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Herpesvirus Transport to the Nervous System and Back Again

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Abstract

Herpes simplex virus, varicella zoster virus, and pseudorabies virus are neurotropic pathogens of the *Alphaherpesvirinae* subfamily of the *Herpesviridae*. These viruses efficiently invade the peripheral nervous system and establish lifelong latency in neurons resident in peripheral ganglia. Primary and recurrent infections cycle virus particles between neurons and the peripheral tissues they innervate. This remarkable cycle of infection is the topic of this review. In addition, some of the distinguishing hallmarks of the infections caused by these viruses are evaluated in terms of their underlying similarities.

Keywords

neuroinvasion; neurotropism; neurovirulence; axon; neuron; sensory ganglion

INTRODUCTION

One of the defining characteristics of viruses belonging to the *Herpesviridae* family is the establishment of lifelong infections by means of a latency program. During latency the infection persists in a dormant state during which the viral genome is stably maintained but no viral particles are assembled. Neurons of the peripheral nervous system (PNS) host the latent infection for a subset of viruses belonging to the *Alphaherpesvirinae*, a subfamily of the *Herpesviridae* (Figure 1). The first demonstration of the neurotropic properties of these viruses came from a study of the veterinary pathogen, pseudorabies virus (PRV), published by Dr. Albert Sabin (145). Since that time, it is widely recognized that instillation of neurotropic herpesviruses such as herpes simplex virus (HSV-1, HSV-2) or PRV into rodents typically results in transmission of infection from the PNS to the central nervous system (CNS), producing a lethal encephalitis. Transmission from the PNS to the CNS occurs across synapse-linked neurons, and the resulting self-amplifying circuit-specific spread has been exploited to trace neural connections in the vertebrate nervous system (47, 158). How these neuroinvasive and neurovirulent properties relate to the more typically benign infections of natural hosts remains an open question. HSV-1 only rarely causes severe encephalitis in humans.

Productive infections consist of a cycle of transmission between latently infected neurons in ganglia of the PNS and the somatic cells to which they project. Virus particles never cross a synapse in recurrent infections of the natural host, and yet these viruses are exquisitely

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capable of doing just that. Why do these viruses maintain this devastating potential, and how is this property suppressed in the natural host, where disease is most often mild or nonexistent? This sets the neurotropic herpesviruses apart from other agents such as poliovirus, rabies virus, measles virus, and Japanese encephalitis virus, which are virulent upon entering the nervous system (175). Nevertheless, because of the high prevalence of these viruses in ourselves and our livestock, the low incidence of severe forms of disease takes a large toll. For example, HSV-1 pathogenesis is generally limited to cold sores but the virus is also the leading cause of infectious blindness in the United States (92). Reactivation of varicella zoster virus (VZV) produces shingles, which can transform into postherpetic neuralgia. And neonatal transmission of HSV-2 results in a high incidence of CNS and disseminated infections in newborns (72).

There are many outstanding questions regarding the neurotropic herpesvirus infectious cycle. “It must be explained, for example, why a virus causing no more than a cold sore in one person can produce fatal encephalitis in another.” This problem, which was articulated by Richard Johnson and Cedric Mims in 1968, remains relevant today (67). I review the current understanding of the neurotropic infectious cycle that has resulted from more than half a century of study.

TRANSMISSION FROM THE SITE OF EXPOSURE TO NERVE ENDINGS

Nerve endings are generally not exposed to the outside world. As such, the neurotropic herpesvirus infectious cycle exhibits dual tropism: replication in somatic cells such as epithelia, followed by transmission into neurons (Figure 2). How these viruses transmit between cell types is poorly understood. While *Betaherpesvirinae* and *Gammaherpesvirinae* can effect changes in cell tropism by modulating the composition of the infectious viral particle, there is currently no evidence that neuroinvasive herpesviruses employ a similar strategy (16, 149).

