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Directional spread of an α-herpesvirus in the nervous system

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Abstract

Pseudorabies virus (PRV), an α-herpesvirus, is capable of spreading between synaptically connected neurons in diverse hosts. In this report, two lines of experimentation are summarized that provide insight into the mechanism of virus spread in neurons. First, techniques were developed to measure the transport dynamics of capsids in infected neurons. Individual viral capsids labeled with green fluorescent protein (GFP) were visualized and tracked as they moved in axons away from infected neuronal cell bodies in culture during egress. Second, the effects of three viral membrane proteins (gE, gI and Us9) on the localization of envelope, tegument, and capsid proteins in infected, cultured sympathetic neurons were determined. These three proteins are necessary for spread of infection from pre-synaptic neurons to post-synaptic neurons in vivo (anterograde spread). Us9 mutants apparently are defective in anterograde spread in neural circuits because essential viral membrane proteins such as gB are not transported to axon terminals to facilitate spread to the connected neuron. By contrast, gE and gI mutants manifest their phenotype because these proteins most likely function at the axon terminal of the infected neuron to promote spread. These two sets of experiments are consistent with a model for herpesvirus spread in neurons first suggested by Cunningham and colleagues where capsids and envelope proteins, but not whole virions, are transported separately into the axon. © 2002 Published by Elsevier Science B.V.

Keywords: Axonal transport; Virus assembly; Membrane proteins; Herpesvirus; Pseudorabies virus

1. Introduction

Most α-herpesviruses (e.g. herpes simplex virus, HSV; varicella-zoster virus, VZV; bovine herpes virus, BHV, and pseudorabies virus, PRV) are parasites of the peripheral nervous system (PNS) in their natural hosts. One remarkable feature of the α-herpesvirus life cycle is that after entering the PNS of their natural hosts, infection rarely spreads to the

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central nervous system (CNS). Instead, a reactivatable, latent infection is established in PNS ganglia. Normally, these PNS infections are relatively benign. However, when neonates or immunocompromised individuals experience infection, disseminated disease, often characterized by fatal CNS invasion, is a common outcome. In the case of PRV, fatal or debilitating CNS infection is a common result of infection of baby pigs and dead-end hosts (e.g. infection of cattle, dogs, and rodents).

In this report, we focus on the mechanisms by which PRV spreads in the nervous system of lab animals (rodents and chicken embryos). Infection of natural hosts usually initiates on exposed mucosal surfaces followed by invasion of PNS nerve termini that innervate the site of infection. When viral DNA reaches the nuclei of PNS neurons in ganglia, a latent infection is established within days. The latent infection can be reactivated and as a result virus almost always spreads back to mucosal surfaces where the primary infection began; spread to the CNS is exceedingly rare. Experiments discussed in this report are directed toward answering several basic questions: What are the virus- and cell-encoded mechanisms that direct virus into and back out of the PNS? Why does the virus only occasionally enter the CNS in its natural host and almost always in non-natural hosts? What are the viral gene products that promote disease and how do they work? How do the PNS and CNS respond to viral infection? These basic questions continue to lead us into exciting (and sometimes unexpected) areas.

2. Material and methods

2.1. Virus propagation

Virus stocks were produced using the porcine kidney cell line PK15 cell line as previously described (Whealy et al., 1993). The laboratory strain PRV Becker (PRV-Be) was used as the wild type and parent for all virus constructions (Card et al., 1990). PRV 160 (Us9 null) has been described (Brideau et al., 2000a,b). PRV 91 (gE null) has been described (Whealy et al., 1993). PRV 160R is a revertant of PRV 160 that restores all tested wild-type virus phenotypes (Brideau et al., 2000a). The PRV-Becker *UL35* gene was cloned and a recombinant virus, PRV-GS443, carrying the fusion allele in place of the wild-type *UL35* gene was made using the pBecker3 infectious *E. coli* clone (Smith and Enquist, 2000; Smith et al., 2001) RV 368 carries both the Us9 null mutation from PRV 160 and the *GFP–VP26* hybrid gene from PRV-GS443 replacing the *VP26* gene. PRV 368 was isolated as a recombinant arising after co-infection of PRV 160 and PRV-GS443 (Tomishima and Enquist, submitted for publication).

