0.6 ml virus inoculum (~2 × 10^7 particles/ml) of Ad5EGFP diluted in binding buffer with 30 μM nocodazole (~20 particles/cell). Following adsorption at 37°C for 60 min, the inoculum was removed and cells were washed twice with complete medium containing 30 μM nocodazole. Fresh medium containing 30 μM nocodazole was added, and cells were returned to 37°C. Mock-treated cells, infected but not exposed to nocodazole, served as controls. At 12 h p.i., Ad5EGFP-infected cells on coverslips were fixed with 4% paraformaldehyde and stained for β-tubulin to confirm the absence of microtubules.

For flow cytometry experiments using colchicine to disrupt microtubules, 293 cells were pretreated with 100 μM colchicine for 2 h on ice and then for 2 h at 37°C prior to infection. The colchicine concentration was maintained at 100 μM during the virus adsorption period at 4°C and the subsequent incubation period at 37°C. Conditions for infection and subsequent processing were the same as those described for the flow cytometry experiments using nocodazole. In the colchicine experiments, cells on coverslips were treated with Triton X (0.1% in PEM buffer [100 mM piperazine-N,N’-bis(2-ethanesulfonic acid), 1 mM EGTA, 1 mM MgCl₂, pH 6.9]) for 5 min and then rinsed three times with PEM buffer to extract soluble tubulin prior to staining residual microtubules with anti-β-tubulin (28).

Ad5.CMV-LacZ was also used to monitor genome delivery in the presence or absence of nocodazole by microscopic examination of infected 293 cells cultured stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The pretreatment and infection conditions were the same as those used with Ad5EGFP. At 12 h p.i., cells were fixed with 4% paraformaldehyde for 5 min at room temperature and then washed with PBS and incubated with X-Gal (dissolved from a stock solution of 20 mg/ml in dimethylformamide to 1 mg/ml in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride) for 1 h at 37°C. The X-Gal solution was replaced with PBS, and cultures were examined, using a Leica DMIL microscope, for blue cells, indicative of β-galactosidase activity and successful genome delivery.

**Intracellular trafficking of labeled virions.** Virions were labeled with 5- (and 6-) carboxyfluorescein, succinimidyl ester (Molecular Probes) as described by Miyazawa et al. (31). Infectivity was not reduced by the labeling process (results not shown). For the intracellular trafficking experiments, cells were seeded in 60-well Terasaki plates (Nunc) as described previously (7).

**Electron microscopy.** Cells fixed with 4% paraformaldehyde were permeabilized with PBT (0.1% Triton X in PBS) for 15 min at room temperature and then blocked with 5% BSA-PBT at room temperature for 45 min before incubation with primary antibody (diluted in 5% BSA-PBT) for 45 to 60 min at 37°C. After 3-5 min washes with PBS at room temperature, cells were incubated with secondary antibody (also diluted in 5% BSA-PBT) for 45 min at 37°C. Cells were then washed with PBT (two 5-min washes), followed by PBS (two 5-min washes). Coverslips were mounted in PBS containing DAPI (0.25 μg/ml) with or without 1,4-diacyzidobenzene (2,2’-octane (20 mM) and 4,6-diamidino-2-phenylindole (DAPI) (0.25 μg/ml) and examined with a Leica DMA fluorescence microscope, using the 63X oil objective and the 1.05, 1.25×, or 1.6 μg magnifier. Images were captured with a Hamamatsu charge-coupled-device camera (C4742-95) and processed using OpenLab imaging software, version 2.0.7 (Improvement, Inc.) and Adobe Photoshop, version 6.0.

**Immunofluorescence microscopy.** Primary monoclonal antibody against the Golgi protein with a molecular weight of 58,000 (SSK) and secondary tetramethyl rhodamine isothiocyanate-conjugated antimurine antibody, as well as Cy3-conjugated monoclonal antibody against β-tubulin, were purchased from Sigma-Aldrich Canada Ltd. Polyclonal antibody against γ-tubulin and Texas Red-conjugated antirabbit antibody were gifts from Andrew Wilde, University of Toronto. Monoclonal antibody (clone FK2) against multibiquitin chains (13) and polyclonal antibody against the 20s proteasomal subunit were gifts from John Brumell, The Hospital for Sick Children.

