Retrograde Axon Transport of Herpes Simplex Virus and Pseudorabies Virus: a Live-Cell Comparative Analysis

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Upon entry, neuroinvasive herpesviruses traffic from axon terminals to the nuclei of neurons resident in peripheral ganglia, where the viral DNA is deposited. A detailed analysis of herpes simplex virus type 1 (HSV-1) transport dynamics in axons following entry is currently lacking. Here, time lapse fluorescence microscopy was used to compare the postentry viral transport of two neurotropic herpesviruses: HSV-1 and pseudorabies virus (PRV). HSV-1 capsid transport dynamics were indistinguishable from those of PRV and did not differ in neurons of human, mouse, or avian origin. Simultaneous tracking of capsids and tegument proteins demonstrated that the composition of actively transporting HSV-1 is remarkably similar to that of PRV. This quantitative assessment of HSV-1 axon transport following entry demonstrates that HSV-1 and PRV share a conserved mechanism for postentry retrograde transport in axons and provides the foundation for further studies of the retrograde transport process.

Herpes simplex virus type 1 (HSV-1) and the veterinary herpesvirus pathogen pseudorabies virus (PRV) establish latent infections within the peripheral nervous systems (PNS) of their hosts. Neurotropic herpesviruses gain access to the PNS at nerve endings present in infected skin or mucosal tissue. Upon entry at the nerve terminal, viral particles are transported in axons toward the neuronal cell body to ultimately deposit the viral genome into the nucleus. This process is referred to as retrograde transport and is critical for the establishment of latency. Following reactivation, progeny viral particles travel anterogradely from the ganglia toward the nerve terminals, resulting in reinfection of the dermis or other innervated tissues. Reactivated infection can manifest in various forms, including asymptomatic virus shedding or mild focal lesions (herpes labialis), or less frequently in more-severe disease (herpes keratitis, encephalitis, and in the case of varicella-zoster virus, shingles).

All herpesviruses consist of an icosahedral capsid that contains the viral genome surrounded by a layer of proteins known as the tegument, which is contained within a membrane envelope (33). HSV-1 and PRV capsids dissociate from the viral envelope (2, 13, 14, 22, 23, 25, 28, 30, 40) and several tegument proteins (13, 16, 21, 25) upon fusion-mediated entry into cells. However, following entry into epithelial cell lines, the VP1/2 and UL37 tegument proteins are detected in association with HSV-1 capsids at the nuclear membrane (16, 22, 23, 25, 28, 30, 40) and several tegument proteins (13, 16, 21, 25) upon fusion-mediated entry into cells. Reactivated infection can manifest in various forms, including asymptomatic virus shedding or mild focal lesions (herpes labialis), or less frequently in more-severe disease (herpes keratitis, encephalitis, and in the case of varicella-zoster virus, shingles).

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Although HSV-1 and PRV share similarities in their neurotropism in vivo (reviewed in reference 12), studies of axon transport have indicated possible mechanistic differences relevant to the underlying cell biology of neural transmission (reviewed in reference 10). As a result, a live-cell analysis comparing PRV and HSV-1 is needed to determine if axon transport mechanisms are conserved between the two neurotropic herpesviruses genera: Simplexvirus (HSV-1) and Varicellaviruses (PRV). In this study, the retrograde transport process that delivers capsids to the nuclei of sensory neurons was compared for HSV-1 (strains KOS and F) and PRV (strain Becker).

