Visualization of an Alphaherpesvirus Membrane Protein That Is Essential for Anterograde Axonal Spread of Infection in Neurons

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ABSTRACT Pseudorabies virus (PRV), an alphaherpesvirus with a broad host range, replicates and spreads in chains of synaptically connected neurons. The PRV protein Us9 is a small membrane protein that is highly conserved among alphaherpesviruses and is essential for anterograde axonal spread in neurons. Specifically, the Us9 protein is required for the sorting of newly assembled PRV particles into axons. However, the molecular details underlying the function of Us9 are poorly understood. Here we constructed PRV strains that express functional green fluorescent protein (GFP)-Us9 fusion proteins in order to visualize axonal transport of viral particles in infected rat superior cervical ganglion neurons. We show that GFP-Us9-labeled structures are cotransported with viral particles in the anterograde direction within axons. Additionally, the vast majority of anterograde-directed capsids (labeled with VP26-monomeric red fluorescent protein) and a viral membrane protein (labeled with glycoprotein M fused to mCherry) are cotransported with GFP-Us9 in the anterograde direction. In contrast, during infection with PRV strains that express nonfunctional mutant GFP-Us9 proteins, cotransport of mutant GFP-Us9 with capsids in axons is abolished. These findings show that axonal sorting of progeny viral particles is dependent upon the association of viral structures with membranes that contain functional Us9 proteins. This association is required for anterograde spread of infection in neurons.

IMPORTANCE Alphaherpesviruses, such as pseudorabies virus (PRV), are parasites of the mammalian nervous system. These viruses spread over long distances in chains of synaptically connected neurons. PRV encodes several proteins that mediate directed virion transport and spread of infection. Us9 is a highly conserved viral membrane protein that is essential for anterograde neuronal spread of infection. In the absence of Us9, newly replicated viral particles are assembled in the cell body but are not transported into or transported within axons. Here, we constructed and characterized novel PRV strains that express functional green fluorescent protein (GFP)-Us9 fusion proteins in order to visualize its localization in living neurons during infection. This enabled us to better understand the function of Us9 in facilitating the spread of infection. We show that all viral particles moving in the anterograde direction are labeled with GFP-Us9, suggesting that the presence of Us9 determines the capacity for directed transport within axons.

Alphaherpesviruses, including the human pathogens herpes simplex virus 1 (HSV-1), HSV-2, and varicella-zoster virus, are neuroinvasive pathogens that replicate and spread within the mammalian nervous system (1, 2). In their natural hosts, these viruses enter the peripheral nervous system (PNS) and establish lifelong persistent infections in sensory ganglia. Periodically, newly replicated viral particles are transported out toward the periphery and cause recurrent disease. In rare cases, infection spreads from the PNS to the central nervous system, an event that is often lethal. Spread of infection, both within and between hosts, is integral to the viral life cycle and requires bidirectional transport of viral particles over long distances in axons. Specifically, retrograde axonal transport of viral particles (toward the neuron cell body) is required to initiate infection and establish latency, while anterograde axonal transport (away from the neuron cell body) is required to spread infection to the periphery. The directional spread of viral infection is controlled by the action of specific viral proteins.

Pseudorabies virus (PRV) is an alphaherpesvirus with a broad host range that is used extensively for studying viral spread in the nervous system (3, 4). PRV proteins that are important for anterograde spread of infection include highly conserved glycoprotein I (gI), gE, and Us9 (5–7). Of these three proteins, only PRV Us9 is essential for anterograde spread of infection in neurons both in vitro and in vivo (5, 8, 9). PRV Us9 is a small (98-amino-acid) type II tail-anchored membrane protein enriched in lipid raft microdomains. These attributes allow Us9 to accumulate in the cellular plasma membrane and trans-Golgi network and incorporate into virion envelopes (5, 10, 11). However, the molecular mechanisms underlying Us9’s role in viral spread and directed axonal transport of viral particles remain poorly understood.