Despite the apparent lack of a neuron-specific tropism switch, viral effectors are required for neuroinvasion. The ICP34.5 protein of HSV-1 was described as a neurovirulence determinant due to its selective requirement for infections in animals (27). However, since its discovery, ICP34.5 has been recognized for its role in evading the host immune response (91, 101, 137). Although ICP34.5 is required for spread to neurons from the mouse cornea, for example, this is in large part due to loss of viral replication in the cornea itself (172). One factor that appears to specifically govern viral transmission into the nervous system without loss of viral replication at sites of peripheral inoculation is the PRV deubiquitinase activity housed within the amino terminus of the pUL36 (VP1/2) tegument protein (17, 89). All herpesviruses consist of a membrane envelope that contains an icosahedral capsid and a collection of additional proteins collectively referred to as the tegument, of which VP1/2 is one component. The capsid and tegument are deposited into cells upon fusion-mediated entry (Figure 3). How the deubiquitinase activity of VP1/2 contributes to the transmission of infection to the nervous system is currently unknown, but the phenotype of the mutant virus indicates that an innate barrier exists between epithelial tissues and innervating neurons that prevents viral transmission into the PNS. In this context, it is perhaps noteworthy that recent studies of poliovirus demonstrate that neuroinvasion is inefficient unless damage has been inflicted on the innervated tissue (86, 130). Whereas poliovirus is an enteric pathogen that infrequently invades the nervous system, the infectious cycles of HSV and PRV efficiently breach the epithelia-neuron barrier. Identifying the relevant substrates of the herpesvirus deubiquitinase will be required to better define the innate barrier function and how these viruses overcome it.

DELIVERY OF VIRAL PARTICLES FROM AXON TERMINALS TO PERIPHERAL GANGLIA

Entering the Nerve Ending

Upon cell contact, three events are triggered in the HSV-1 virion: There is an internal restructuring of the tegument, the virion orients so that the dense pole of the particle faces away from the cell surface, and the fusion apparatus is triggered (54, 104, 109). Virion restructuring results in redistribution of tegument proteins in the virion that were initially symmetrically proportioned around the capsid to an asymmetric distribution, and its significance is unknown. Similar morphologic changes in HSV-1 virions can occur simply by allowing the particles to age (123). While the meaning of these findings can only be speculated on, they are suggestive of an internal virion trigger mechanism that is required at the moment of entry. Consistent with this, binding of HSV-1 virions to cells induces the disassociation of the pUL16 tegument protein from the capsid (109). This event is required for infection (108). Whether release of pUL16 from the capsid contributes to the larger morphological changes visualized in the tegument is currently unknown but seems likely.

How HSV-1 enters cells, including neurons, by membrane fusion is extensively reviewed elsewhere (31, 62, 70, 157). It should be noted here, however, that the principal HSV-1 envelope protein that triggers entry into cells is glycoprotein D (gD), which binds several cell membrane receptors including Nectin-1, HVEM (herpesvirus entry mediator), and 3-*O*-sulfated heparan sulfate (55, 114, 152). HSV-1 is dependent upon gD to enter cells, with Nectin-1 serving as the primary entry receptor on neurons and HVEM playing a supporting role (69, 77, 93, 164). However, the importance of gD in the neurotropic herpesviruses is not conserved. The prototypic varicellovirus, VZV, does not encode a gD homolog and instead may enter neurons through a different receptor interaction (160). In PRV, gD is dispensable for cell-cell spread and neuroinvasion, and gD mutant viruses spread independently of Nectin-1 and HVEM (25, 61, 134, 135, 140). Because PRV cannot produce extracellular plaque-forming units in the absence of gD, it can be inferred that PRV spread in the nervous system does not require the release of cell-free virions from infected cells.

Although all herpesviruses enter cells by membrane fusion, the fusion event can occur at either the plasma membrane or an endosomal membrane (111, 126). Several lines of evidence indicate that HSV-1 and PRV enter axon nerve endings by fusion at the plasma membrane: (*a*) entry is pH independent, (*b*) entry at the plasma membrane has been observed by transmission electron microscopy, and (*c*) capsids are not associated with fluid phase markers following entry (98, 125, 154).