MATERIALS AND METHODS

Plasmid construction. Several plasmids were used as templates for PCR in a two-step bacterial artificial chromosome (BAC) recombination protocol (46). Plasmids pPE-EGFP-in and pPE-mRFP1-in, a kind gift from Nikolaus Osterrieder, were used to insert the monomeric fluorescent proteins GFP (green fluorescent protein) and mRFP1 (monomeric red fluorescent protein 1) into herpesvirus BAC clones. The pPE-mCherry-in plasmid was derived from pRSET-B/mCherry (35) by duplicating a portion of the mCherry open reading frame (ORF) and inserting the aphA1 gene (encoding resistance to kanamycin) and an I-SceI cleavage site between the duplicated sequences. This was achieved by amplifying nucleotides 1 to 472 of the mCherry ORF with primers 5'-GGG GATCCGATGACGGATGACGATGATAAGGTGAGCAAGGGC GAGG (BamHI site underlined) and 5'-GGATCCATG-GATTACAAGGATGACGACGATGATAAGGTGAGCAAGGGC GAGG (BamHI site underlined) and 5'-GGATCCATG-GATTACAAGGATGACGACGATGATAAGGTGAGCAAGGGC GAGG (BamHI site underlined) and 5'-GGATCCATG-GATTACAAGGATGACGACGATGATAAGGTGAGCAAGGGC GAGG (BamHI site underlined). The first primer encodes a FLAG epitope (DYKDDDDK) fused to the amino terminus of mCherry, allowing the optional inclusion of the epitope tag when one is inserting mCherry into BAC plasmids. The NsiI site derived from the second primer was used to clone the PCR product into an endogenous PstI site in the mCherry ORF, producing the optional inclusion of the epitope tag when one is inserting mCherry into BAC plasmids. The NsiI site derived from the second primer was used to clone the PCR product into an endogenous PstI site in the mCherry ORF, producing a 121-nucleotide duplication with a BglII restriction site at the center. The aphA1 gene and I-SceI cleavage site cassette from pPE-EGFP-in was subcloned into the BglII site by PCR amplification using primers carrying 5' BglII sites.

Virus construction. PRV-GS847 is a monofluorescent virus that encodes mRFP1 (6) fused to the VP26 capsid protein and has been described previously (39). All HSV-1 recombinants constructed for this study were made using a two-step recombination protocol (46) performed in the Escherichia coli strain GS1783, which encodes inducible Red and I-SceI activities. GS1783 was transformed with either the pKOS37 BAC clone (15) or the pYBac102 BAC clone (44), and recombination was targeted by homology sequences encoded in the 5' ends of PCR primer pairs. Primers for insertion of the mCherry ORF as a translational fusion to the 5' end of the HSV-1 UL35 ORF were 5'-CCGACA

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TABLE 1. Virus strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Fusion 1</th>
<th>Fusion 2</th>
<th>Titer (PFU/ml)</th>
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</thead>
<tbody>
<tr>
<td>PRV-GS847</td>
<td>mRFP1-VP26</td>
<td></td>
<td>3.0 \times 10^8</td>
</tr>
<tr>
<td>HSV KOS-37</td>
<td></td>
<td></td>
<td>1.0 \times 10^8</td>
</tr>
<tr>
<td>HSV KOS-GS3223</td>
<td>mCh-VP26</td>
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<tr>
<td>HSV F-YEbac102</td>
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<td></td>
<td>7.5 \times 10^6</td>
</tr>
<tr>
<td>HSV F-GS2822</td>
<td>mRFP1-VP26</td>
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<td>2.8 \times 10^8</td>
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<tr>
<td>HSV F-GS3351</td>
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<tr>
<td>HSV F-GS3330</td>
<td>GFP-VP11/12</td>
<td>VP1-2-GFP</td>
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<td>mRFP1-VP26</td>
<td></td>
<td>1.6 \times 10^8</td>
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</table>

F at a multiplicity of infection (MOI) of 2.0. Lysates were harvested in 500 μl of 2× final sample buffer (10 ml of 625 mM Tris [pH 6.8], 10 ml glycerol, 10 mg bromophenol blue, 20 ml 10% sodium dodecyl sulfate [SDS], 50 μl β-mercaptoethanol) at 1 day postinfection (dpi). Due to the low level of expression of the VP1-2-GFP fusion, 3.5 \times 10^9 Vero cells seeded in a 10-cm diameter dish were infected at an MOI of 0.3 with HSV F-GS2945 and were harvested in 500 μl of 2× final sample buffer at 5 dpi. All samples were boiled for 3 min; 15 μl of each was separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 4-to-20% polyacrylamide gel and was subsequently transferred to a polyvinylidene fluoride membrane (Pall). The membrane was incubated with mouse anti-GFP antibody B-2 (Santa Cruz Biotechnology) diluted 1:1,000, followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) diluted 1:10,000. The horseradish peroxidase signal was detected with luminol-coumaric acid-H_2O_2 chemiluminescence, and the exposed film was digitized using an EDAS 290 documentation system (Kodak).