2.2. Neuron culture and infection

For video confocal microscopy: dissociated sensory neurons from the dorsal root ganglia (DRG) of E8–E10 chick embryos were seeded on 22 mm square glass coverslips pretreated with polyornithine at ~100 neurons per coverslip as described by Smith et al. (2001). The neurons were cultured for 3–5 days to allow for axon outgrowth at which time they were infected with PRV-GS443. The number of neurons on each coverslip was low to prevent

axons from contacting each other. As a result, most of the input virions bound directly to the coated coverslip and never came into contact with the neurons.

Rat sympathetic neurons from the superior cervical ganglia (SCG) of rat embryos (15–16 day gestation) were dissociated according to the methods of DiCicco-Bloom et al. (1993). The 35 mm plastic dishes were coated with 100 ug/ml poly-D-lysine, and approximately 80,000 neurons were put down on each dish. Dividing cells were eliminated by adding cytosine-ß-D-arabino-furanoside, to 2 μM from days 2–3 and 5–6.

Experimental protocols were approved by the Animal Welfare Committee at Princeton University and were consistent with the regulations of the American Association for Accredition of Laboratory Animal Care and those in the Animal Welfare Act (public law 99-198).

2.3. Confocal video microscopy

For time-lapse recording of living cells, individual coverslips of infected chick embryo neurons were sealed onto a glass slide as described by Smith et al. (2001). GFP emissions from infected neurons at 37 °C were detected with a Zeiss 510 laser scanning confocal microscope fitted with a heated stage and a heated 63× 1.4NA oil objective. Excitation was at 488 nm with an argon laser, and up to 1000 frames were captured per recording.

2.4. Indirect immunofluorescence microscopy

Chick neurons were cultured as described above. Rat neurons were cultured in 35 mm plastic dishes for at least 3 weeks as described above. Cultures were infected as described above for chick neurons. At various times after infection, neurons were rinsed with phosphate buffered saline (PBS), fixed with 3.2% paraformaldehyde, and then rinsed three times with PBS. The neurons were permeabilized for 3 min in PBS containing 3% BSA and 0.5% saponin. Fixed cells were then incubated with the appropriate primary antibody for 1 h in a humidified chamber at 37 °C, incubated with Alexa 488- or Alexa 568-conjugated secondary antibodies (Molecular Probes) for 1 h in a humidified chamber at 37 °C, and rinsed three times with PBS/BSA/saponin and once with distilled water. A drop of Vectashield Mounting Medium (Vector Laboratories Inc., H 1000) was placed in the floor of the dish, and then a coverslip was placed on the Vectashield and sealed with nail polish. GFP-capsids were excited with a 488 nm argon laser, and Alexa 546 was excited with a 543 nm HeNe laser. Single optical sections were captured using a Nikon Optiphot-2 microscope equipped with a BioRad MRC600 scan head.

3. Results

3.1. Visualizing axonal transport of newly replicated capsids in living sensory neurons

This work has been described recently in Smith et al. (2001). The DNA of all α-herpesviruses is packaged in an icosahedral capsid shell made up of four virally encoded proteins, VP5, VP26, VP19C and VP23. VP5 is the major capsid protein and forms the

capsid hexons and pentons. VP26 binds to the outer surface of hexons, but not pentons, while VP19C and VP23 are part of the heterotrimeric complex joining VP5 hexons (Conway et al., 1996; Schrag et al., 1989; Zhou et al., 1999). An assortment of additional viral proteins, collectively called the tegument, surround the capsid, which in turn is enclosed by a lipid bilayer containing many virally encoded membrane proteins. When green fluorescent protein (GFP) is fused to the N-terminus of VP26 in HSV-1 or PRV, the fusion protein not only is incorporated into capsids, but also has no detectable effect on assembly and growth of infectious virus in cultured cells (Desai and Person, 1998; Smith et al., 2001). The GFP-VP26 containing capsids are stable, easily purified, and, indeed, individual capsids emit sufficient fluorescence to be observed as point sources of light that can be visualized with the short exposure times necessary to perform rapid time-lapse microscopy (Smith et al., 2001).