Tegument Disassociation

Following fusion-mediated entry into cells, the herpesvirus envelope is lost and the tegument and capsid are deposited into the cytosol (Figure 3). A recent analysis of tegument protein associations with capsids based on resistance to detergent and salt extraction classified seven proteins as components of the inner tegument: pUL14, pUL16, pUL21, pUL36 (VP1/2), pUL37, pUs3, and ICP0 (139). The inner tegument consists of proteins in close juxtaposition to the capsid that likely are acquired prior to the final budding event that produces the enveloped virion. Although this study was not a comprehensive examination of all tegument proteins, it seems a reasonable first approximation that the identified proteins may remain bound to capsids upon entry as part of a retrograde transport complex. This is the case for at least three of these proteins. VP1/2 and pUL37 cotransport with capsids upon entry into sensory neurons according to fluorescence time-lapse imaging of HSV-1 and PRV infections (9, 96). The retention of VP1/2 and pUL37 on capsids postfusion is also evident by imaging fixed cell lines by electron microscopy and fluorescence methods (33, 58). The

same approaches have confirmed that the pUs3 protein kinase is also cotransported with capsids following entry into neurons and nonneuronal cells infected with PRV (30, 58). Because tegument proteins that remain capsid bound may modulate retrograde transport, determining which proteins are retained on the capsid is of great interest. Consistent with this, a truncated version of VP1/2 that is poorly retained on HSV-1 capsids following entry fails to deliver genomes to nuclei (148). Of the seven recognized inner tegument proteins, four have been implicated in capsid delivery to the nucleus (Table 1).

HSV-1 and PRV tegument disassociation appears to be similar: both retain VP1/2 and pUL37 and remove pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16), and pUL49 (VP22) (9, 96). However, whereas removal of the last four tegument proteins appears to be efficient for PRV, HSV-1 retains small amounts of VP11/12 and VP16 on capsids postfusion (9). Although this seems to be a subtle difference between the two viruses, it may have relevance to the infectious cycle, as discussed below.

Cortical Actin

Binding of herpesvirus to cells triggers Rho GTPases that can in turn induce rearrangements in the actin cytoskeleton (28, 36, 129). There is increasing evidence that these signaling events may increase cell susceptibility to infection (reviewed in References 171 and 183). Whether these events pertain to infection at axon terminals has not been addressed. Applying cryo-electron tomography to image HSV-1 capsids deposited in the cytosol after fusion into synaptosomes, which are axon terminals severed from neurons, reveals capsids underneath the plasma membrane surrounded by dense meshworks of actin filaments (104). Although fusion-mediated entry occurred in less than one minute, cytosolic capsids remained near the plasma membrane for more than one hour. This suggests actin may be a barrier to initial infection.

Evidence that virus-induced signal cascades assist capsid translocation through cortical actin can be inferred from time-lapse imaging. Following contact with a terminal of an intact sensory axon, fluorescently tagged PRV capsids display motion that is biphasic (154). Initially, capsids move retrograde with slow kinetics as they enter the axon shaft. Fast retrograde transport consistent with dynein motion (see below) engages shortly thereafter. Capturing recordings of entry events remains challenging and has so far precluded an in-depth analysis of initial intracellular transport dynamics. Nevertheless, the available observations are consistent with navigation of capsids from the plasma membrane to microtubules via a directed process that may be promoted by alterations in axonal actin. In natural infections, nerve endings are generally not freely accessible to extracellular virions as they are in culture models of infection. Although highly speculative, the transmission of infection across epithelia-neuron contacts may promote virus-induced rearrangements of axon actin in a process analogous to the virological synapses reported for HIV and HSV (12, 115).

Retrograde Axon Transport

Time-lapse imaging of fluorescently tagged capsids of HSV-1 and PRV in cultured neurons has revealed that retrograde transport is a robust and sustained process that efficiently delivers capsids to the distant nucleus (9, 50, 94, 154). Transport occurs along microtubules at rates in excess of 1 $\mu m\ s^{-1}$ (9, 80, 168). The fast retrograde axonal transport exhibited by HSV-1 and PRV capsids can be mediated only by active microtubule transport by the dynein motor complex (reviewed in References 71 and 170). Retrograde capsid motion is not continuous; brief reversals in transport direction occur in the axon (154). This transient anterograde motion implies the presence of a kinesin motor on the capsid in addition to the dynein motor complex. The presence of opposing microtubule motors is consistent with

endogenous cellular cargoes as well as other viruses in axons (59, 147). Although not intuitive, the opposing pull of the kinesin motor may act to promote dynein retrograde transport of the capsid (6).