Fluorescence microscopy and image analysis. All images were acquired with an inverted wide-field Nikon Eclipse TE2000-U microscope using automated fluorescence filter wheels (Sutter Instruments, Novato, CA), a Cascade 650 camera (Photometrics, Roper Scientific), and a 60× 1.4-numerical-aperture oil objective (Nikon). The microscope was housed in an environmental box maintained at 37°C (In Vivo Scientific). The Metamorph software package was used for image acquisition and processing (Molecular Devices, Downingtown, PA).

To image extracellular viral particles, Vero cells were seeded 1:10 onto coverslips and were infected the following day with 1 \times 10^5 PFU per coverslip. The MOI was difficult to calculate, because the cells were sparse at the time of infection. Two to three days postinfection, static fluorescence images of viral particles released from Vero cells onto bare regions of the coverslip were captured with sequential exposures to mRFP1 (1 s) and GFP (4 s). The presence of GFP-tagged emissions from individual capsid-containing extracellular viral particles was scored using a custom automated image-processing algorithm that detected particles based on GFP emissions and determined the presence or absence of corresponding GFP emissions from the same diffraction-limited emission source. Typically, 10 to 100 puncta were scored per image, with multiple images analyzed for each sample.

Live-cell imaging of infected primary sensory neurons or SK-N-SH cells was performed in sealed chambers as previously described (38). Viral transport in axons following entry was captured in either chick or mouse DRG explants or in differentiated SK-N-SH cells for up to 1 h postinfection. Neurons were infected with 6 \times 10^5 PFU per coverslip, and time lapse monofluorescent imaging of mRFP1 emissions was acquired at 10 frames/s (100-ms streaming exposures). Individual capsid transport velocities and run lengths were calculated by kymograph analysis. Runs were defined as uninterrupted diagonal lines and were measured for the distance traveled (length) and the average velocity (slope). The frequency of viral particle retrograde axonal transport was determined by counting the number of red fluorescent capsids entering a fixed field of view per minute.

Retrograde transport of capsids in axons was monitored by continuous sequential imaging of mRFP1 and GFP emissions using automated excitation and emission filter wheels (Sutter Instruments, Novato, CA) and 100-ms exposures for each channel. In some experiments, endocytic vesicles were labeled by incubating DRG explants with tetramethylrhodamine (TMR)-dextran as previously described (39). Labeled neurons were infected with a GFP-tagged monofluorescent virus, and time lapse imaging of TMR-dextran and GFP emissions was sequentially captured continuously for the first hour postinfection.

Nuclei present in the center of chick sensory DRG explants were imaged from 2 to 3 h following infection with dual-fluorescent viral strains. Capsid and tegument proteins that had accumulated at the nuclear rim were captured with sequential exposures to mRFP1 (1 s) and GFP (4 s).

RESULTS

Isolation of monofluorescent capsid-tagged virus strains. To study the dynamics of HSV-1 capsids in axons, a red fluorescent protein was fused in frame to the 5' end of the gene encoding VP26 (UL35) in two commonly studied strains: HSV-1 KOS (mCherry-capsid) and HSV-1 F (mRFP1-capsid). To directly compare HSV-1 capsid transport with that of PRV, a previously described mRFP1-capsid-encoding strain of PRV Becker was included in the study (39). All viruses were derived...
from bacterial artificial chromosome (BAC) clones of the respective viruses (15, 36, 44). The PRV Becker mRFP1-capsid virus replicates to wild-type titers (39), while the titer of the HSV-1 F mRFP1-capsid strain was reduced 3-fold and that of the HSV-1 KOS mCherry-capsid was reduced 8-fold relative to the respective unmodified strains (Table 1).

Viral capsid transport dynamics following entry into cultured neurons. Primary sensory DRG neurons isolated from E8-to-E10 chick embryos were infected with PRV Becker, HSV-1 F, or HSV-1 KOS encoding red capsids, and individual transported capsids were tracked at 10 frames/s (100-ms exposures) in axons by time lapse fluorescence microscopy during the first hour postinfection. Avian neurons were initially used for these experiments due to their consistent availability and ease of isolation, which allowed for routine imaging of transport events for the multiple viral isolates. Capsids were readily detected undergoing retrograde transport in chick sensory neurons, allowing for the tracking and analysis of more than 900 uninterrupted transport events, or runs, for each virus. As previously described for PRV, HSV-1 capsids displayed predominantly retrograde motion that was occasionally interrupted by pauses and short reversal events (39). Upon direct comparison, HSV-1 and PRV capsids were determined to move with strikingly similar dynamics (Fig. 1). The velocities of retrograde runs ranged from 0.5 to 5.0 \( \mu \text{m/s} \), with averages between 2.1 to 2.6 \( \mu \text{m/s} \) for all viruses (Fig. 1A). In previous reports, retrograde capsid transport velocities for PRV were reported to be closer to 1.0 \( \mu \text{m/s} \) (1, 7, 39). The reason for the