Cells fixed with 4% paraformaldehyde were permeabilized with PBT (0.1% Triton X in PBS) for 15 min at room temperature and then blocked with 5% BSA-PBT at room temperature for 45 min before incubation with primary antibody (diluted in 5% BSA-PBT) for 45 to 60 min at 37°C. After 3-5 min washes with PBS at room temperature, cells were incubated with secondary antibody (also diluted in 5% BSA-PBT) for 45 min at 37°C. Cells were then washed with PBT (two 5-min washes), followed by PBS (two 5-min washes). Coverslips were mounted in PBS containing DAPI (0.25 μg/ml) with or without 1,4-diacyzidobenzene (2,2’-octane (20 mM).

**Electron microscopy.** 293 cells were seeded onto 18-mm coverslips in six-well tissue culture dishes (3 × 10^5 cells/well). The next day, cells were rinsed three times with ice-cold binding buffer and infected with 0.6 ml per well of the same labeled virus suspension used in the tracking experiments (~10^7 particles/ml; ~10^6 particles/cell) at 4°C for 60 min to synchronize infection. Unbound virions were then removed by rinsing the cells with ice-cold binding buffer. Subsequently, cells were incubated in warm binding buffer at 37°C for 30 min. After infection, cells were rinsed twice with PBS, fixed with 2% glutaraldehyde for 30 min at room temperature, washed with PBS and then 0.1 M phosphate buffer, pH 7.4, and stored in the same buffer until further processed.

To continue processing, cells were fixed with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 30 min and then washed twice (5 min each time) in 0.1 M phosphate buffer, pH 7.4, followed by two washes (5 min each) in distilled water. Cells were stained for 30 min in 1% aqueous uranyl acetate, followed by three washes (5 min each) in distilled water. The cells were dehydrated through a graded series of ethanol and then infiltrated and embedded in Epon. Sections were stained with 2% uranyl acetate and 0.2% lead citrate. The samples were examined with a FEI Tecnai 20 electron microscope at 80 kV. Processing of the samples from the osmium tetroxide fixation step onwards and assistance with viewing the stained sections were provided by Robert Temkin, Advanced Bioimaging Centre, Mount Sinai Hospital, Toronto.
RESULTS

Trafficking patterns. The trafficking pattern of labeled Ad5 virions in HeLa, HEp-2, and A549 cells was consistent with published images of HeLa cells (19) and A549 cells (23, 31), showing virus bound to the cell surface after adsorption at 4°C (Fig. 1b to d) and accumulation of virions around the nucleus after warming to 37°C for 60 to 90 min (Fig. 1f to h). In the case of 293 cells, virions were bound to the cell surface at 4°C (Fig. 1a), as seen with the other cell lines, but the trafficking pattern of labeled Ad5 virions at 37°C was strikingly different. The nuclei of 293 cells were found to have an irregular shape, most with an “indented” region (Fig. 1a and e and 2A to C), and 60 min after warming the cells to 37°C, the majority of virions had accumulated in a cluster within the “indented” region (Fig. 1e). A minor population of virions could be seen in a perinuclear distribution around the nucleus in some cells following enhancement of the signal (Fig. 2A).

Immunospecific staining of 293 cells, following internalization of labeled virus, showed that the clusters are found near the Golgi apparatus (Fig. 2B) and the microtubule organizing center (MTOC) (Fig. 2C). In cells which were infected and then underwent mitosis, the signal remained with the centrioles (Fig. 2D). Time course experiments showed that clusters were beginning to form by 20 min after warming to 37°C, and by 40 min, they were comparable in appearance to clusters seen after 60 min (not shown). By 6 h after warming, clusters were still apparent, and signal could be detected up to 16 h (not shown).

To determine whether the juxtanuclear clusters represented intact virions rather than degradation products, thin sections of infected cells were examined by electron microscopy. Virions which appeared to be intact were readily found in the “indented region” of the nucleus. Figure 3 (upper and lower panels) shows two representative fields, each at low, medium, and high magnification. In one field, multiple virions are contained within a single vesicle (Fig. 3a to c), whereas in the other field, virions appear to be in the cytoplasm in close proximity to small empty vesicles (Fig. 3d to f) whose identity is unknown but which might represent Golgi vesicles sectioned on a tangent.

Effect of microtubule disruption on virion trafficking. When cells were treated with 30 μM nocodazole (to disrupt microtubules) prior to infection and were maintained in the presence of 30 μM nocodazole during virus adsorption and incubation, virions did not accumulate in juxtanuclear clusters but were predominantly scattered throughout the cytoplasm (Fig. 4). Homogeneous staining for β-tubulin confirmed that microtubules were not intact. Under these conditions, a perinuclear distribution of virions around the nuclei of 293 cells was more evident than in cultures whose microtubules were intact (Fig. 4A). In A549 cells, often used for trafficking studies because they are flat and provide high-quality images in fluorescence microscopy, perinuclear accumulation of labeled virions was particularly striking in the absence of microtubules (Fig. 4B).