Anterograde spread involves the sequential steps of sorting...
newly synthesized progeny virions to the axon, long-distance transport within axons, and subsequent egress and entry into a susceptible cell. The assembly state of the transported virion moving in the anterograde direction has been highly contested. Evidence that fully assembled, mature PRV particles undergo anterograde transport comes from several studies using live-cell imaging of fluorescently tagged PRV mutants replicating in dispersed neuronal cultures or in Campenot chambers coupled with transmission electron microscopy (8, 12–17). Us9 is also predicted to be on the surface of anterograde-directed vesicles containing virions (10, 11, 18). Notably, Us9-null mutants assemble and release virions from the cell body but do not sort or transport these newly synthesized virions into axons.

In this study, we constructed and characterized PRV strains that express a novel green fluorescent protein (GFP) fusion to the amino terminus of Us9 (GFP-Us9). We demonstrate that GFP-Us9 retains the capacity for anterograde-directed spread of PRV. We also generated PRV strains expressing the GFP-Us9 fusion protein or GFP-Us9 missense protein in combination with other fluorescent viral structural proteins. These strains enabled us to visualize the transport dynamics of PRV particles in relation to GFP-Us9 using live-cell imaging. We found that the functional GFP-Us9 fusion protein is associated with nearly all virions moving in the anterograde direction, suggesting that Us9 may directly engage viral or host factors that facilitated axonal sorting and transport of intracellular virions.

RESULTS
Construction and validation of PRV expressing GFP-Us9. In this study, we constructed PRV recombinants that express a GFP fusion to the amino terminus of Us9 (GFP-Us9). We retained the capacity to promote the sorting and transport of viral structural proteins into axons. We were guided by previous work with other viral homologs of Us9 which suggested that the amino terminus could accommodate large amino acid additions without compromising anterograde spread function (19). In addition, a carboxy-terminal GFP fusion to Us9 (Us9-GFP) could not promote axonal sorting and anterograde spread of infection in vitro or in vivo (20). The presence of the bulky GFP on the Us9 carboxy terminus appears to disrupt the sorting and transport of viral structures (20) (Fig. 1A), presumably through interference with protein-protein interactions. To limit steric hindrance at the amino terminus, we added a flexible linker between GFP and Us9 (Fig. 1A). To test whether PRV expressing GFP-Us9 was capable of anterograde spread, we used a well-characterized compartmentalized chamber system adapted by Ch’ng and Enquist (13, 21, 22) (Fig. 1B and Materials and Methods). This system has been used to examine the Us9-mediated anterograde spread of PRV from neuronal cell bodies (soma or S compartment) into susceptible epithelial PK15 cells. Wild-type virus infections typically spread to the N compartment via axonal transport and reach a maximum titer within 24 h of S compartment infection. In contrast, chamber infection with PRV strains that do not express Us9 or express a nonfunctional mutant Us9 protein results in essentially no infectious virus in the N compartment (8, 18–20).

The S compartments of compartmentalized neuronal cultures were infected with PRV strains expressing wild-type Us9 (PRV 328) (20), Us9-GFP (PRV 164) (20), or GFP-Us9 (PRV 340) (this study). At 24 h postinfection, viral titers in the S compartment
were equivalent for all three PRV strains, indicating similar production of infectious progeny (Fig. 1C). However, titers in the N compartment at this time were markedly different, depending on the PRV strain. PRV expressing wild-type Us9 spread efficiently to the N compartment, producing viral titers ranging between $10^7$ and $10^8$ PFU/ml. PRV 164, the mutant expressing Us9-GFP, spread poorly or not at all to the N compartment, as noted previously (20). In contrast, the PRV strain expressing the GFP-Us9 fusion protein (PRV 340) spread to the N compartment in all chambers, with viral titers near $10^7$ PFU/ml, an approximately 1-log decrease from wild-type Us9 titers at the same time (Fig. 1C). The decrease in the N compartment titer of GFP-Us9 expressing PRV is not due to reduced viral amplification; wild-type-expressing and GFP-Us9-expressing PRV strains have equivalent replication kinetics in PK15 cells (Fig. 1D). We conclude that GFP-Us9 protein is functional for promoting significant anterograde spread of infection in our in vitro system.

