Pseudorabies Virus Us9 Directs Axonal Sorting of Viral Capsids

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Pseudorabies virus (PRV) mutants lacking the Us9 gene cannot spread from presynaptic to postsynaptic neurons in the rat visual system, although retrograde spread remains unaffected. We sought to recapitulate these findings in vitro using the isolator chamber system developed in our lab for analysis of the transneuronal spread of infection. The wild-type PRV Becker strain spreads efficiently to postsynaptic neurons in vitro, whereas the Us9-null strain does not. As determined by indirect immunofluorescence, the axons of Us9-null infected neurons do not contain the glycoproteins gB and gE, suggesting that their axonal sorting is dependent on Us9. Importantly, we failed to detect viral capsids in the axons of Us9-null infected neurons. We confirmed this observation by using three different techniques: by direct fluorescence of green fluorescent protein-tagged capsids; by transmission electron microscopy; and by live-cell imaging in cultured, sympathetic neurons. This finding has broad impact on two competing models for how virus particles are trafficked inside axons during anterograde transport and redefines a role for Us9 in viral sorting and transport.

MATERIALS AND METHODS

Cells and virus. Porcine kidney cells (PK15) were maintained at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and PenStrep (HyClone) in a 5% CO₂ environment. All PRV strains were propagated on PK15 cells at a low multiplicity of infection for 48 h and then collected by scraping cells into the conditioned medium. To normalize the pH of the virus sample, sterilfiltered 1 M HEPES buffer was added to a final concentration of 10 mM before freezing aliquots at −80°C. The wild-type PRV strain Becker and its derivatives PRV 160, PRV GS443, PRV 368, and PRV 180 were described previously (3, 6, 11, 25, 28). PRV 160 (Us9-null) contains a nonsense stop mutation at position 4 in the Us9 open reading frame. GS443 expresses green fluorescent protein (GFP) fused to the capsid protein VP26. PRV 368 was constructed by recombination between PRV 160 and GS443, yielding a Us9-null virus expressing GFP-VP26. PRV 180 expresses red fluorescent protein (RFP) fused to the capsid protein VP26.

Antibodies. The antibodies used here include polyclonal rabbit antisera recognizing the gE cytoplasmic domain (27) (used at 1:400 by immunofluorescence [IF]); polyclonal goat antisera recognizing gB (31) (used at 1:200 by IF); mouse monoclonal antibody to PRV major capsid protein (made by Alex Flood at the Princeton Monoclonal Antibody Facility; used at 1:100 for IF). All secondary Alexa fluorophores (used at 1:500) were purchased from Molecular Probes.

Neuronal cultures. Detailed protocols for dissecting and culturing neurons are found in Ch’ng et al. (8). Briefly, sympathetic neurons from the superior cervical ganglia were dissected from embryonic day 15.5 (E15.5) to E16.5 pregnant Sprague-Dawley rats (Hill-Top Labs, Inc., Pennsylvania) cut in half with dissection knives and plated within a 35-mm plastic tissue culture dish on top of a square of Aclar (Electron Microscopy Sciences, Pennsylvania) coated with 300...
µg of poly-€-ornithine (Sigma Aldrich) is used to prepare the surface for growing neurons.

**Confocal microscopy and live imaging.** Fixed samples were imaged with a Leica SP5 laser scanning confocal microscope. Images were taken with a 40x oil immersion objective, and z-stacks of 10-12 images were taken at 0.2-µm intervals. Images were processed using ImageJ software or Adobe Photoshop. The number of puncta undergoing axonal transport in neurons infected with PRV Becker was calculated using the number of puncta as a ratio to the total number of cell bodies in a field of view. Results were analyzed using one-way ANOVA followed by Tukey’s post hoc test.