FIG. 1. Capsid transport dynamics in axons. Neurons were infected with red fluorescent capsid strains, and retrogradely transported capsids were imaged at 10 frames/s (100-ms exposures) in a 58.5-\( \mu \text{m} \) by 78-\( \mu \text{m} \) field for as long as 1 h following infection. (A) Histograms of capsid transport velocities in which each data point is the average velocity of a moving capsid during a single run. Values on the x axis are expressed in micrometers per second. The solid curve in each panel represents the Gaussian best fit, and the dashed curves represent the 95% confidence interval. The goodness of fit is indicated by the \( R^2 \) value. (B) Histograms of capsid run lengths. Values on the x axis are expressed in micrometers. The solid curve is the best-fit decaying exponential, and the dotted curves represent the 95% confidence interval. The goodness of fit is indicated by the \( R^2 \) value. Values below 2 \( \mu \text{m} \), which were underrepresented because our temporal resolution was insufficient to resolve short runs, were excluded from histograms and curve-fitting analyses but were included in the average run length (bar graph). Run lengths are underestimates due to the movement of particles out of the focal plane. Values above 30 \( \mu \text{m} \) were infrequent, in part due to the field size, and were not included in the histogram plots but were included in the average run length (bar graph). All histograms are labeled with the cell type at the top left and the viral strain at the top right. Bar graphs represent average capsid velocities (A) and run lengths (B). Letters above bars indicate the neuronal cell type (C, chick DRG; M, mouse DRG; H, human SK-N-SH).
increase in average velocity in the current study is unclear, but it may be explained by changes in the neuron medium formulation and improved neuron health following dissection. The rates reported here are consistent with rates of HSV-1 retrograde transport observed in squid axons (4).

Consistent with the previous PRV studies, transport velocity profiles for HSV-1 and PRV were accurately modeled by Gaussian distributions (an $R^2$ value of 1 would indicate a perfect fit), indicating that transport occurred at a single optimal rate (Fig. 1A). The average run lengths of individual capsid transport events ranged from 7.0 to 8.8 μm, with large standard deviations and frequency distributions that were well modeled by decaying exponentials ($R^2$ values ranged from 0.96 to 0.99 [Fig. 1B]). The rapid velocities and decaying exponential profiles of run lengths are both consistent with productive microtubule-based transport, and the tight Gaussian velocity profiles are indicative of the involvement of a single species of microtubule motor, most likely dynein (11, 42). When the data sets from PRV Becker, HSV-1 KOS, and HSV-1 F were combined and reanalyzed as a single data set, the transport velocity remained well modeled by a Gaussian distribution ($R^2, 0.99$) and the run lengths by a decaying exponential ($R^2, 0.99$), consistent with the equivalence of the retrograde transport dynamics of these viruses.

Although chick DRG neurons are productively infected by HSV-1 (17), infections in mammalian neurons were next examined in order to assess if avian neurons accurately modeled HSV-1 retrograde axon transport. HSV-1 F capsid trafficking was examined in primary DRG sensory neurons isolated from mice and in differentiated neuron-like human SK-N-SH cells. SK-N-SH cultures had fewer neuronal cells than primary explants, resulting in less-frequent capsid transport and fewer runs for analysis ($n = 105$). HSV-1 transport dynamics, in terms of both velocity and run length, were equivalent in avian and mammalian neurons, indicating that chick DRG are a good model of the HSV-1 retrograde trafficking in axons (Fig. 1).

