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Us9-Independent Axonal Sorting and Transport of the Pseudorabies Virus Glycoprotein gM

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Axonal sorting and transport of fully assembled pseudorabies virus (PRV) virions is dependent on the viral protein Us9. Here we identify a Us9-independent mechanism for axonal localization of viral glycoprotein M (gM). We detected gM-mCherry assemblies transporting in the anterograde direction in axons. Furthermore, unlabeled gM, but not glycoprotein B, was detected by Western blotting in isolated axons during Us9-null PRV infection. These results suggest that gM differs from other viral proteins regarding axonal transport properties.

Pseudorabies virus (PRV) is a member of the family *Herpesviridae* and infects the peripheral nervous system (PNS) (1). Spread of PRV infections within the nervous system is directional, with virions moving from peripheral sites to neuronal cell bodies (retrograde) or from the neuronal cell body to the periphery (anterograde) (2, 3). Anterograde spread requires the sorting of virions from the cell body into axons with subsequent transport away from the cell body. The PRV protein Us9, a nonglycosylated viral type II membrane protein, is essential for anterograde transport and spread. In the absence of Us9 expression, PRV virions are excluded from the axon (4, 5). Us9 interacts with the molecular motor Kif1A to direct sorting and transport (6). While Us9 is known to mediate transport of infectious virions, its role in axonal transport of other viral assemblies is unclear.

Alphaherpesvirus assembly is a complex multistep process involving interactions of virus proteins with cellular membranes as well as several membrane budding and fusion events that produce distinct structures besides infectious virions (7, 8). Herpesviral proteins can assemble into noninfectious particles called light particles, or L particles (9, 10). These noninfectious particles, which have an envelope and tegument proteins but lack a capsid, can be detected in axons *in vitro* and *in vivo* (11–14). Furthermore, in HSV-infected neurons, glycoproteins C and D have been detected in axons on structures devoid of capsid protein during infection by Us9-null mutants (15). While distinct structures can be sorted into axons and transported, their functional relevance to anterograde spread of infection and the requirement of Us9 for their transport is unknown. In this study, we focused on the Us9-independent axonal transport of viral glycoprotein M (gM).

We constructed a plasmid encoding mCherry-tagged glycoprotein M (gM) through *de novo* synthesis, designated pML124 (11). Two single fluorescent PRV strains expressing this fusion were then derived: PRV 347 (gM-mCherry/wild-type Becker) and PRV 437 (gM-mCherry/Us9-null). PRV 347 and 437 were isolated following cotransfection of linearized pML124 and nucleocapsid DNA from PRV Becker (WT) or PRV 161 (Us9-null) (16), as previously described (11). We performed live-cell imaging of PRV 347 and PRV 437 in dissociated rat superior cervical ganglion (SCG) cultures under conditions previously described (11). As for other PRV membrane proteins (6, 17), substantially more fluorescent signal was detected in neuronal cell bodies than on puncta in axons. As expected, mCherry-labeled puncta trafficked in the anterograde direction after infection with PRV 347, similar to the previously characterized gM-mCherry/GFP-Us9 dually labeled puncta (11). However, we also observed anterograde transport of gM-mCherry puncta after infection with PRV 437 (Us9-null) (see Movie S1 in the supplemental material). Puncta moving in an anterograde direction were detected beginning at 8 h postinfection (Fig. 1A). The motility of these structures was qualitatively different between PRV 347 and PRV 437, with Us9-null mCherry puncta appearing less motile with an apparently higher stall frequency due to the high number of immobile mCherry puncta. To our knowledge, this is the first observation of definitive anterograde transport of a PRV membrane protein in the absence of Us9 expression.

Previous work indicated that glycoproteins gB, gC, and gE as well as bulk virion envelope epitopes could not be detected in axons during Us9-null infections by immunofluorescence (4, 18, 19). To reconcile these observations with our finding of Us9-independent transport of gM, we verified the Us9-null phenotype and Us9 expression levels of PRV 437. We measured expression of Us9 by PRV 347, 437, and 161 during infection of PK15 cells by Western blotting (WB) using a polyclonal rabbit Us9 antiserum (5). No Us9 was detected for the Us9-null mutants PRV 437 and PRV 161 (Fig. 1B). The gM-mCherry fusion protein was detected using a polyclonal rabbit gM antiserum (20) as two distinct bands at 70 and 50 kDa for PRV 347 and PRV 437, showing the expected