3.2. Kinetic analysis of capsids moving in axons

As reported by Smith et al. (2001) newly replicated PRV capsids entering axons have net anterograde movement (i.e. from the cell body to the axon terminus; Fig. 1; Smith et al., 2001). Despite this net anterograde movement, capsid motility is bi-directional. Indeed, at any given instant, about 1 capsid in 7 moves back toward the cell body (retrograde movement). About 1/3 of the capsids move, stop and move again in the same direction. Reversals of movement are near instantaneous; there is no obvious lag before reverse movement begins (Fig. 2). The velocity of capsid movement in the anterograde direction is 1.97 μm/s and the reverse velocity in the retrograde direction is 1.28 μm/s. These speeds are compatible with known motion produced by kinesin motors (fast anterograde movement) and dynein motors (fast retrograde movement). Therefore, not only do anterograde motors associate with each moving capsid, retrograde motors also must be present to account for rapid reversals. A noteworthy finding was that the distance traveled in either the anterograde or retrograde direction was rather long (about 10 μm on the average for anterograde and 7 μm for retrograde movement). When the distance traveled in the anterograde direction was analyzed for many capsids, the data was best fit by a single decaying exponential equation. This data suggests that at any given time, a capsid has an equal probability of stalling or reversing no matter how far it has moved. Such processivity is well known for kinesin and dynein motor movement (Block et al., 1990; Howard et al., 1989; King and Schroer, 2000). The average distance calculated for anterograde capsid travel predicts a rather long run length of 10.7 ± 1.3 μm. A single kinesin motor moving cargo on microtubules in vitro has a processivity factor of about 1 μm (Block et al., 1990).

3.3. Anterograde flux of capsids

The average number of capsids entering the field of view (per unit time) moving in an anterograde direction varied significantly from axon to axon, but did not change appreciably with time post infection. The observed anterograde flux within individual axons displayed a roughly Gaussian distribution and ranged from 0.010 to 0.067 capsids/s, with a mean of 0.047 ± 0.005 capsids/s (i.e. on average one capsid entered the field every 21 s). The average retrograde flux was 0.0072 ± 0.0015 capsids/s (i.e. one capsid every 139 s).