The frequency of capsid retrograde transport events, or flux, was assessed for PRV Becker, HSV-1 KOS, and HSV-1 F in chick sensory DRG. Infected neurons were continually imaged during the first hour following infection, under the conditions described above, and the flux was determined by averaging the number of capsids entering the field of view per minute (Fig. 2). Because retrograde capsid flux is proportional to the amount of input viral inoculum, a normalized multiplicity of infection was maintained for these experiments. The HSV-1 KOS capsid transport frequency was reduced relative to those of PRV Becker and HSV-1 F, which showed similar flux. Whether the reduced HSV-1 KOS flux resulted from a defect in transport or a defect in an earlier stage of infection, such as entry, could not be differentiated in this assay.

Isolation and characterization of dual-fluorescent HSV-1 strains. In previous studies, simultaneous imaging of PRV capsids and tegument in axons identified a subset of tegument proteins that remain associated with the capsid during retrograde trafficking in neurons (7, 21). To similarly assess which tegument proteins are cotransported with HSV-1 capsids in axons, a panel of dual-fluorescent recombinant strains of HSV-1 F was produced. The coding sequence for GFP was inserted into one of several different tegument-encoding genes of the HSV-1 F BAC that was previously modified to encode RFP-capsids (mRFP1-VP26). Six of the major tegument proteins were examined: VP1/2 (pUL36), pUL37, VP11/12 (pUL46), VP13/14 (pUL47), VP16 (pUL48), and VP22 (pUL49) (Table 1). Dual-fluorescent recombinant strains with $>1$-log reductions in titer from that of the monofluorescent mRFP1-capsid virus were excluded from the study. Analysis of plaque size revealed minor reductions in the cell-cell spread of dual-fluorescent viral strains from that of the wild type, with the exception of VP1/2-GFP (36.7% of the wild type) and GFP-VP22 (55% of the wild type), which had more-significant spreading defects (Fig. 3). These two isolates were nevertheless included in the study, with the caveat that the results obtained with the VP1/2-GFP and GFP-VP22 viruses would be less conclusive, particularly if these recombinant viruses proved to behave differently than the previously described PRV equivalents (21).

Several assays were used to ensure that the GFP-tegument fusions were properly expressed. First, the presence of the fusion proteins in infected cell lysates was assessed by Western blot analysis using an antibody directed against GFP. Equal
lysate volumes were loaded onto denaturing polyacrylamide gels with the exception of VP1/2-GFP, which required a more concentrated sample to detect the full-length protein. The presence of two VP1/2-GFP-reactive bands was consistent with posttranslational modification of VP1/2 (27). Two additional faster-migrating bands were also detected for VP1/2-GFP, at ~90 kDa and ~45 kDa (Fig. 4); these may result from proteolytic cleavage (18). The other five viruses had prominent bands at the expected molecular mass for the encoded GFP fusion protein (Fig. 4). Second, the localization of each GFP-tegument protein following infection of Vero cells was consistent with the findings of previous immunofluorescence analyses (data not shown) (26, 27, 29, 31, 34, 50). Third, structural incorporation of the GFP-tegument fusions was monitored by fluorescence imaging of individual extracellular viral particles in situ on a coverslip that had been released from nearby infected Vero cells, as described previously (2, 21). For each virus strain, red fluorescence from individual capsid-containing particles was frequently coincident with GFP fluorescence emissions, indicating the presence of the GFP-tegument fusion protein in the extracellular viral particle (Fig. 5). A minority of particles were observed to emit red fluorescence only, reflecting a population in which the GFP-labeled protein is not present or is incorporated at levels below detection. Consistent with this, GFP-VP1/2, which had the lowest level of virion incorporation (76.5% of the total), also had the dimmest GFP emissions of the dual-fluorescent HSV-1 recombinants (data not shown) and the weakest reactivity in infected cell lysates (Fig. 4). In addition, several GFP-tegument proteins (GFP-VP11/12, GFP-VP13/14, VP16-GFP, and GFP-VP22) expressed from the dual-fluorescent HSV-1 viruses displayed particle-to-particle variability in fluorescence emission intensities, similar to the heterogeneity in tegument incorporation previously described for PRV (9, 21). Also like PRV, all HSV-1 dual-fluorescent viruses produced a subset of particles emitting GFP fluorescence only (Fig. 5), which we interpret as light particles (43).