Subcellular localization and transport of GFP-Us9. Since the new GFP-Us9 fusion protein was capable of supporting anterograde spread of PRV, we next determined the localization of GFP-Us9 in infected cells. Previously, Us9 and Us9-GFP have been characterized to accumulate in both the plasma membrane and intracellular puncta in infected cells (9, 10, 19, 23). We imaged superior cervical ganglion (SCG) neurons infected with a PRV strain expressing GFP-Us9 fusion protein (PRV 340) spread to the N compartment in all chambers, with viral titers near $10^7$ PFU/ml, an approximately 1-log decrease from wild-type Us9 titers at the same time (Fig. 1C). The decrease in the N compartment titer of GFP-Us9 expressing PRV is not due to reduced viral amplification; wild-type-expressing and GFP-Us9-expressing PRV strains have equivalent replication kinetics in PK15 cells (Fig. 1D). We conclude that GFP-Us9 protein is functional for promoting significant anterograde spread of infection in our in vitro system.

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In the absence of Us9 protein or when the defective Us9-GFP fusion protein has been expressed, no viral capsids are found in axons of infected neurons (8). However, consecutive frames from a representative movie of GFP-Us9 and VP26-mRFP puncta show cotransport of the two virus fluorescent fusion proteins in the anterograde direction within axons (Fig. 2). As we previously observed, axons contain abundant GFP-Us9 puncta with a subset of these puncta colabeled with VP26-mRFP. In the selected frames, two VP26-mRFP structures are colabeled with GFP-Us9 and moving in the anterograde direction (Fig. 2B; see Movie S1 in the supplemental material). When we quantified axons for fluorescent structures moving in the anterograde direction, the majority of GFP-Us9 puncta, over 60%, did not colocalize with detectable VP26-mRFP puncta. When we looked exclusively at anterograde-directed VP26-mRFP positive puncta, 96.5% of these puncta were colabeled with GFP-Us9 (Fig. 2C). We conclude that the VP26-mRFP puncta are capsid assemblies that move in the anterograde direction only when complexed with GFP-Us9.

A mutant Us9 is not cotransported with anterograde-directed capsids. We next constructed a PRV recombinant expressing a previously characterized Us9 missense protein fused to the amino terminus of GFP. This mutant carries alanine substitution mutations in the conserved dityrosine motif located at the start of the acidic protein cluster in the cytoplasmic domain (Y49-50A). These residues are essential for Us9-mediated anterograde-directed viral spread of infection in a rat eye model (24). We tested
the anterograde spread capacity of PRV expressing GFP-Us9 Y49-50A (PRV 440) in the compartmentalized neuronal culture system (Fig. 3A). As predicted, PRV strains expressing either the native or the GFP-fused version of the dityrosine missense protein were completely incapable of supporting spread to the N compartment, confirming that these strains are defective in anterograde spread.

The anterograde spread deficiency phenotype observed with GFP-Us9 may be caused by either an inability of the missense protein to sort viral proteins into axons or an inability to engage anterograde transport machinery. To determine if virion transport in axons was blocked or reduced, we constructed PRV strains that expressed both the GFP-Us9 Y49-50A and VP26-mRFP fusion proteins (PRV 442) and infected dissociated SCG cultures as before. In axons of PRV 442-infected neurons, there were no anterograde-directed VP26-mRFP puncta compared to PRV 341 (wild-type GFP-Us9/VP26-mRFP)-infected neurons at comparable times after infection (see Movies S2 and S3 in the supplemental material). To quantify the numbers of colabeled virion structures, the cultures were fixed and VP26-mRFP puncta were counted (Fig. 3B). The number of colabeled puncta in PRV 442-infected axons was markedly lower than that of those found after comparable PRV 341 infection (Fig. 3C). We suspect that the small numbers of dually labeled structures observed in PRV 442 infections are similar to immobile dually labeled structures in the movies in the supplemental material. Interestingly, while VP26-mRFP puncta were not moving in the anterograde direction after PRV 442 infection, substantial numbers of singly labeled GFP-Us9 Y49-50A puncta continued to move in the anterograde direction.