**RESULTS**

We have studied extensively the directionality, transneuronal spread of PRV in the rat visual system (6, 7). In these studies, virus is injected into the vitreous humor of the rat eye where it infects retinal ganglion cells. Infection then spreads in an anterograde fashion (presynaptic to postsynaptic neurons) to all regions of the brain that receive retinal input. Immunohistochemical staining of viral antigens is then performed on sliced, fixed tissue from infected animals, typically a tedious process. Feierbach et al. recently reported the use of a facile in vitro chamber system that recapitulates the transneuronal spread phenotypes of several PRV mutants (15). Ganglion explants are plated and allowed to extend axons for 1 week. A nonseptated, Teflon chamber disk is placed on top of the axons thereby capturing a subpopulation of axon ends. Dissociated neurons are then plated inside the chamber ring and allowed to form connections with the explant axon termini. This anisolated population of both presynaptic and postsynaptic neurons can be established.

We have reported that deleting Us9 precludes anterograde, transneuronal spread through anterograde circuits in vivo, while trafficking through retrograde circuits is unaffected (3). To test whether a Us9-null mutant was unable to spread from primary to secondary neurites using the in vitro chamber system, we infected the SCG explants on the outside of the chamber either with wild-type PRV Becker or with Us9-null mutants. After 24 h, the dissociated SCG neurons inside the chambers were examined by indirect IF (illustrated in Fig. 1A). After infection with PRV Becker, >70% of second-order neurites inside the chamber reacted readily with antisera specific for viral glycoproteins gE and gB, as well as the major capsid protein VP5 (Fig. 1B, top row). This was determined by calculating the number of immunopositive cell bodies as a ratio to the total number of cell bodies in a field of view (n = 20). In contrast, when explants were infected with the Us9-null virus,
dissociated neurons inside the chamber ring showed no reactivity with gE, gB, or VP5 (Fig. 1B, middle row). However, Us9-null mutants were capable of efficient infection of explant neurons as determined by strong reactivity with antiserum against gE, gB, and VP5 (Fig. 1B, bottom row). We did note a uniform, nonspecific “speckle” pattern in some samples labeled with the anti-gE antibody. This pattern was present in mock-infected cells and corresponded to areas of high cell density (e.g., near the SCG explant). However, it was not a confounding factor in the interpretation of our results. Overall,
these findings are consistent with the inability of Us9-null infections to spread through anterograde circuitry of the rat visual system (3).

Tomishima and Enquist speculated that the Us9 transneuronal spread phenotype reflected the lack of sorting of viral glycoproteins into the axon of infected neurons. However, they reported that in Us9-null infections, capsids, and tegument proteins entered the axon unimpeded (28). To confirm the finding in our in vitro chamber system, we imaged the axon shafts emanating from the SCG explant physically isolated inside the chamber (Fig. 2A, arrowhead). Axons originating from Becker-infected explants showed strong reaction with antisera specific for gE, gB, and VP5 (Fig. 2B, top row). Some puncta labeled with VP5 antibody also costained with gE and...
gB antisera, suggesting that capsids were transported together with viral glycoproteins (Fig. 2B, inset). In contrast, axons originating from explants infected with the Us9-null mutant did not react with antisera against gE or gB, which is consistent with previous observations (28). Surprisingly, we did not detect any reaction with VP5 antibodies, suggesting that sorting of viral capsids into axons is, in fact, dependent on Us9 (Fig. 2B, bottom row).

To confirm and extend these unexpected findings, we first infected SCG explants on the outside of the chamber with PRV GS443, a recombinant PRV strain that expresses GFP fused to VP26, a capsid protein (25). This fusion protein efficiently assembles into capsids that can be visualized as uniform green puncta in live imaging and fixed preparation studies. We and others have used PRV GS443 for assessing capsid trafficking kinetics in axons (25), virus particle composition during anterograde and retrograde transport (1), and actin/assembly body formation in the nucleus of infected neurons (16). After 24 h, capsid puncta had traveled from the explant and were readily detected in axons inside the chamber (Fig. 3B). Importantly, when explants were infected with PRV 368, a GFP-VP26-expressing mutant with Us9 deleted, no green puncta were detected inside the chamber, although an extensive network of axons was visible (Fig. 3C). This was determined by analyzing multiple fields of view (n = 20) of duplicate samples at high magnification.