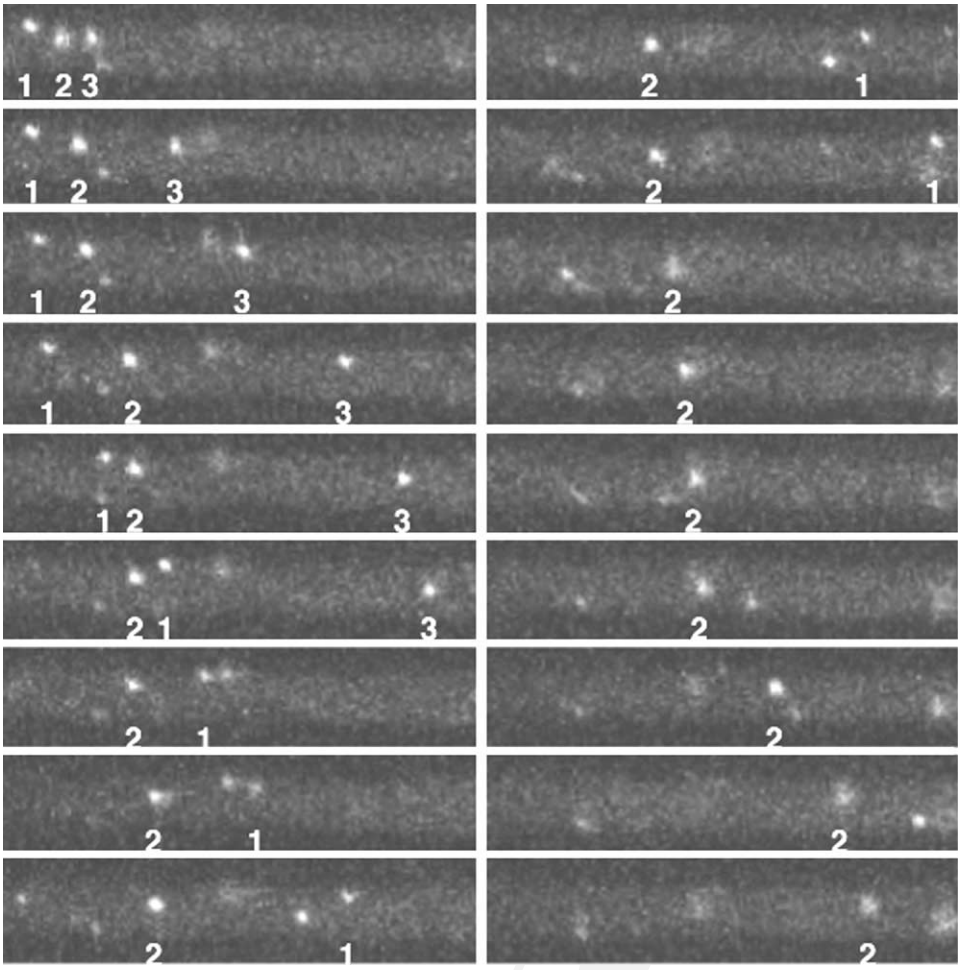

Fig. 1. Capsid transport during egress is saltatory. Time-lapse recording of GFP-capsids during viral egress in an axon of a cultured sensory neuron (11 h after infection). Frames are at 2-s intervals, and are ordered from top to bottom, and left to right. Several capsids progressed through the axon in the anterograde direction (from left to right—the cell body was to the left of the recorded region). Three capsids (numbered 1–3) remained in the focal plane throughout the majority of the recording. The motion of capsids nos. 1 and 2 was saltatory, showing periods of no movement, while capsid no. 3 maintained continuous movement.

3.4. Capsids stall in axons as infection proceeds

Up to about 8 h after infection, despite copious synthesis of viral proteins in the neuronal cell bodies, few, if any virion proteins can be detected in axons. After 10 h of infection, capsids were easily detected in axons. Many of these capsids were moving, but some were stalled (see Fig. 3, Smith et al., 2001). At 10 h after infection about 5 capsids were stalled (not moving during observation) per 30 μm of axon. By 14 h after infection the number of stalled capsids had increased about 5- or 6-fold. On average, about 3% of the total