**HSV-1 particle composition following entry into sensory neurons.** To determine if HSV-1 tegument proteins remain associated with capsids during retrograde axon transport, the dual-fluorescent strains of HSV-1 F were used to infect cultured chick DRG sensory neurons. Upon infection, mRFP1-capsids were readily detected moving retrogradely in axons.
with dynamics that were consistent with the monofluorescent viruses described in Fig. 1 (data not shown). Continuous sequential 100-ms exposures of mRFP1 and GFP emissions revealed that both VP1/2-GFP and GFP-pUL37 were cotransported with capsids. The frequencies of cotransport with mRFP1-capsids for VP1/2-GFP (75.5%) and GFP-pUL37 (92.2%) were consistent with the proportions of extracellular viral particles that had detectable GFP emissions: 76.5% for extracellular VP1/2-GFP particles and 93.1% for GFP-pUL37 particles (Fig. 5 and 6). Therefore, no evidence of dissociation of these two tegument proteins from capsids upon infection was obtained. In contrast, the remaining four tegument proteins examined were frequently observed in extracellular virions (Fig. 5) but were rarely associated with capsids that were being transported in axons (Fig. 6). Therefore, a subset of HSV-1 tegument proteins that includes VP1/2 and pUL37 remained associated with capsids that moved retrogradely in axons, and these proteins are good candidates for effectors of capsid transport. These results are consistent with those previously obtained with PRV, which also specifically retains VP1/2, pUL37, and US3 on capsids during transport, while losing VP11/12, VP13/14, VP16, and VP22 (7, 16, 21).

In contrast to PRV transport, a small subpopulation of HSV-1 mRFP1-capsids was transported together with GFP-VP11/12 (7.5% of the total) and VP16-GFP (12.2% of the total) (Fig. 7A) (21). In the case of VP11/12, the frequency of cotransport increased to 28.4% when GFP was fused to the C terminus (data not shown). The VP11/12-GFP dual-fluorescent virus was, however, excluded from this study due to a 125-fold reduction in virus titer from that of the wild type. In addition to the infrequent detection of GFP-VP11/12 and VP16-GFP cotransport with capsids, infection with these dual-fluorescent HSV-1 strains occasionally produced GFP signals that were moving retrogradely independently of capsids (Fig. 7B). This tegument-only retrograde transport was observed at the greatest frequency in the less-tolerated C-terminal fusion, VP11/12-GFP. To determine if GFP-VP11/12 and VP16-GFP signals resulted from endocytosis, sensory neurons labeled with the endosomal marker TMR-dextran were subsequently infected with monofluorescent strains of HSV-1 encoding either GFP-VP11/12 or VP16-GFP. Although this approach can detect endocytosed virus particles (39), no occurrences of TMR-dextran cotransport with GFP emissions from either of these viruses were observed (Fig. 7C).

To examine capsid and tegument association following transport, nuclei located in the cell bodies of chick DRG ex-
plants were imaged from 2 to 3 h following infection with dual-fluorescent strains of HSV-1. At this time point, individual mRFP1-capsid emissions had accumulated at nuclear rims. VP1/2-GFP and GFP-UL37 emissions were coincident with docked mRFP1-capsid signals at frequencies consistent with their incorporation into extracellular viral particles (Fig. 5 and 8). The remaining GFP-labeled tegument proteins were not detected in association with capsids that had accumulated at nuclear rims (Fig. 8).

**DISCUSSION**

Herpesviruses must deliver their DNA to the host nucleus in order to establish infection. This process is particularly challenging for neurotropic herpesviruses, which have to travel long distances in axons in order to reach the neuronal cell body. HSV-1 retrograde transport requires intact microtubules in neurons as well as in nonneuronal cells (20, 24, 42, 47, 48), suggesting a role for the cellular minus-end-directed motor dynein. In nonneuronal cells, newly entering HSV-1 capsids colocalize with cytoplasmic dynein, and capsid targeting to the nucleus is reduced when dynein function is disrupted by overexpression of the dynamitin component of the dynactin complex (11).

In this study, time lapse fluorescence microscopy was used to track individual translocating HSV-1 capsids following infection in neurons. Direct comparisons between PRV and two commonly studied strains of HSV-1 (KOS and F) revealed that retrograde transport properties are conserved between these representatives of the two neuroinvasive herpesvirus genera: *Simplexvirus* (HSV-1) and *Varicellovirus* (PRV). In addition, HSV-1 capsids moved with the same velocities and run lengths in primary sensory neurons from chickens and mice, as well as in a human neuroblastoma cell line that was differentiated in culture to extend axonal projections. These results indicate that the retrograde transport mechanism is sufficiently conserved across species to allow for productive interactions between the virus and the cellular transport machinery and that avian and rodent neurons accurately model the retrograde axon transport of HSV-1 that occurs in human cells.