FIG. 3. GFP-tagged capsids do not enter the axon in the absence of Us9. (A) A chamber ring was placed on top of preformed axons emanating from the SCG explant to physically separate the site of infection from the site of imaging. No dissociated SCG neurons were plated inside the chamber. The arrowhead highlights the region where images were taken at high magnification within the chamber. (B and C) Explants were infected for 24 h with PRV GS443 (GFP-VP26) (B) or PRV 368 (GFP-VP26, Us9 null) (C) and fixed with 4% paraformaldehyde. Direct fluorescence of explants outside the chamber (×20 magnification) and capsids inside the chamber (×60 magnification) was visualized by using spinning-disk confocal microscopy.

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We next used transmission electron microscopy to determine whether capsids were present in the distal axons of infected explants. Explants on the outside of the chamber were infected with PRV Becker or PRV 160 (Us9-null) and then imaged directly inside the chamber ring 24 h postinfection. A total of 128 GFP-VP26 puncta were observed moving in the anterograde direction in PRV GS443-infected neurons, entering the field of view by, on average, 4.6 ± 1.6 capsids/min (Fig. 5A; see also Movie S1 in the supplemental material). Despite extensive analyses, no capsid puncta were observed in the axon of neurons infected with PRV 368, although GFP-VP26 signal was clearly visible in the soma (Fig. 5B; see also Movie S2 in the supplemental material). To test whether the axonal sorting defect in PRV 368 was due to the lack of Us9 protein, we coinfected neurons with PRV 368 and PRV 180 (a Becker recombinant expressing mRFP-VP26) (11). We could easily detect green, red, and yellow puncta moving in the anterograde direction in coinfected neurons (Fig. 6 and see also Movie S3 in the supplemental material). We conclude that PRV 368-infected neurons can sort and move GFP-VP26 capsid puncta in their axons when complemented in trans by a Us9-expressing virus recombinant.

**DISCUSSION**

We report here that the axonal targeting of PRV capsids is dependent on the viral membrane protein Us9. We confirmed this observation by indirect IF (Fig. 2), direct fluorescence of GFP-tagged capsids (Fig. 3), transmission electron microscopy (Fig. 4), and live-cell imaging in cultured, sympathetic neurons (Fig. 5 and 6). Our findings raise a key question: why did we obtain such disparate results using essentially the same reagents as Tomishima and Enquist (14, 28)? In short, the answer is that imaging the proximal axon of infected neurons, as was done in the previous studies, can be misinterpreted, particularly when indirect IF is used at late times after infection. For example, Tomishima and Enquist fixed neurons infected with PRV GS443 and PRV 368 at 17 hpi and used anti-EGFP antibodies to enhance GFP signal in axons (28). We believe that the signal observed by Tomishima and Enquist was unassembled GFP-VP26 protein and not assembled capsids. This free GFP-VP26 in the proximal axon confounded interpretation of “capsid” localization in axons infected with a Us9-null virus, especially when images were obtained at low magnification (14, 28). We first came to this idea from live neuron imaging studies with PRV GS443 and PRV 368. We consistently observed a diffuse green fluorescent signal building in the proximal axon late in infection (>12 h postinfection). Indeed, by 16 h postinfection, this diffuse signal was strong enough in the proximal segment that we could visualize the connection of cell bodies to cognate axons. The general phenomenon of entry of unassembled structural proteins into the proximal segment of axons during infection also may explain the bright, but noticeably diffuse signal of the major capsid protein VP5 reported by Tomishima and Enquist (28).

Furthermore, in our studies, a serendipitous advantage of using the in vitro chamber system was that the site of imaging was several millimeters from the SCG explant, automatically forcing us to image in the mid-distal axon instead of the proximal axon of infected neurons. We consistently observed a diffuse green fluorescent signal building in the proximal axon late in infection (>12 h postinfection). Indeed, by 16 h postinfection, this diffuse signal was strong enough in the proximal segment that we could visualize the connection of cell bodies to cognate axons. The general phenomenon of entry of unassembled structural proteins into the proximal segment of axons during infection also may explain the bright, but noticeably diffuse signal of the major capsid protein VP5 reported by Tomishima and Enquist (28).