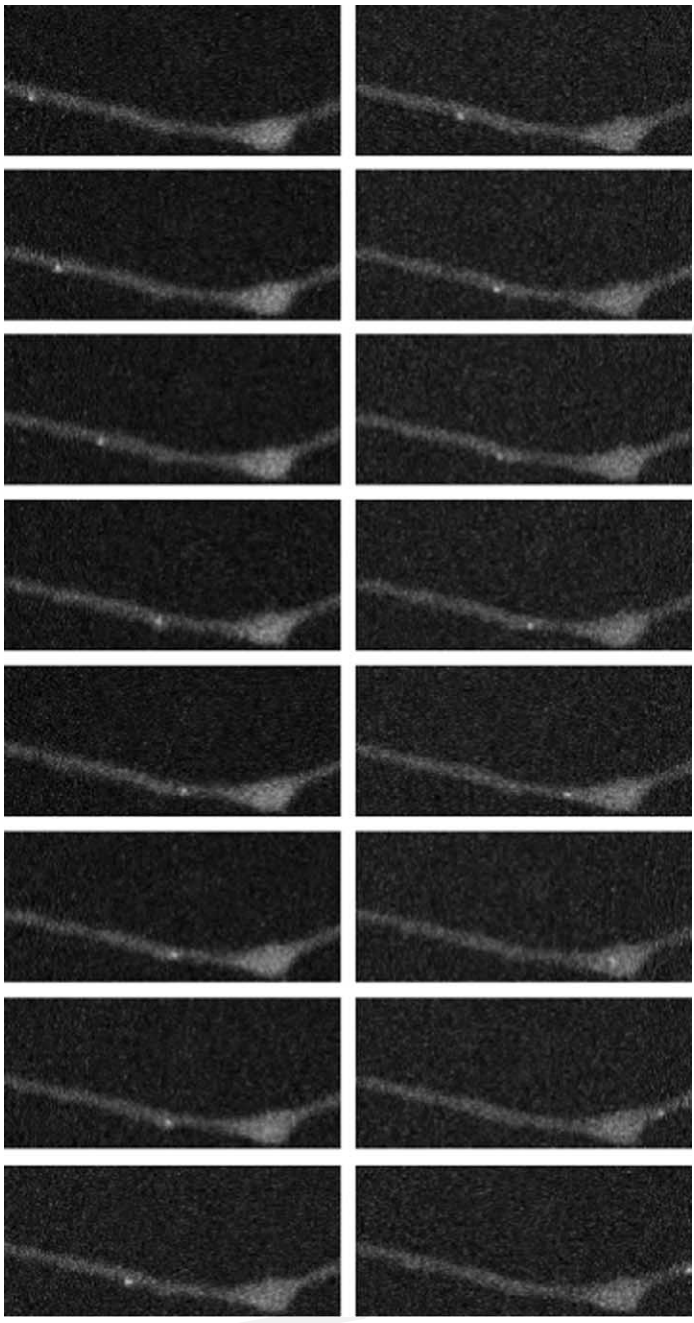

Fig. 2. Capsids reverse direction during egress. Time-lapse recording of a single GFP-capsid undergoing egress in an axon of a cultured sensory neuron (12 h after infection). Frames are 1-s intervals, and are ordered top to bottom, and left to right. The capsids motion begins in the anterograde direction, suddenly reverses but maintains processivity, then returns to anterograde motion leaving the field of view to the right.

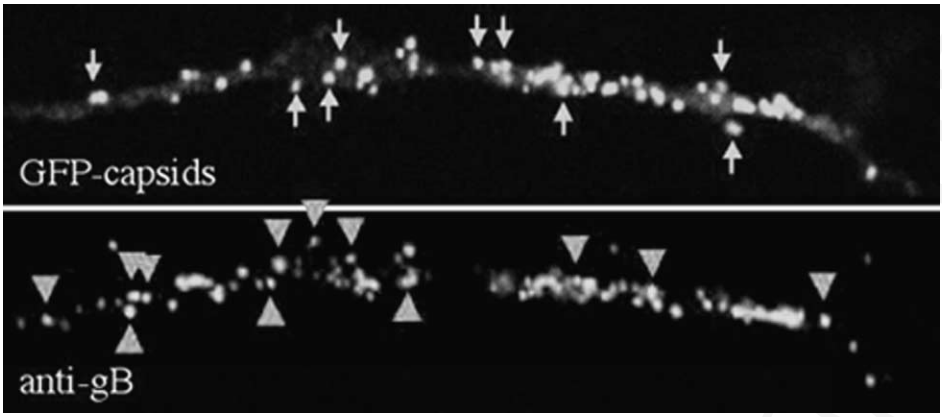

Fig. 3. Localization of capsids and a viral membrane protein in an axon terminal. An axon terminal of a cultured sensory neuron infected with the GFP-capsid virus (upper panel is endogenous GFP signal from capsids) was fixed at 12 h after infection and processed for immunofluorescence for the gB viral membrane protein (lower panel). The axon ends in a growth cone at right. The capsid and gB signal are often colocalized, especially towards the growth cone. In the axon shaft, capsid and gB signals often do not colocalize. Arrows indicate capsids lacking gB signal (upper panel); arrowheads indicate gB punctae not associated with a capsid (lower panel).

anterograde capsid flow stop moving during an infection. The majority of capsids tended to stall and accumulate in the proximal axon (near the cell body), at varicosities in axons, and at axon terminals (see Fig. 4; Smith et al., 2001). An important conclusion from these studies is that when observing fixed preparation, one cannot be sure if any given capsid is stalled or moving in axons.

3.5. Capsids accumulate at axon terminals

As noted above, capsids move from cell bodies toward axon termini. These termini are not sites of synaptic contact as there are no targets for synapse formation in these cultures (Smith, 1998). Therefore, it was of some interest to observe what happens when capsids reach the axon terminus. As described by Smith et al. (2001) every terminal examined, newly arriving capsids were retained and accumulated over time. No capsids reversed direction to enter axons after reaching the terminal, and unexpectedly, no capsids could be observed emerging from the terminal at any time. The significance of this observation will be discussed later.