Although formation of the juxtanuclear clusters was dependent on intact microtubules, it appeared that a population of virions was transported to the perinuclear region even in the absence of detectable microtubules. Ad5EGFP was then used to determine whether infectivity was compromised under these conditions. Both 293 and A549 cells were treated with 30 μM nocodazole prior to infection with Ad5EGFP at a MOI sufficient to infect less than 50% of the cells. The nocodazole concentration was maintained at 30 μM during virus adsorption and after the cells were warmed to 37°C. It was reasoned that if microtubules were important for transport of virions along the infectious pathway to the nucleus, then the number of cells expressing EGFP should be lower in cultures treated with nocodazole. When infected cultures were examined microscopically 12 h p.i., the numbers of EGFP-expressing cells were comparable in treated and nontreated cultures (Fig. 5),
even though microtubules were disrupted by nocodazole treatment. Moreover, a brighter signal was observed in the treated cultures (i.e., an absence of microtubules) by microscopy and was confirmed by flow cytometry (Fig. 5). Nocodazole treatment had no effect on the number of fluorescent cells, but the curves representing both 293 and A549 cells treated with nocodazole were shifted to the right, indicating an increase in fluorescence intensity. The mean fluorescence intensities of nocodazole-treated and untreated cells infected with Ad5EGFP are compared in Table 1. In both 293 and A549 cells, the mean fluorescence intensity of infected, treated cells was about double that of infected, untreated cells.

When experiments were repeated with 293 cells, using colchicine to disrupt microtubules, the proportions of EGFP-positive cells were again comparable in treated and nontreated cultures (Table 1). However, the increased intensity of fluorescence seen with nocodazole treatment was not seen with colchicine treatment (Table 1). Extraction of soluble tubulin from colchicine-treated cells prior to staining of the cells with anti-β-tubulin showed that there were still some surviving microtubules immediately prior to infection, but most of these appeared as disconnected fragments (Fig. 6).

Genome delivery in 293 cells was also monitored microscopically after X-Gal staining of nocodazole-treated and untreated cells.
cultures infected with Ad5.CMV-LacZ. As seen with Ad5EGFP, using the same pretreatment conditions and the same low MOI, the numbers of cells expressing signal were comparable in the presence or absence of nocodazole (Table 2), indicating that genome delivery was not compromised by disruption of microtubules.

**DISCUSSION**

This study draws attention to the irregular shape of 293 cell nuclei and to an intracellular trafficking pattern for Ad5 which is strikingly different from the perinuclear accumulation characteristic of HeLa, HEp-2, and A549 cells. Accumulation of virions in clusters near the MTOC proved to be dependent on microtubule-mediated transport, but microtubules were not essential for successful genome delivery to the nucleus.

Localization of virions near the MTOC, within the “indented” region formed by the nucleus, in 293 cells is consistent with the minus end-directed transport of incoming virions along microtubules, as shown by Suomalainen et al. (41) and Leopold et al. (24). However, it is not clear why the majority of incoming virions remain in stable clusters near the MTOC in 293 cells (Fig. 1e) and some A549 cells (14; data not shown) but not other cells. Initially, it was thought that clustering of the virions might reflect a cytoskeletal organization related to the irregularly shaped nuclei or to the neuronal origin of 293 cells (35). However, Shayakhmetov et al. (37) showed juxtanuclear clusters of chimeric Ad5 (with Ad35 fiber knobs) in hematopoietic cells with regular oval-shaped nuclei. This observation is meaningful in that the fiber knobs from Ad35 (species B) confer a binding specificity for CD46, rather than CAR, as the primary receptor. Clustering at the MTOC therefore is not unique to cells with irregularly shaped nuclei or to infections mediated by virion binding to CAR.