Purified extracellular virions stripped of their envelopes bind dynein and kinesin motors, and this binding is enhanced by extracting outer tegument proteins from the particles (139, 180). These findings implicate either the capsid or tegument proteins closely juxtaposed to the capsid surface as microtubule motor binding sites. Several genes encoding tegument proteins can be deleted without impeding retrograde axon transport, but the inner tegument proteins have critical roles in virion assembly, making the study of mutant viruses during the initial stages of neuron infection difficult (7). Among these, VP1/2 is considered a top candidate for recruiting microtubule motors to capsids. Antibodies directed against VP1/2 or truncation of the VP1/2 carboxyl-terminus interferes with capsid delivery to the nucleus and VP1/2 is required for microtubule transport during late infection in cell lines (33, 97, 148). In addition, VP1/2 carrying an amino acid change that confers temperature sensitivity dramatically accumulates in aggresomes at the nonpermissive temperature (1, 3). While this phenotype may simply result from an abundance of misfolded VP1/2 proteins inside cells, a recent model of virus retrograde transport posits that viruses may mimic protein aggregates to recruit dynein and effect their transport to the microtubule organizing center (MTOC) (176).

Viruses lacking the inner tegument proteins ICP0, pUL14, or pUL37 suffer from delayed delivery of capsids to the nucleus in cell lines (Table 1). Whether these proteins contribute to dynein recruitment and retrograde transport or another step in capsid delivery is also of great interest. The pUL37 tegument protein was examined in a nuclear delivery assay between nuclei in syncytia and in this context was dispensable (143). This finding indicates that pUL37 does not function at nuclear pore complexes (NPCs), but rather at an earlier step in infection: perhaps overcoming the cortical actin barrier or microtubule transport.

Dynein recruitment does not have to be mediated by a tegument protein. The pUL35 (VP26) capsid protein can bind dynein and was implicated in capsid retrograde transport (46). However, in the absence of VP26, PRV capsids transport at wild-type velocity, and transport of HSV-1 is unimpeded both in culture and in a mouse model of infection (7, 40, 44). Therefore, if VP26 binding to dynein is biologically relevant, it must be redundant with another dynein recruitment mechanism. Furthermore, de novo–assembled capsids isolated from the nucleus of infected cells do not bind to dynein in vitro, arguing that capsid proteins may not be involved in dynein recruitment; however, the possibility of posttranslational modifications to capsids in the cytosol cannot be ruled out (139, 180). While studies of HSV-1 and PRV have propelled our understanding of retrograde axon transport, a new neuron chamber infection model has the promise of extending these studies to VZV (103).

From Microtubule Organizing Center to Nuclear Pore Complexes

Minus-end-directed transport along microtubules is expected to end at the MTOC. However, HSV-1 capsids only transiently accumulate at the MTOC prior to moving to NPCs in the nuclear membrane (156). The MTOC is typically located adjacent to the nucleus. Whether the MTOC is close enough to allow subsequent passive diffusion of capsids to NPCs is debated. For adenovirus, capsid translocation from the MTOC to NPCs is dependent upon the nuclear export factor CRM1 (159). Whether CRM1 or a factor exported from the nucleus by CRM1 is necessary for proper adenovirus targeting is unknown, but the dependence on CRM1 argues for a facilitated process. Evidence of a plus-end kinesin motor activity associated with retrogradely trafficking HSV-1 and PRV capsids has led to the suggestion that movement from the MTOC to NPCs may be mediated by plus-end transport along perinuclear microtubules (45).

Nuclear Injection of the Viral Genome

By transmission electron microscopy, HSV-1 capsids are observed docked at NPCs following entry into cell lines (14, 156). The interactions between capsids and NPC proteins that mediate this coupling are not fully defined. Capsid docking is dependent on importin-β and Ran GTPase in an in vitro reconstituted model, and the Nup358 component of the NPC is required for capsid docking in intact cells (33, 131). Tegument components of the retrograde transport complex remain capsid bound once docked at nuclear pores, where the genome is released into the nucleus. Once docking occurs, the VP1/2 tegument protein is proteolytically processed, and this cleavage is required for subsequent release of the DNA genome from the capsid into the nuclear pore (68). A recent study determined that pUL37, and to a lesser extent VP1/2, is lost from docked capsids by 4 h postinfection (5). Whether this loss is of functional significance is not clear. Although the proteins may simply turn over after genome injection, their loss could be a functional consequence of VP1/2 proteolytic processing and genome release from the capsid. HSV-1 encoding a temperature-sensitive mutation in VP1/2 is not processed at the nonpermissive temperature and fails to release its genome upon NPC docking (14, 68, 75). VP1/2 is not expected to maintain the encapsidated genome, so its cleavage likely triggers a conformational change that allows escape of the DNA through the portal vertex of the capsid (122). VP1/2 is bound directly to the capsid surface through an interaction with the pUL25 capsid protein and additionally makes contact with the pUL17 and VP5 capsid proteins (23, 29, 132). Notably, pUL25 is required for stable genome encapsidation during assembly and packaging in the nucleus (107). Although deletion of UL25 prevents production of infectious virions, HSV-1-encoding UL25 mutations that are defective for genome injection after NPC docking have been isolated (138). In addition to binding VP1/2, pUL25 binds two NPC components: Nup214 and hCG1 (132). VP1/2 encodes a nuclear localization signal that is essential for productive infection (2). It will be of great interest to learn whether capsid-bound VP1/2 binds importin-β via the essential nuclear localization signal, and how this pertains to capsid docking and genome injection.