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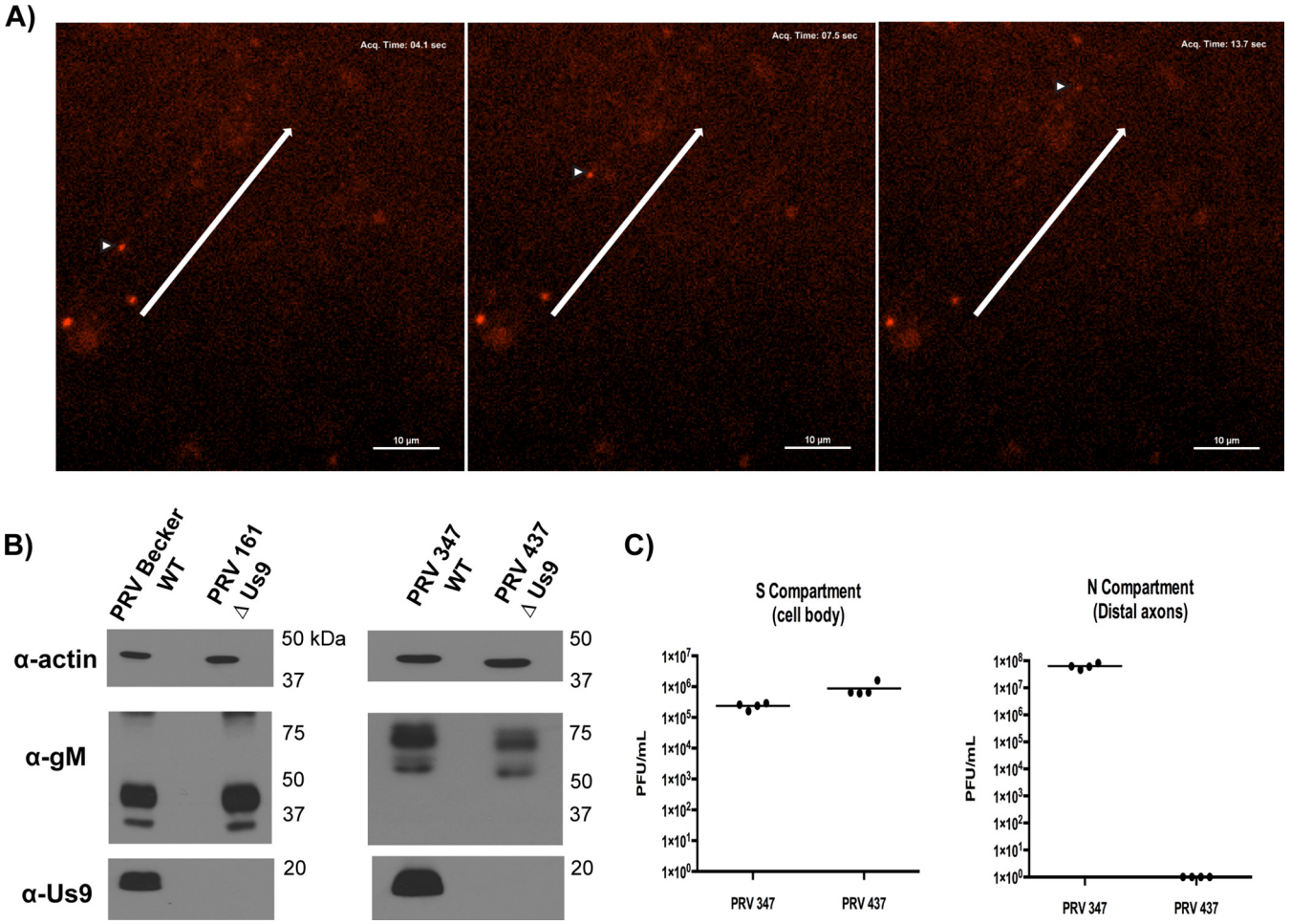

FIG 1 Characterization of a Us9-independent pathway for anterograde axonal transport of gM. (A) Live cell imaging stills of anterograde transport of gM-mCherry in rat SCG neurons at 8 h postinfection with PRV 437. Triangles indicate punctate structures moving in an antergrade direction. Arrows indicates anterograde directionality. (B) WB assessment of Us9 and gM expression in PK15 whole-cell extracts infected with PRV Becker, PRV 161 (Us9-null parental strain), PRV 347 (gM-mCherry), and PRV 437 (gM-mCherry, Us9-null). (C) Anterograde spreading capacities of PRV 347 and PRV 437 in chambered neuronal cultures at 24 h postinfection. Point estimates reflect viral titers in the N compartment for infections performed in quadruplicate for each viral strain. Lines denote median titers.

increase in size over the untagged gM protein. We then assessed the anterograde-spread capacity of PRV 347 and PRV 437 by infecting primary cultures of rat SCG neurons in modified Campenot chambers as described previously (21). No anterograde spread into the isolated N compartment was observed at 24 h postinfection with PRV 437, compared to the robust spread of PRV 347 (Fig. 1C), confirming the Us9-null phenotype.