It is not known whether the fluorescent clusters represent virions contained within vesicles or free in the cytoplasm, since both types were seen by electron microscopy (Fig. 3). Given the current model for adenovirus entry (29), the Ad5 clusters likely represent virions released from vesicles and then carried along microtubules to the MTOC. No colocalization was evident by immunostaining with antibody against EEA-1, transferrin receptor, or LAMP-1 as markers for early endosomes, recycling endosomes, and late endosomes/lysosomes, respectively (data not shown). Formation of virion clusters near the MTOC may reflect the activation of signal transduction pathways by high levels of input virus binding to the surfaces of 293 cells. Increasing dilution of the labeled Ad5 inoculum gave a

![FIG. 3. Examination of infected 293 cells by electron microscopy. Cells were infected and processed for electron microscopy as described in Materials and Methods. (a to c) One field at low, medium, and high magnification, respectively. (d to f) A different field at low, medium, and high magnification, respectively. Arrows are positioned to indicate the same site within the cell at different magnifications. An asterisk marks an individual virion in panels c and f. The nucleus (N) is identified where visible.](http://jvi.asm.org/)...
fluorescent signal that was less intense and still localized to the “nuclear indentation” of 293 cells, but even at the limit of detection, the adsorbed MOI was several hundred particles per cell (not shown).

The virion clusters are similar in appearance to aggresomes, which represent accumulations of protein near the MTOC, often in association with components of the protein degradation pathway (15, 21). Aggresome formation is microtubule dependent and typically is accompanied by a major reorganization of vimentin filaments to form a cage around the protein inclusion (15, 21, 22). Although aggresome formation is enhanced in the presence of proteasome inhibitors or when certain proteins are overexpressed (44), the adenovirus E1B 55K protein, along with p53, has been reported to form aggresomes in uninfected 293 cells at normal expression levels (25). In the current study, formation of the virion clusters, like that of aggresomes, was microtubule dependent (Fig. 4), but vimentin remained as extended filaments throughout the cells and did not form a cage around the virion cluster (not shown). Moreover, the virion clusters did not recruit the 20s proteasome subunit, nor were they ubiquitinated, as determined by immunofluorescent staining with anti-20s and anti-FK2 antibodies, respectively (not shown). Brown et al. (6) reported that the p53/E1B 55K inclusions, which form at the MTOC, dissociate from the spindle poles in mitotic 293 cells. In contrast, virions that have already become associated with the MTOC remain attached at both spindle poles during mitosis (Fig. 2). Taken together, the properties of the virion clusters suggest that they likely do not represent classical aggresomes.

Stable association of Ad5 virions with spindle poles of dividing 293 cells (Fig. 2D) is consistent with strong association of virions with the MTOC. Other studies have reported virion clusters at the spindle poles of mitotic A549 cells (17, 24) and at the MTOC of enucleated A549 cells (4). Virions were more likely to accumulate at the nuclei of A549 cells rather than the MTOC when the nuclear membrane was intact (4), but even so, virion clusters were noted at the MTOC in some cells (17). Strunze et al. (40) have linked virion accumulation at the MTOC to the absence of functional CRM1, a nuclear export factor proposed to facilitate detachment of virions from microtubules at the nuclear periphery and thus allow attachment to the nuclear pores. In the case of 293 cells, most of the virions clustered at the MTOC even in the presence of intact nuclei and with no inhibitors (Fig. 1 and 2). The stable association of virions with the MTOC might be expected to compromise infectivity, making 293 cells less sensitive to infection, but in fact, 293 cells are no less sensitive than other cells to productive infection (8). This observation, coupled with the ability to detect clusters up to 16 h p.i., a time when progeny virus is already being produced (8), suggested that the clusters did not

![FIG. 4. Formation of virion clusters is dependent on intact microtubules.](http://jvi.asm.org/)

(A) 293 cells were mock treated or treated with 30 μM nocodazole for 1 h prior to infection and maintained in 30 μM nocodazole during virus adsorption and internalization as described in Materials and Methods. Following internalization of virions at 37°C for 60 min, cells were fixed with 4% paraformaldehyde and stained with Cy3-labeled antibody against β-tubulin. (a) Overlay of virus signal (green) with DAPI signal (blue) in the absence of nocodazole. (b) Overlay of virus signal (green) with DAPI signal (blue) in cells treated with nocodazole (+ Noc). Perinuclear distribution of virions is indicated with an arrow. (c) β-Tubulin in cells shown in panel a. (d) β-Tubulin in cells shown in panel b. (B) A549 cell from a culture pretreated with 30 μM nocodazole, infected and processed as described for A. Overlay of virus signal with DAPI signal is shown in panel c. Perinuclear distribution of virions is indicated with an arrow in panels a and c.
represent virions en route to successful genome delivery. The use of nocodazole and colchicine showed that intact microtubules are required for cluster formation but not for successful genome delivery, thereby confirming that the clusters are not an essential part of the infectious pathway in 293 cells and showing that the pathway leading to genome delivery could be uncoupled from bulk virion transport along microtubules.