Like PRV capsids, HSV-1 capsids were cotransported with the VP1/2 and pUL37 tegument proteins, and these complexes remained intact after reaching the nuclear membrane, while the VP11/12, VP13/14, VP16, and VP22 tegument components were predominantly lost prior to the onset of retrograde movement. Consistent with these findings, VP1/2 and pUL37 colocalize with HSV-1 capsids at the nuclear membranes of epithelial cells (8), and VP1/2 is required for DNA deposition into the nucleus (3, 18, 32). The data presented here complement these findings and further show that HSV-1 undergoes a disassembly process similar to that of PRV, with the resulting transport complexes conserved between the two viruses (21). However, subtle differences between HSV-1 and PRV were noted. In contrast to the findings for PRV in previous studies, the dissociation of HSV-1 VP16 and VP11/12 was not complete; a minority of HSV-1 capsids were cotransported with these proteins when they were fused to GFP. Coincidentally, GFP-VP11/12 and VP16-GFP fusions were also seen to move in axons independently of capsid signals.

Although the significance of these observations is unclear, they may be of value for considering the process by which HSV-1 establishes latency. The presence of a small amount of cotransported VP16 may help to explain why, upon initial seeding of the nervous system, some neurons establish an acute feedback loop that sends progeny virions back to peripheral innervated tissues, while other neurons become latently in-
fect ed (5, 45). The difference in neuron fate could be attributed to small doses of VP16 delivered to the neural soma by variable numbers of retrogradely moving capsids. Consistent with such a model, VP16 was absent from capsids docked at the nuclear membrane, indicating that this tegument protein is released from capsids following axon transport. Additional studies will be needed to determine the fate of the input VP16 and if, in fact, it is nuclear. However, alternative explanations of the VP16 and VP11/12 retrograde transport must also be considered. The finding that fusing GFP to the opposite end of VP11/12 both attenuates the propagation of the virus more than 100-fold and results in a nearly 4-fold increase in GFP emissions from capsids in transport suggests that the disassociation of VP11/12, and possibly that of VP16, can be impaired when the protein is fused to GFP.

The source of the transport of VP11/12 and VP16 in axons independently from capsids is mysterious. Our initial suspicion was that L-particles may be endocytosed occasionally at the terminal axon. However, the other tegument proteins examined (UL37, VP1/2, VP13/14, and VP22) are all constituents of L-particles yet were never observed moving in axons apart from capsids, and an endocytic tracer further helped to rule out this possibility. Whether this phenomenon has biological significance will require further study. We note that, consistent with the observations reported here, an earlier study of a truncated HSV-1 VP16-GFP fusion construct saw retrograde motion following microinjection into squid axons (4).

The conservation of VP1/2 and pUL37 association with HSV-1 and PRV capsids in transport suggests a functional role for these proteins in the recruitment of the dynein microtubule motor complex. Consistent with this, detergent-extracted HSV-1 virions having exposed VP1/2 and pUL37 bind dynein motor components when mixed with cytosolic extracts and show enhanced movement in an in vitro motility assay (49). However, because VP1/2 is required for HSV-1 and PRV propagation, its role in retrograde transport has not been directly assessed. The pUL37 tegument protein is also required for propagation of HSV-1, but low titers of PRV lacking pUL37 can be obtained and were recently found to deliver HSV virions having exposed VP1/2 and pUL37 bind to the nuclei of nonneuronal cells poorly, which may indicate a role for pUL37 in retrograde transport (19).

This study demonstrates that the processes of retrograde axon transport for HSV-1 and PRV are strikingly conserved and host species independent. Whereas the dynamics of HSV-1 retrograde transport in axons had not been examined in detail prior to this study, our understanding of the mechanism of HSV-1 egress from neurons is complicated by incongruous reports (reviewed in reference 10). We are pursuing a follow-up live-cell study comparing HSV-1 and PRV anterograde axon transport in order to better understand this late stage of neuron infection.

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