Our results suggest that a vesicle containing gM-mCherry enters and is transported in axons without Us9. Moreover, the live-cell imaging assay confirmed that gM-mCherry labeled puncta were mobile and therefore not extracellular inoculum or stalled particles. To exclude confounding effects on gM localization due to the mCherry fluorophore tag, we analyzed native untagged proteins in PRV-infected SCG cultures using the modified Campenot chamber system as described above. Cultures were infected with PRV Becker or PRV 161, and the contents of each compartment were harvested separately for WB analysis. The S compartment samples demonstrated expression of the viral membrane proteins gB and gM, indicative of productive infection and new protein synthesis above input virion material (Fig. 2). Us9 was detected in PRV Becker- but not PRV 161-infected chambers. Robust gM signal was detected in the N compartment axons at 18 and 24 h postinfection with both PRV Becker and PRV 161, confirming our observations with the live-cell imaging assay. These results verified the Us9-independent entry of untagged, native gM protein into axons. Furthermore, the viral glycoprotein gB was not detected in axons after PRV 161 (Us9-null) infection, while the mature cleaved gB form was detected in axons after wild-type infection (Fig. 2). These findings confirmed the dependence of glycoproteins like gB on Us9 for transport (18) and further suggested that Us9-independent transport of gM constitutes a process that does not involve all viral membrane proteins.

It remains unclear if gM is unique in its ability to undergo Us9-independent axonal sorting and transport. gM is a type III transmembrane protein expressed as a heterodimer with gN (22) that mediates secondary envelopment through interactions with gE and tegument (23–25). gM also facilitates endocytic retrieval and relocalization of both viral and host proteins from the plasma

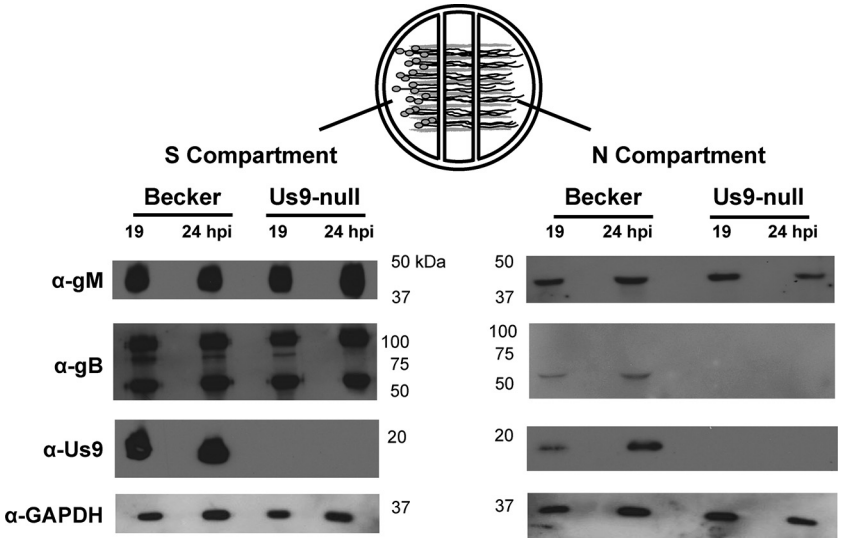

FIG 2 Western blot assessment of axonal entry of viral membrane proteins during PRV infection of chambered rat SCG cultures. Samples from chambered neuronal cultures infected with PRV Becker or PRV 161 were assessed by WB to determine the axonal targeting properties of various membrane proteins in the presence/absence of Us9 expression. S and N compartment samples were extracted separately at 18 or 24 h postinfection. Results are representative of two independent biological replicates.

membrane (20, 26). It is possible that the membrane topology of gM targets it to *trans*-Golgi-derived vesicles that form part of the secretory pathway and are sorted constitutively into axons by host adaptor proteins such as AP3 (27, 28). The host secretory pathway has already been implicated in transport and egress of infectious virions (14, 29, 30). It is also possible that gM actively directs the axonal targeting of these currently uncharacterized vesicles to modulate the viral life cycle. However, it is difficult to establish a more precise functionality for gM in anterograde transport, as deletion of this glycoprotein severely impacts overall viral replication/assembly (31), resulting in a pleiotropic effect on other steps of the viral life cycle. Future experiments are needed to detail the kinesin motor that moves these vesicles in axons and to characterize the cohort of gM binding partners.

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