These results support the notion put forward by Glotzer et al. (17), based on studies with A549 and HeLa cells, that virions can be transported to the nucleus along a microtubule-independent pathway. As noted by many authors, microtubule disruption resulted in dispersion of the majority of virions throughout the cell (11, 17, 24, 26) (Fig. 4). Even so, a detectable proportion of the virion population could still be found at the nuclear periphery, as determined by tracking fluorescent virions (17) (Fig. 4 of current study) and by electron microscopy (11). Indeed, nuclear targeting of the bulk virion population appeared to improve in the absence of microtubules following nocodazole treatment (Fig. 4). Importantly, viral gene expression was not compromised when microtubules were disrupted (17), as reported for 293 and A549 cells in the current study (Fig. 5; Tables 1 and 2). The increased fluorescence intensity from EGFP expression in repeated experiments with nocodazole-treated cells is unexplained; a similar increase was not seen when microtubules were disrupted with colchicine. Giannakakou et al. (16) reported enhanced nucleus-directed transport of labeled Ad, as well as enhanced nuclear delivery of p53, in A549 and HeLa cells when microtubule dynamics were suppressed by low doses of nocodazole (1 to 3 nM). In that case, however, the low dose of nocodazole (1 to 3 nM) was not sufficient to depolymerize microtubules, whereas in the current study, a higher concentration of nocodazole (30 μM) clearly disrupted the microtubules.

A high MOI, typically used for trafficking experiments, facilitates tracking of the bulk virion population but can obscure the pathway taken by the minor virion population en route to successful genome delivery. For this reason, experiments to determine the effect of microtubule disruption on infectivity were done with a low MOI, sufficient to infect only a small proportion of the cells, and cells were scored for genome delivery before progeny virus could spread to infect neighboring cells. The rationale was that if intact microtubules are required for virion transport, depolymerization of microtubules should result in a lower proportion of productively in-

FIG. 5. Infection with Ad5EGFP in the presence or absence of nocodazole. 293 and A549 cells were treated with 30 μM nocodazole or left untreated and then infected with Ad5EGFP at a low MOI and fixed 12 h p.i., as described in Materials and Methods. Representative fields are shown. (A) Nuclei were stained with DAPI. (a) A549 cells in the absence of nocodazole. (b) EGFP signal from the field shown in panel a. (c) EGFP signal from the field shown in panel e. (g) EGFP signal from the field shown in panel h. (h) 293 cells treated with 30 μM nocodazole. (f) EGFP signal from the field shown in panel b. (d) 293 cells in the absence of nocodazole. (e) A549 cells treated with 30 μM nocodazole. (i) EGFP signal from the field shown in panel c. (d) 293 cells treated with 30 μM nocodazole. (B) FACS analysis of cells from the same experiment shown in A. Cells were processed for flow cytometry as described in Materials and Methods. Each curve represents the cell population from one well of a six-well plate.
processed from the flow cytometry data shown graphically in Fig. 5. The total number of cells counted per sample was used to determine the cutoff between background fluorescence and fluorescence due to EGFP. The value for mean fluorescence is based on the population detected above the cutoff. The number of cells counted per sample was ~90,000 for A549 cells and ranged from ~30,000 to 70,000 for 293 cells. The values for nocodazole are taken from the flow cytometry data shown graphically in Fig. 5.

### Table 1: Fluorescence-activated cell sorting analysis of cells infected with Ad5EGFP in the presence or absence of nocodazole or colchicine

| Drug and cell line | Infection | Value for treated cells | Value for untreated cells | MF T/U |
|--------------------|-----------|-------------------------|---------------------------|--------|
|                    |           | Fluorescent mean        | Fluorescent mean          |        |
|                    |           | (%)                     | (%)                       |        |
| Nocodazole         | –         | 0.6 ± 1.7               | 0.7 ± 0.1                 | 1      |
|                    | +         | 40.3 ± 1.7              | 36.2 ± 1.5                | 2.4    |
| A549               | –         | 0.7 ± 1.7               | 0.4 ± 1.3                 | 1      |
|                    | +         | 4.4 ± 0.4               | 5.8 ± 0.3                 | 1.9    |
| Colchicine         | 293 –     | 0.2 ± 0.1               | 0.2 ± 0.1                 | 1.0    |
|                    | 293 +     | 31.1 ± 7.1              | 41.7 ± 2.0                | 1.3    |

**Values for mean fluorescence and percent fluorescent cells represent the mean ± standard deviation for triplicate samples. Uninfected, untreated cells were used to determine the cutoff between background fluorescence and fluorescence due to EGFP.**