GFP-Us9 is cotransported with other viral membrane proteins. To determine if the GFP-Us9 puncta not associated with VP26-mRFP contain other viral membrane proteins, we fused mCherry to the carboxy terminus of PRV gM, a conserved glycoprotein present in viral and cellular membranes with known involvement in secondary envelopment (25, 26). The gM-mCherry fusion protein replaced endogenous gM and was expressed from the endogenous gM promoter. We imaged dissociated SCG neurons infected with a double-recombinant virus expressing both GFP-Us9 and gM-mCherry (PRV 348). At distal axon sites, many doubly positive punctate structures were observed moving in the anterograde direction (Fig. 4; see Movie S4 in the supplemental material), 96% of the GFP-Us9-positive structures moving in the anterograde direction were also positive for gM-mCherry. Conversely, very few structures were singly labeled with GFP-Us9 or gM-mCherry. The high concordant localization of these two viral
membrane proteins suggests that they are alternative products of virion production which could include light particles or vesicles carrying viral membrane proteins only (Fig. 5). The full composition of these structures and their role in anterograde viral spread remain to be resolved.

**DISCUSSION**

Viral protein Us9 is essential for anterograde-directed spread of PRV infection in vitro and in vivo. Our goal was to visualize the transport dynamics, localization, and function of PRV Us9 by using fluorescent fusion proteins and live-cell imaging of infected neurons. We could not use a previously characterized carboxy-terminal fusion of GFP to Us9 (Us9-GFP) because it was defective for anterograde-directed spread of infection. However, we characterized an N-terminal fusion (GFP-Us9) and found that it retains the ability to promote anterograde spread of PRV. GFP-Us9 was associated with anterograde moving viral particles (labeled with VP26-mRFP or gM-mCherry). A GFP-Us9 missense mutant incapable of anterograde spread (Y49-50A) did not sponsor anterograde-directed capsids, but the missense fusion protein itself moved into axons and was transported in the anterograde direction. These fluorescent viral fusion proteins have allowed the visualization of viral processes associated with the directed spread of infection in neurons.

Our data are in concordance with the married model of PRV transport: fully assembled virions, enclosed in a membranous transport vesicle, are transported in axons in an anterograde direction. This model has been validated using a variety of experimental techniques for PRV, including fluorescent and electron microscopy coupled with compartmentalized neuronal culturing (12, 13). The significant coassociation of GFP-Us9 with almost all anterograde-directed capsids is greater than results reported for other viral membrane proteins (12, 27). In contrast, the structures being transported during HSV infection remain controversial (16, 17, 27–30). There are conflicting reports about the role of Us9 in HSV spread (31, 32) and that Us9 does not associate with HSV membrane proteins (33). The development of a functional HSV GFP-Us9 fusion may help resolve the disparate observations and models for HSV transport.

Intracellular membrane localization of Us9 is important for sorting newly assembled virions from the cell body into axons. Us9 in lipid rafts is essential for axonal sorting and anterograde spread of infection but not for incorporation into virion membranes or for virion egress from the cell bodies of neurons (18). Us9 is predicted to localize within the membranes of the virion transport vesicle such that the functional domains of Us9 are exposed to the intracellular environment. This would allow Us9 to directly engage proteins that are required for axonal sorting and anterograde transport of vesicles containing virions (9, 34). Us9 is incorporated into the virion envelope (11, 35), but the GFP-Us9 labeling of extracellular virions is at or below the limit of detection under optical conditions identical to those used in this study (unpublished results). This raises the possibility that the concentration of GFP-Us9 within virions is variable and reduced compared to that of intracellular virions surrounded by a transport vesicle membrane. The fact that we can observe GFP-Us9 as fluorescent puncta associated with capsids in axons suggests that Us9 may be preferentially incorporated into the membrane of the intracellular transport vesicle surrounding virions rather than virion envelope proteins.
lopes. Regulation of Us9 activity may also occur by posttranslational modifications such as phosphorylation (11). However, a simple model is that differential incorporation of GFP-Us9 into transport vesicles could regulate whether or not virions are sorted into axons. Vesicles with lower GFP-Us9 activity would egress from the cell body, while vesicles with increased GFP-Us9 activity would be sorted into axons.