3.6. Us9 mutants provide evidence for separate transport of envelope proteins and capsids in axons

Inactivation of any one of three viral membrane proteins (gE, gI and Us9) blocks spread of infection from pre-synaptic to post-synaptic neurons in vivo (anterograde spread) (Enquist et al., 1998; Mettenleiter, 2000). Brideau et al. (2000a) argued that Us9 affects a different process of virus spread than gE or gI because Us9 null mutants have no phenotype

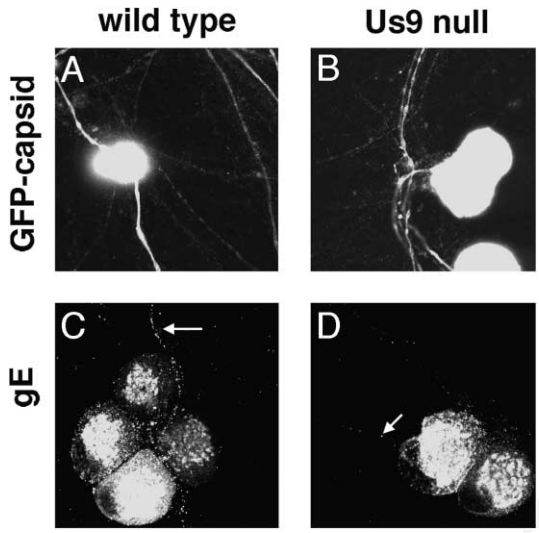

Fig. 4. Us9 is necessary to transport membrane proteins but not capsid into the axon. Dissociated neurons from the rat superior cervical ganglia were allowed to mature in culture for 21 days. In the first experiment, all the neurons in a culture were infected with either GS448 (wild type with GFP-VP26 capsid fusion—A) or PRV 368 (Us9 null virus with GFP-capsid fusion—B) for 8 h, and then were fixed and permeabilized. An antibody against GFP revealed the subcellular localization of capsids. The loss of Us9 expression does not change the localization of capsids (compare A and B). In the second experiment, approximately 10% of the neurons in a culture were infected for 12 h with the wild type or Us9 null virus, and an antibody against gE was used. Us9 null infections led to a defect in the accumulation of gE in the axon compared to the wild type (compare C and D). The arrow in C points to an axon is filled with vesicles containing gE. The arrow in D points to a vesicle that localizes to the axon in Us9 null infections. These vesicles are rare, and do not travel far from the cell body of infected neurons.

in tissue culture cells whereas gE/gI null mutants often form smaller plaques. Recently, we have obtained evidence that Us9 acts at a different step from gE and gI during virus spread in cultured neurons (Tomishima and Enquist, submitted for publication).

Us9 is an abundant 98 amino acid type II membrane phosphoprotein. In addition, to being a structural protein found in virion envelopes, Us9 is found in infected cell membranes, accumulating in small vesicles in or near the trans-Golgi network (Brideau et al., 1999). We considered two models to explain the inability of Us9 mutants to spread from pre-synaptic to post-synaptic neurons. In the first model, Us9 functions at the synapse to promote egress of virions so that the post-synaptic cell can be infected. In the second model, the Us9 protein functions at the cell body to promote virion entry into axons. If the first model has merit, all viral structural proteins will appear in axons after Us9 mutant infection. If the second model is correct, no virion proteins will appear in axons after Us9 infections. Neurons were infected and at various times after infection with the wild type or Us9 mutants and the presence or absence of viral structural proteins in axons was determined by immunofluorescence microscopy.

The data were consistent with the second model: viral membrane proteins were absent from axons after Us9 null mutant infection. However, the second model is not strictly correct, as capsid and tegument proteins were in axons after Us9 null mutant infection

(Fig. 4). Thus, Us9 protein is required for appearance only of viral membrane proteins in axons, but not capsid or tegument proteins.

We now can understand to a first approximation why Us9 mutants cannot spread to post-synaptic cells in animals: critical membrane proteins such as gB known to be required for spread from neuron to neuron (Babic et al., 1993) do not move to axon terminals to promote viral spread. In addition, these data also support and extend the Cunningham model (Penfold et al., 1994). Viral membrane proteins not only are transported separately from capsids in axons, but also specific viral proteins regulate transport of membrane proteins.

4. Discussion

4.1. The first analysis of anterograde movement of individual capsids in infected axons

Viral components leaving the neuronal cell body move toward the plus-end of microtubules to reach the axon terminal (Smith et al., 2001). By direct observation of individual egressing capsids in axons of cultured sensory neurons, transport was found to be bi-directional with net transport in the plus-end direction. While capsid transport velocity was similar in both directions (average plus-end velocity = 2.0 μm/s; average minus-end velocity = 1.3 μm/s), transport in the plus-end direction was more frequent and processive (capsids tended to travel longer distances before stopping) than in the minus-end direction. Because individual capsids can reverse direction seemingly instantaneously (observations are currently limited to temporal resolutions of 10 frames/s), and in some instances at high frequency, capsids must simultaneously associate with both plus- and minus-end directed microtubule motors. Further work is required to determine if the plus- and minus-end motors associate with the capsid independently of each other (e.g. do they bind at separate penton vertices or do they bind as a complex?). In addition, the role of bi-directional movement during egress remains to be elucidated.