*For mean fluorescence is based on the population detected above the cutoff.*

**Table 2: Numbers of cells expressing β-galactosidase in 293 cell cultures infected with Ad5.CMV-LacZ in the presence or absence of nocodazole**

| Expt no. (n=4) | No. of counts per field with inoculum (virions/ml) of: | Untreated | Noc | Untreated | Noc |
|----------------|------------------------------------------------------|-----------|-----|-----------|-----|
|                | 4 × 10⁷                                               |           |     |           |     |
| 1 (20)         | 66 ± 16                                               | 71 ± 13   | 20 ± 5 | 16 ± 4    |
| 2 (20)         | 71 ± 9                                                | 76 ± 8    | 25 ± 5 | 36 ± 9    |
| 3 (10)         | 57 ± 10                                               | 73 ± 10   | 24 ± 6 | 28 ± 6    |

*Cells were infected with Ad5.CMV-LacZ, as described in Materials and Methods, and then fixed at 12 h p.i. and stained with X-Gal. Cells were examined microscopically, and the number of blue cells per field was counted with the 10× objective. Data are shown as mean no. of counts per field ± standard deviation.

**Noc. treated with nocodazole.**

### Figures

**Fig. 6.** Tubulin in colchicine-treated 293 cells immediately prior to infection and at 12 h.p.i. with Ad5EGFP. As part of the flow cytometry experiments (Table 1, colchicine), cells seeded on coverslips were fixed with or without extraction of soluble tubulin immediately prior to infection (a to d) or at 12 h.p.i. (e to h). (a and c) Untreated cells with total tubulin. (b and f) Treated cells with total tubulin. (d, g, and h) Treated cells with residual tubulin following extraction of soluble tubulin.

### Table 1

| Drug and cell line | Infection | Value for treated cells | Value for untreated cells | MF T/U |
|--------------------|-----------|-------------------------|---------------------------|--------|
|                    |           | Fluorescent mean        | Fluorescent mean          |        |
|                    |           | (%)                     | (%)                       |        |
| Nocodazole         | –         | 0.6 ± 1.7               | 0.7 ± 0.1                 | 1      |
|                    | +         | 40.3 ± 1.7              | 36.2 ± 1.5                | 2.4    |
| A549               | –         | 0.7 ± 1.7               | 0.4 ± 1.3                 | 1      |
|                    | +         | 4.4 ± 0.4               | 5.8 ± 0.3                 | 1.9    |
| Colchicine         | 293 –     | 0.2 ± 0.1               | 0.2 ± 0.1                 | 1.0    |
|                    | 293 +     | 31.1 ± 7.1              | 41.7 ± 2.0                | 1.3    |

**Values for mean fluorescence and percent fluorescent cells represent the mean ± standard deviation for triplicate samples. Uninfected, untreated cells were used to determine the cutoff between background fluorescence and fluorescence due to EGFP.**
Ad5LacZ and assessed infectivity by expression of the use of different reporter genes. Since Mabit et al. (26) used thermore, diffusion seems unlikely to account for the nuclear the infected cells treated with nocodazole or colchicine. Fur- thermore, diffusion seems unlikely to compensate for microtubules in all of might explain successful infection of some cells in the popula-
tance of an alternative, less-efficient pathway and suggested tubules were disrupted. The same authors did note the exis-
tions along microtubules to accumulate near the MTOC and
viruses are transported along microtubules towards the center for nuclear targeting of virions, but they do not rule out the possibility that microtubules normally play a role in virus targeting to the nucleus in untreated cells.
It is clear from multiple reports in the literature that adeno-
viruses are transported along microtubules towards the center of the cell (11, 24, 41). In the current study, the striking formation of juxtanuclear virion clusters in 293 cells highlights a seemingly nonproductive minus-end directed transport of viri-
ons along microtubules to accumulate near the MTOC and raises the question as to whether much of the documented transport along microtubules might also be nonproductive. It is interesting to speculate whether this pathway might represent a host defense mechanism by which virions are diverted from the infectious pathway. Segregation of virion accumulation at the MTOC from successful genome delivery, using nocodazole to disrupt microtubules, emphasizes that microtubule-medi-
ated trafficking of the bulk virion population to the MTOC should not necessarily be interpreted as the route taken by virions en route to genome delivery. The current study indi-
cates that successful genome delivery does not require intact microtubules for virus transport, at least in 293 and A549 cells. These findings reflect the complex nature of the adeno-

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**REFERENCES**

1. Arnberg, N., K. Edlund, A. H. Kidd, and G. Wadell. 2000. Adenovirus type 5 uses sialic acid as a cellular receptor. J. Virol. 74:42–48.