The number of GFP-Us9 puncta without capsids moving in the anterograde direction is striking, with over 60% of the total fluorescently labeled puncta lacking a detectable VP26-mRFP signal. The coassociation of wild-type GFP-Us9 with gM-mCherry in almost all anterograde-traffic particles suggests that viral membrane protein assemblies are involved. These structures might include capsid-less light particles (36), as well as vesicle precursors involved in virion formation (Fig. 5). The relevance and impact of nonviral structures on the spread of infection remain to be determined. While the number of structures observed might be influenced by in vitro culture conditions, light particle production does occur in vivo (37). Interestingly, the GFP-Us9 missense mutant Y49-50A moves efficiently in the anterograde direction in the absence of full virions. It is possible that membrane-associated Us9 facilitates transport for any vesicular membrane, and the Y49-50A mutation alters a second function involved in the recruitment of mature virions into axons. Alternatively, the Y49-50A mutant may be incorporated into membranes and passively carried along a cellular secretion pathway. Further experimentation is required to understand the nature and importance of these nonviral structures.

One caveat of our study is that the GFP-Us9 fusion protein does not completely restore viral spread to wild-type levels. Further observation of viral transport and spread using different mutant Us9 proteins will allow us characterize the modest deficiency related to the GFP fusion, as well as to dissect Us9 function.

In conclusion, this fluorescent fusion protein enables us, for the first time, to visualize Us9-dependent axonal sorting events. Further goals of studies utilizing this functional fusion protein include the identification of protein interaction partners, understanding of the functional defects of Us9 point mutations, and testing of the model of differential Us9 incorporation into a subset of viral membranes. GFP-Us9 is an important reagent leading to the eventual understanding of Us9's function in alphaherpesvirus spread and disease.

MATERIALS AND METHODS

Plasmids and viruses. The PRV mutant expressing GFP-Us9 (PRV 340) was constructed by cloning the PRV Us9 opening reading frame into the EcoRI site of pEGFP-C1. A flexible hydrophilic linker was placed between the GFP moiety and the Us9 protein (38); the nucleotide sequence of the EcoRI site of pEGFP-C1 was altered to introduce a homologous arm of the NsiI fragment containing the cytomegalovirus promoter and the 3' homologous arm containing sequence downstream of the simian virus 40 poly(A) site. Cells and medium were then collected together, serially diluted, and plated onto PK15 cells. Individual plaques expressing GFP-Us9 were identified using a Nikon Eclipse TE300 inverted epifluorescence microscope. Initial isolates were subjected to three further rounds of plaque purification and examined by Western blot analysis to confirm the expression of PRV GFP-Us9 and the loss of expression of bovine herpesvirus Us9. This virus strain was designated PRV 340. The construction of GFP-Us9 Y49-50A was done in a similar manner using de novo synthesis methods to generate Us9 with alanine codon substitutions at tyrosines 49 and 50 (24) also containing the flexible linker sequence. This construct was cloned into pEGFP-C1 as described above, which was then used to generate a PRV strain expressing GFP-Us9 Y49-50, designated PRV 440. To construct the recombinant PRV strain expressing GFP-Us9 and VP26-mRFP (14), PK15 cells were coinfected at a multiplicity of infection (MOI) of 10 PFU per cell with 1 × 10⁶ PFU of PRV 340 and PRV 325 (Us9-null/VP26-mRFP virus isolated as described in reference 8) and allowed to undergo full CPE; the isolated virus was designated PRV 337. Individual fluorescent plaques expressing both GFP and mRFP were isolated and purified for three subsequent rounds of plaque purification as described above and designated PRV 341. Coinfection of PRV 440 and PRV 325 was also used to isolate GFP-Us9 Y49-50A/VP26-mRFP, designated PRV 442.