SELECTIVE LOSS OF VP16 DURING NEURONAL INFECTION

Once in the nucleus, a single viral genome can establish a replication compartment as an early step to viral amplification (76). In neurons of the natural host, this potential is suppressed and latency is instead established. The decision to replicate or enter latency is tied to the fate of the VP16 tegument protein. VP16 is a transactivator of immediate early gene expression that enters the nucleus of somatic cells in a complex with host cell factor C1 (HCF-C1) to promote productive infection (84, 85). Upon entering a cell, VP16 is removed from the capsid (Figure 3) (58). In the context of a neuron, this disassociation eliminates an obvious means to deliver VP16 to the neural soma upon entry at the distal axon (9, 96). Input VP16 may be otherwise unable to reach neuronal nuclei, as retrograde axon transport of cytosolic proteins targeted for nuclear import can be blocked in healthy neurons (60). These findings suggest that the neuron polar architecture is predisposed to favor latency establishment. However, VP16 expression in neural soma is not sufficient to trigger productive infection, indicating that there is a second barrier to VP16 nuclear delivery in neurons (150). This second block appears to be at the level of nuclear import. HCF-C1 is sequestered from the nucleus in neurons, which is expected to prevent VP16 nuclear import (81). These two properties functioning in tandem may serve as the primary determinant of latency establishment in neurons. In accordance with this model, VP16 is also a critical determinant for reactivation of HSV-1 from latency (165).

In the case of HSV-1, the presence of a small amount of capsid cotransported VP16 may help explain why, upon initial seeding of the nervous system, some neurons establish an acute productive infection that sends progeny virions back to peripheral innervated tissues,

whereas other neurons become latently infected (15, 166). 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 once docked at the nuclear membrane, indicating that this tegument protein is released from capsids following axon transport (9). Whether capsid-dependent trafficking of VP16 to nuclear pores requires HCF-C1 for subsequent nuclear import is unknown.

POSTREPLICATIVE SPREAD

Herpesvirus replication in the nucleus is followed by capsid assembly and genome encapsidation. There is a long-standing debate on how nucleocapsids egress to the cytosol, but current consensus supports a budding event through the inner nuclear membrane followed by a fusion event at the outer nuclear membrane (reviewed in References 66 and 110). Prior to envelopment at the *trans*-Golgi network (TGN), PRV capsids acquire inner tegument proteins, including VP1/2, pUL37, and pUs3 (57, 74). In fact, the composition of PRV capsids newly deposited into the cytosol, whether from extracellular virions or newly replicated from the nucleus, is remarkably similar. How capsids are differentially targeted to the nucleus early in infection and to the TGN postreplication is an open issue. Because the complement of tegument proteins that are capsid bound in the cytosol is not fully known during either stage of infection, differences in the composition of the capsid-tegument complexes may exist.

For HSV-1, this conundrum could be explained by observations that a subset of outer tegument proteins are acquired on capsids in the nucleus prior to reaching the cytosol; however, these observations are generally inconsistent. VP16 was detected by immunogold electron microscopy on capsids that had budded into the inner nuclear membrane (118). This reactivity was quite weak, however, and in several independent studies VP16 was not detected on purified nuclear capsids (139, 141, 180, 182). The pUL41 outer tegument protein (virion host shutoff, Vhs) also has mixed reports regarding whether it is present on nuclear capsids (139, 141, 180). Similar to reports on these outer tegument proteins, reports vary regarding the site of acquisition for HSV-1 inner tegument proteins, including VP1/2 and pUL37 (20, 124, 139, 169, 180).