**FIG. 4.** Axons are devoid of enveloped virus particles during a Us9-null infection. Explants on the outside of the chamber were infected for 24 h with PRV Becker or PRV 160 (Us9-null), and axons inside the chamber were visualized by transmission electron microscopy. Samples were examined in duplicate. A 1-mm square with high axonal density inside the chamber was selected for sectioning by the ultramicrotome. Six serial sections (70 nm apart) were scrutinized for the presence of virus particles. (A) Enveloped virus particles were detected in the distal-axon of Becker-infected explants but were not present in explants infected with PRV 160 (B).
imal segment. In addition, live-cell imaging was also a key approach in determining the number of green capsid puncta undergoing anterograde transport, regardless of background fluorescence. Indeed, the first experiments by Smith et al. addressing anterograde transport kinetics of PRV capsids show green puncta traversing a “sea of green” in the proximal segment of chicken embryo DRG neurons (25).

Our work demonstrates that PRV Us9 is essential for the anterograde spread of infection in the rat visual system, as well as for neuron-to-cell spread in a compartmentalized chamber system (3, 10). Studies examining the closely related HSV Us9 homolog also support a role for this protein in anterograde spread of infection (19, 22). Polcicova et al. showed that an HSV Us9 mutant was severely impaired in its ability to spread from the murine eye to retinorecipient centers in the brain, as well as the anterograde circuit from the trigeminal ganglia to the mouse cornea (22). LaVail et al. concluded from studies in vivo that viral capsid and DNA failed to enter the axon in the absence of Us9, although gD and somewhat less gC were detected (19).

Our PRV Us9 data support a model in which viral capsids and membrane proteins assemble in the cell body and are trafficked together inside vesicles toward the axon terminus (5, 18, 20, 21). Recent live-cell imaging experiments examining anterograde and retrograde PRV transport using dual-color viruses expressing fluorescent capsid, tegument, and viral glycoproteins supports this notion (1). Capsids (visualized as fluorescent mRFP-VP26 puncta) moving away from the cell body were associated with the viral glycoprotein gD (visualized as fluorescent gD-GFP puncta). On the other hand, no capsids were present in the axon terminus.
hand, RFP-VP26 puncta moving toward the cell body no longer contained detectable gD-GFP but did associate with the inner tegument protein VP1/2 (1). In contrast, Snyder et al. recently concluded HSV capsids were transported in axons independent of viral membrane proteins (26). These studies were performed on fixed neurons that were subsequently stained with HSV antibodies against capsid and viral glycoproteins. This finding is also well established for PRV-infected neurons: capsid proteins and glycoproteins do not always colocalize in axons fixed and stained with antibodies (see Fig. 2 and images in references 14 and 29). In fact, this finding provided much of our initial enthusiasm for the idea that PRV structural components are trafficked separately inside the axon. However, recent reports using live-cell imaging with dual-color fluorescent PRV recombinants (1, 15), electron microscopy coupled with chamber technology (10, 11, 15), and the data from the present report indicating that PRV Us9 is required for axonal sorting of capsid and membrane proteins are more consistent with a model where assembled particles enter and move as complexes in membrane vesicles.

In summary, we report that the PRV membrane protein Us9 directly affects the axonal sorting of capsids in SCG explants and dissociated neurons. Future studies will focus on the mechanism by which Us9 mediates such axonal sorting and targeting. Our current hypothesis is that Us9 directs host transport vesicles carrying assembled virus particles to varicosities and axon termini where egress occurs. The cellular and viral proteins involved in these processes remain to be elucidated.

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ADDENDUM IN PROOF

Similar studies involving HSV gE- and US9- mutants have demonstrated that gE/gI and US9 promote anterograde transport of HSV capsids into rat and human neuronal axons (A. Snyder and D. C. Johnson, personal communication). HSV glycoprotein transport into axons was also reduced with gE- and US9- mutants, although not as severely as with capsids.

REFERENCES