2. Arnberg, N., A. H. Kidd, K. Edlund, F. Olfat, and G. Wadell. 2000. Initial interactions of subgenus D adenoviruses with A549 cellular receptors: sialic acid versus α-sialosiglycoproteins. J. Virol. 74:7691–7693.

3. Arnberg, N., P. Pring-Akerblom, and G. Wadell. 1997. Microtubule-independent trafficking and p53 nuclear accumulation by suppression of micro-
tubule dynamics. Proc. Natl. Acad. Sci. USA 94:1239–1254.

4. Blagoslonny, U. F. Greber, and T. Fojo. 2002. Enhanced microtubule-de-
dependent trafficking and p53 nuclear accumulation by suppression of micro-
tubule dynamics. Proc. Natl. Acad. Sci. USA 99:10855–10860.

5. Glotzer, J. B., A. I. Michou, A. Baker, M. Saltik, and M. Cotten. 2001. Microtubule-independent motility and nuclear targeting of adenoviruses with fluorescein labeled genomes. J. Virol. 75:2421–2434.

6. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1973. Microbiology, 2nd ed., p. 1042–1044. Harper and Row Publishers, Inc., Hagerstown, MD.

7. Fujimuro, M., H. Sawada, and H. Yokosawa. 1994. Production and charac-
terization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. FEBS Lett. 349:173–180.

8. Cashman, S. M., D. J. Morris, and R. Kumar-Singh. 2004. Adenovirus type 5 pseudotyped with adenovirus type 37 uses sialic acid as a receptor. Virology 324:129–139.

9. Chang, L., and R. D. Goldman. 2004. Intermediate filaments mediate cy-
toskeletal crosstalk. Nat. Rev. Mol. Cell Biol. 5:601–613.

10. John, J. A., C. L. Ward, and R. R. Kopito. 1999. Character-
ization and dynamics of aggresome formation by a cytosolic GFP-chimera. J. Cell Biol. 146:1239–1254.

11. Giannakakou, P., M. Nakano, K. C. Nicolaou, A. O’Brate, J. Yu, M. V. Blagoslonny, U. F. Greber, and T. Fojo. 2002. Enhanced microtubule-de-
dependent trafficking and p53 nuclear accumulation by suppression of micro-
tubule dynamics. Proc. Natl. Acad. Sci. USA 99:10855–10860.

12. Glotzer, J. B., A. I. Michou, A. Baker, M. Saltik, and M. Cotten. 2001. Microtubule-independent motility and nuclear targeting of adenoviruses with fluorescein labeled genomes. J. Virol. 75:2421–2434.

13. Gaggar, A., D. M. Shayanakhetov, and A. Lieder. 2003. CD46 is a cellular receptor for group B adenoviruses. Nat. Med. 9:1408–1412.

14. Garcia-Mata, R., Z. Bebok, E. J. Sorscher, and E. Sztul. 2003. CD46 is an alternative receptor for group B adenoviruses. J. Cell Physiol. 160:47–60.

15. Brown, M. 1985. Selection of nonfastidious adenovirus species in 293 cells inoculated with stool specimens containing adenovirus type 40. J. Clin. Mi-

16. Brown, M., H. L. Wilson-Friesen, and F. Doane. 1992. A block in release of progeny virus and a high particle-to-infectious ratio contribute to poor growth of enteric adenovirus types 40 and 41 in cell culture. J. Virol. 66:3196–3205.

17. Glotzer, J. B., A. I. Michou, A. Baker, M. Saltik, and M. Cotten. 2001. Microtubule-independent motility and nuclear targeting of adenoviruses with fluorescein labeled genomes. J. Virol. 75:2421–2434.

18. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36:59–72.
23. Leopold, P. L., R. Ferris, I. Grinberg, S. Worgall, N. R. Hackett, and R. G. Crystal. 1998. Fluorescent virions: dynamic tracking of the pathway of adenovirus gene transfer vectors in living cells. Hum. Gene Ther. 9:367–378.

24. Leopold, P. L., G. Kreitzer, N. Miyazawa, S. Rempel, K. K. Pfister, E. Rodriguez-Boulan, and R. G. Crystal. 2000. Dynacin- and microtubule-mediated translocation of adenovirus serotype 5 occurs after endosomal lysis. Hum. Gene Ther. 11:151–165.

25. Liu, Y., A. Shevchenko, A. Shevchenko, and A. J. Berk. 2005. Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex. J. Virol. 79:14004–14016.