In contrast to the varying reports for HSV-1, tegument proteins have generally not been detected on PRV nuclear capsids (57). However, the PRV VP1/2 tegument protein is expressed as multiple isoforms, one of which is a carboxyl-terminal fragment that associates with capsids in the nucleus and expedites nuclear egress (90). Although this new finding has not yet been examined in the context of HSV-1 infection, there is evidence of a carboxyl-terminal species of VP1/2 associated with nuclear HSV-1 capsids (139). Given the technical challenges inherent to these studies, a case can be argued for the need of side-by-side experiments comparing HSV-1 and PRV tegument acquisition to determine whether divergence in assembly pathways of these neurotropic herpesviruses truly exists. A report that HSV-1 uses gB and gH to egress from the nucleus, whereas a separate report found no role for these proteins in PRV nuclear egress, further emphasizes the need for direct comparative analysis to define the assembly and egress pathways used by these viruses (49, 73). This is especially important given that HSV-1 and PRV assembly and egress are essentially indistinguishable when viewed by transmission electron microscopy (56).

Transport to the Cytoplasmic Envelopment Site

While the carboxyl-terminal species of VP1/2 plays an accessory role in the egress of capsids from the nucleus, full-length VP1/2 is essential for cytoplasmic envelopment and egress (41, 53). In addition, time-lapse imaging has shown that UL36-null PRV capsids fail to transport along microtubules. UL37-null PRV envelopes in the cytoplasm poorly but

retains vestigial intracellular transport (97). Because UL37-null PRV acquires capsid-bound VP1/2, the directed motion exhibited by this mutant supports a role for VP1/2 in microtubule transport that is enhanced by pUL37 (74). As discussed above, VP1/2 and pUL37 also contribute to capsid delivery to the nucleus, very likely by recruiting the dynein motor to capsids. Implicit in these observations is a puzzle. Capsid-VP1/2-pUL37-pUs3 complexes are present in the cytosol following both entry and postreplication (Figures 3 and 4), with both potentially recruiting the dynein motor, and yet the trafficking of these particles is quite different.

Two broad possibilities that could explain how capsids target to the nucleus during early infection and to the cytoplasmic envelopment site during egress require further examination. First, capsid-associated kinesin activities could be specifically enhanced during the egress stage of infection. Capsid-tegument complexes bind both dynein and kinesins (139). During retrograde axon transport, kinesin-based motion is weak and the predominating dynein-based motion results in capsid delivery to the neural soma (154). The presence of kinesin activity during retrograde axon transport is not intuitive but may be explained if kinesin and dynein recruitment also occurs during egress. In this scenario, capsid-associated kinesin activities could be enhanced to traffic the capsids to the site of envelopment. Second, capsids may recruit the dynein motor and move retrograde at both stages of infection. The presence of viral tegument and glycoproteins in the biosynthetic pathway during late infection could redirect capsid trafficking to the TGN for envelopment, as capsids move by dynein-based motion toward the MTOC. Similar to that observed in nonneuronal cell lines, capsids bud into the TGN in neurons and are released from the neural soma by exocytosis (113).

AXON TARGETING

In the absence of either gE, gI, or the pUs9 envelope proteins, PRV cannot transport through neural circuits in the anterograde direction (13, 18, 19, 21, 82, 83, 116, 117, 174). In cultured neurons the phenotype of the deletion mutants is not absolute, but PRV or HSV-1 lacking any one of these proteins is dramatically reduced for anterograde transmission in cultured neurons (24, 100, 155). When the genes for all three proteins are simultaneously deleted, anterograde transmission is eliminated (24). The gE, gI, and pUs9 viral envelope proteins coordinate the delivery of cytoplasmic viral particles from the neural soma into the axon. However, the requirement for these proteins in cultured axons is present only in neurons that have been cultured for prolonged periods: two weeks is typically used. In short-term cultures, anterograde axon transport of PRV is unimpeded in the absence of pUs9 (G. Smith & L.W. Enquist, unpublished data). Therefore, the barrier overcome by these proteins is slow to manifest following axon outgrowth and is consistent with the maturation of the axon initial segment (178).