A PRV recombinant expressing a gM-mCherry fusion protein was constructed by de novo synthesis (GenScript USA Inc., Piscataway, NJ.) of a plasmid containing the terminal 500 bp of the UL10 open reading frame (gM) with an in-frame fusion of mCherry fluorescent protein and 500 bp of downstream flanking sequence. This plasmid was linearized by EcoRI and Sall restriction enzyme digestion and directly cotransfected with PRV Becker nucleocapsid DNA. Virus was harvested, and limiting dilutions were made to isolate individual plaques on PK15 cells. Individual plaques expressing gM-mCherry were identified using a Nikon Eclipse TE300 inverted epifluorescence microscope. These initial isolates were subjected to three subsequent rounds of plaque purification to generate PRV 347. To isolate a virus expressing gM-mCherry and GFP-Us9, gM-Cherry plasmid DNA was linearized and cotransfected with PRV 340 nucleocapsid DNA. Doubly positive gM-mCherry- and GFP-Us9-expressing plaques were isolated and subjected to three rounds of plaque purification. This virus was designated PRV 348.

Neuronal culturing and infection. PK15 cells were maintained in 10% fetal bovine serum (FBS)-1% penicillin-streptomycin in Dulbecco's modified Eagle's medium (DMEM). Primary neuronal cultures of SCG neurons were maintained in neuronal medium, which consists of Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 1% penicillin-streptomycin-glutamine (Invitrogen), B27 Supplement (Invitrogen), and 50 ng/ml neuronal growth factor 2.5 S (Invitrogen). Prior to neuron plating, cell culture surfaces were coated with polyornithine (Sigma-Aldrich, St. Louis, MO) at 500 μg/ml in borate buffer, pH 8.2, followed by murine laminin (Invitrogen) at 10 μg/ml in calcium- and magnesium-free Hanks balanced salt solution. Compartmentalized neuronal cultures were prepared as described in references 13 and 39. Briefly, plastic tissue culture dishes were treated as described above and air dried. Parallel grooves were etched into the surface, and a 1% methylcellulose-1× DMEM solution was spotted onto the grooves. CAMP320 Tellen isolator rings (Tyler Research; Edmonton, Alberta, Canada) were coated with autoclavable vacuum grease on one side and gently applied to the treated culture surface such that the parallel grooves extended across the three compartments. The compartments were individually filled with neuronal medium and tested for leaks. Dissected SCG neurons were plated into one side (S) compartment. Cultures were maintained until axon extensions had robustly penetrated and grown into the far-side (N) compartment, typically 17 to 21 days after dissociation.

Viral infections of PK15 cells were performed in 2% FBS-containing medium. Viral titer determination was similar to infection, but the me-
dium was supplemented with 1% methylcellulose to limit virus diffusion. Three days postinfection, methylcellulose medium was aspirated and cells were stained with methylene blue solution to visualize the cell monolayer.

For infection of dissociated or compartmentalized SCG neuronal cultures, approximately 1 × 10^6 PFU of virus was used per dish, diluted in 100 to 200 µl of conditioned neuronal medium. SCG neurons were not infected until 17 to 24 days of culturing to allow full neuronal maturation.

Infection of compartmentalized neurons and quantitation of virions that have undergone anterograde-directed transport require specific prepara-

Supplemental Material

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00063-12/-/DCSupplemental.  

Movie S1, AVI file, 1 MB.  
Movie S2, AVI file, 3.1 MB.  
Movie S3, AVI file, 3.4 MB.  
Movie S4, AVI file, 4.2 MB.  

References


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