26. Mabot, H., M. Y. Nakano, U. Prank, B. Saam, K. Dohner, B. Sodeik, and U. F. Greber. 2002. Intact microtubules support adenovirus and herpes simplex virus infections. J. Virol. 76:9962–9971.

27. Maizel, J. V., Jr., D. O. White, and M. D. Scharff. 1968. The polypeptides of adenovirus II. Soluble proteins, cores, top components and the structure of the virion. Virology 36:126–136.

28. Marceiller, J., A. Drechou, G. Durand, F. Perez, and C. Pouys. 2005. Kinesin is involved in protecting nascent microtubules from disassembly after recovery from nocodazole treatment. Exp. Cell Res. 304:483–492.

29. Meier, O., and U. F. Greber. 2004. Adenovirus endocytosis. J. Gene Med. 6:S152–S163.

30. Mittereder, N., K. L. March, and B. C. Trapnell. 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J. Virol. 70:7489–7509.

31. Miyazawa, N., P. L. Leopold, N. R. Hackett, B. Ferris, S. Worgall, E. Falek-Pedersen, and R. G. Crystal. 1999. Fiber swap between adenovirus subgroups B and C alters intracellular trafficking of adenovirus gene transfer vectors. J. Virol. 73:6056–6065.

32. Miyazawa, N., C. G. Ronald, and P. Leopold. 2001. Adenovirus serotype 2 retention in a late endosomal compartment prior to cytosol escape is modulated by fiber protein. J. Virol. 75:1387–1400.

33. Segerman, A., N. Arnberg, A. Erikson, K. Lindman, and G. Wadell. 2003. There are two different species B adenovirus receptors: sBAR, common to species B1 and B2 adenoviruses, and sB2AR, exclusively used by species B2 adenoviruses. J. Virol. 77:1157–1162.

34. Segerman, A., J. P. Atkinson, M. Marttila, V. Dennerquist, G. Wadell, and N. Arnberg. 2003. Adenovirus type 11 uses CD46 as a cellular receptor. J. Virol. 77:9183–9191.

35. Shaw, G., S. Morse, M. Ararat, and F. L. Graham. 2002. Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK293 cells. FASEB J. 16:869–871.

36. Shayakhmetov, D. M., Z.-Y. Li, V. Ternovoi, A. Gaggar, H. Gharwan, and A. Lieber. 2003. The interaction between the fiber knob domain and the cellular attachment receptor determines the intracellular trafficking route of adenoviruses. J. Virol. 77:3712–3723.

37. Shayakhmetov, D. M., Z.-Y. Li, A. Gaggar, H. Gharwan, V. Ternovoi, V. Sandig, and A. Lieber. 2004. Genome size and structure determine efficiency of postinternalization steps and gene transfer of capsid-modified adenovirus vectors in a cell-type specific manner. J. Virol. 78:10009–10022.

38. Shortt, J. J., A. V. Pereboev, Y. Kawakami, C. Vasu, M. J. Holterman, and D. T. Curiel. 2004. Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. Virology 322:349–359.

39. Siriena, D., B. Lilienfeld, M. Eisenhut, S. Kalin, K. Boucke, R. R. Beerli, L. Vogt, C. Ruedl, M. F. Bachmann, U. F. Greber, and S. Hemmi. 2004. The human membrane cofactor CD46 is a receptor for species B adenovirus serotype 3. J. Virol. 78:4454–4462.

40. Strunze, S., L. C. Trotman, K. Boucke, and U. F. Greber. 2005. Nuclear targeting of adenovirus type 2 requires CRM1-mediated nuclear export. Mol. Biol. Cell 16:2999–3009.

41. Suomalainen, M., M. Y. Nakano, K. Boucke, S. Keller, and U. F. Greber. 1999. Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus. J. Cell Biol. 144:657–672.

42. van Regenmortel, M. H. V., C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Esteves, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.). 2000. Virus taxonomy. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, CA.

43. Wadell, G. 1999. In A. Granoff and R. G. Webster (ed.), Encyclopedia of virology, 2nd ed.. Academic Press, San Diego, CA.

44. Wojcik, C., and G. N. DeMartino. 2003. Intracellular localization of proteasomes. Int. J. Biochem. Cell Biol. 35:579–589.

45. Wu, E., S. A. Trauger, L. Pache, T. M. Mulle, D. J. von Seggern, G. Siuzdak, and G. R. Nemerow. 2004. Membrane cofactor protein is a receptor for adenoviruses associated with epidemic keratoconjunctivitis. J. Virol. 78:3897–3905.