The transmembrane domain and cytosolic tail of gE are dispensable for anterograde trafficking (167). This last point is remarkable for two reasons. First, gE is not structurally incorporated into virions in the absence of the transmembrane anchor and cytosolic tail. Second, the truncated protein is secreted. Taken together, these findings argue that gE is facilitating the delivery of cytoplasmic viral particles by interacting with extracellular proteins away from the viral particle.

Although the mechanisms by which these proteins function need to be further addressed, pUs9 appears to function in a way that is distinct from gE and gI. PRV lacking pUs9 is far more attenuated for anterograde transmission than are gE or gI mutants, yet the pUs9-null virus has no defect in plaque size, whereas gE-null and gI-null viruses do (24). The function of pUs9 is discussed further below.

Another puzzling facet to these proteins is that they are all selected against during serial passage in culture. PRV spontaneously loses gE expression during serial passage, and an extensive passage regime that was used to make an attenuated vaccine strain of PRV resulted in the deletion of all three genes. Similarly, isolates of HSV-1 with spontaneous mutations in gI or pUs9 have been reported (121, 161).

Anterograde Axon Transport

Trafficking of viral particles to the distal axon occurs by fast microtubule-based transport (153). In a remarkable case report of a nine-year-old boy that experienced herpetic pain from the sciatic nerve and zoster lesions six days later, a crude estimate of herpes anterograde spread in its natural setting was found to be consistent with the measured transport velocities of individual PRV capsids in culture neurons (162). This observation provides a unique validation of the use of neuron culture models in the study of herpesvirus infection. Unfortunately, studies of infection in neural culture models have resulted in a debate regarding the mechanics of anterograde axon transport. Two types of viral particle could potentially enter axons and engage in anterograde transport: cytosolic capsids that emerge from the nucleus (referred to as naked capsids owing to the absence of a membrane envelope) and enveloped virions in secretory vesicles resulting from the budding of naked capsids into the TGN. In both instances tegument proteins would be positioned to recruit motors and effect transport of the particle, whether it is a naked capsid or an enveloped virion (Figure 4). Prior to budding, naked capsids become decorated with inner tegument proteins, which together have the capacity to bind dynein and kinesin motors (139, 180). Upregulation of kinesin-bound motors would provide a hypothetical mechanism by which the distal axon could be targeted. On the other hand, enveloped virions are readily detected in neuronal cell bodies (22, 37, 113). Because enveloped virions reside in a membrane vesicle derived from the host biosynthetic pathway, these vesicles could intrinsically be targeted to the distal axon without the need for viral effectors. Alternatively, viral proteins, including tegument proteins and the cytosolic tails of envelope proteins, could serve as a platform to recruit microtubule motors to the cytosolic surface of the vesicle. Support for the latter comes from the observation that envelope and tegument proteins transport anterograde in axons in the absence of capsids (8, 37, 50, 112). In fact, tegument proteins transport to distal axons in advance of capsid-containing particles (96). Therefore, membrane-bound envelope-tegument complexes have the capacity to transport efficiently in axons. In further support of enveloped virion transport, TGN-derived vesicles containing viral particles isolated from HSV-1-infected cells exhibit kinesin-based motion in a reconstituted in vitro microtubule transport model (88).

Enveloped virions are observed by electron microscopy in axons in animal and culture models of infection, for both HSV-1 (32, 64, 79, 87, 99, 120) and PRV (24, 50, 51, 102), adding further support to the vesicle model of axon transport. However, this model was challenged by a study of HSV-1 in cultured human neurons that reported that all viral particles seen throughout the entire length of axons were naked capsids; enveloped virions were universally restricted to the neuronal cell body (136). This dramatic result had the explicit implication that capsids, or capsid-tegument complexes, directly recruited a kinesin motor independent of the neuronal secretory pathway. However, a subsequent reassessment of this finding concluded that the majority of capsids in axons were in fact enveloped but were restricted to axon terminals and varicosities along the axon (146). Therefore, the debate evolved into a discussion of why naked and enveloped viral particles were coresident in axons and which of these particles represented the species that transports anterograde in axons. Interested readers are directed to the many reports describing the debate that have presented differing viewpoints and have also provided descriptions of additional studies that

contribute to aspects of the debate that go beyond the current overview (for example, see References 34 and 43).