4.2. Viral egress from infected neurons

Based on experience with non-neuronal cell lines, we expected that virus would egress from the terminals and this event would be seen by capsids disappearing from the terminal. However, capsids accumulated at terminals and there was no apparent egress of virus. We determined that the lack of egress did not reflect lack of membrane proteins; Smith et al. (2001) observed that gB, gC, gE and gI were transported to and accumulated at terminals (see Fig. 4 in Smith et al. (2001)). Herpesviruses are often considered to spread from cell to cell by releasing infectious virions; however, propagation of infection can also occur by a less well understood mechanism of direct cell-to-cell transmission that is independent of the release of infectious virions from cells. If fully assembled virions do, in fact, accumulate in axon terminals, this may indicate that spread from the axon terminal is restricted to the direct cell-to-cell mechanism. In the absent of a target cell in close contact to the axon terminal, virions may be blocked for release. Thus, this fortuitous observation could provide important insights into the mechanism of direct cell-to-cell transmission, as well as viral spread in the vertebrate nervous system.

4.3. Membrane proteins and capsids are often separate in axons

One model for how virions move from cell body into axons and then to axon terminals requires that virions be completely assembled in the neuronal cell body, engulfed by a transport vesicle, moved into axons, and transported long distances to the axon terminus for egress by exocytosis. An alternative model, proposed by Penfold et al. (1994), is that assembly of virions destined for the axon terminal does not take place in the cell body. Rather, viral membrane proteins are moved into axons and transported separately from virion capsid and tegument proteins. Assembly of infectious virions then occurs at or near the axon terminus. Our observations are compatible with the second model: by immunofluorescent microscopy, membrane proteins often do not colocalize with capsids in the axonal shaft (Fig. 3); by genetic analysis, Us9 mutants transport capsids and tegument into axons, but not viral envelope proteins.

5. Conclusions

The work of Smith et al. (2001), as summarized in the first part of this report, represents the first direct measure of viral anterograde transport kinetics in infected neurons for any virus. Newly replicated individual herpesvirus capsids (~125 nm diameter) bearing multiple copies of the GFP can be visualized and tracked in infected neurons by laser-scanning confocal time-lapse microscopy. Viral transport in axons is bi-directional and highly processive (continues over large distances without stopping). Based on processivity measurements and rapid reversals of movement, both plus- and minus-end motors are likely to interact simultaneously with moving capsids and membrane proteins. Capsids travel long distances to ultimately accumulate at axon terminals, suggesting that virions do not bud out of cultured chick embryo sensory axon terminals by a cell autonomous mechanism.

Our studies of wild-type and mutant PRV infections in rats, mice, and chicken embryos demonstrates that directional spread of virus in the nervous system is regulated, in part, by several viral genes. In particular, our lab has focused on the gE, gI and Us9 proteins, all of which affect anterograde spread of virus in a defined neural circuit. Based in part on work presented here, we propose that at least two distinct processes are involved in directional spread: (1) movement of virion components into axons and long distance movement of these components to axon terminals; and (2) interactions between the infected axon terminals and connected cells that promote assembly, virus release, or both. The work of Tomishima and Enquist, described in the second part of this report, indicates that the small type II membrane protein called Us9 affects the first process: it promotes the movement of virion envelope (but not tegument or capsid) components into axons. The type I viral membrane proteins gE and gI also are required for directional spread in the nervous system (for review, see Enquist et al., 1998; Mettenleiter, 2000), but the mechanism for their action is distinct from that of Us9. We speculate that gE and gI affect the second process, spread between the infected neuron and cells in close contact based on known roles of these proteins in cell–cell spread between non-neuronal cells. gE expression also exacerbates symptoms and speeds time to death in vivo, but this phenotype is not correlated with virus

spread (Yang et al., 1999). Ongoing experiments with fluorescent protein fusions to Us9, gE, gI, and other membrane proteins, as well as capsid and tegument proteins enable us to test our predictions.

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