Recently, work from several labs, including new electron microscopy studies and time-lapse imaging of viral particle composition during active anterograde transport (10, 65, 120, 179), has provided compelling data that enveloped capsids of HSV-1 are transported anterograde in axons. The only remaining question is whether naked capsids also transport anterograde in axons. Because the tegument and envelope of herpes virions are heterogeneous, with the copy number of protein species varying widely from one particle to the next, this question will be difficult to conclusively answer (37). Viral particles containing low amounts of a tagged envelope protein will often be scored as lacking an envelope, particularly with the added challenge of imaging the particles while they are moving at speeds in excess of 1 μm s^{-1}. Although use of sensitive electron-multiplying charge-coupled device cameras coupled with bright light sources has allowed detection of dim emissions from rapidly moving viral particles in axons, detecting all viral particles by means of a heterogeneous antigen such as an envelope protein will likely require total internal reflection microscopy or a new technique of equivalent sensitivity to determine whether all capsids actively moving in axons are associated with envelope components (10). Several different investigations have provided additional insight into the nature of viral particle association with the transport vesicle. HSV-1-containing vesicles isolated from infected cell cytoplasm frequently do not contain a fully budded virion within the lumen of the vesicle, but instead have a capsid that is partially wrapped in the vesicle membrane (88). Similar structures are seen in axons of neurons infected with HSV-1 (146). Close inspection of transmission electron micrographs of PRV-containing vesicles in axons further hints at these structures (24). There is also functional evidence for axon transport of partially budded PRV (30). Although more studies are necessary to confirm the presence of stable budded intermediates serving as cargoes of kinesin-based anterograde transport, these preliminary observations may have implications for the spread of virions from axons to neighboring cells (see below). Moving forward, additional characterization of the transport vesicle hijacked by HSV-1 and PRV to reach the distal axon is needed. The cellular Vamp2 protein, which functions at presynaptic axon terminals, is often cotransported with HSV-1 and PRV transport vesicles and may indicate that virus-containing vesicles are predisposed to target the axon terminal (10). VP1/2 may be one protein that contributes to this targeting (151). In addition, the pUs9 protein is an intriguing candidate as a membrane-bound effector of anterograde axon transport (163).

A GFP-pUs9 fusion protein expressed by a recombinant of PRV cotransports with capsids to the distal axon, consistent with PRV anterograde transport occurring in a vesicle. Although this finding in itself is not unexpected, it is more notable that GFP-pUs9 was significantly diminished in extracellular viral particles. The reduction in GFP-pUs9 incorporation in extracellular particles relative to viral particles actively undergoing anterograde axon transport can easily be explained only if the GFP-pUs9 protein is enriched in the transport vesicle membrane that surrounds the viral particle (Figure 4). A mutation in GFP-pUs9 that prevents anterograde spread of PRV remained competent to move anterograde in axons, but capsids were no longer cotransported with the GFP signal. These findings provide a compelling case for pUs9 as an anterograde transport effector in neurons. Because pUs9 is dispensable for anterograde capsid transport in short-term neuron cultures, its contribution may be to overcome the barrier function of a mature axon initial segment. Alternatively, if pUs9 recruits a kinesin motor to effect anterograde transport, then redundant mechanisms of kinesin recruitment must be available in short-term neural cultures that become restricted as neurons mature.

Release from the Axon Terminal

Using chambered neuron culture models, investigators cannot detect release of free HSV-1 and PRV virions from axons of infected neurons (24, 106). This is due, not to a lack of anterograde transport in axons, but to virions remaining tethered to the axon surface after emerging from the axon (35). Infection by the surface-bound virions requires axon-cell contact and is dependent upon gB in the virions.

As PRV infection in cultured neurons progresses, the neurons display changes in electrophysiology (105). Action potentials fire at increased frequency and eventually neurons display synchronous firing. This electrical coupling of infected neurons is promoted by axon-axon fusion pores that form in part by the action of gB. Currently, no evidence indicates that the activity of gB that forms axon-axon fusion pores is the result of the population of gB present in virions at the axon surface. But if cell surface virions promote electrical coupling, it may hint at the presence of a pore between the axon and surface virion that would allow for subsequent connectivity upon fusion to a neighboring axon. In this context, the previously discussed observations that viral particles may not fully bud into transport vesicles could be relevant to electrical coupling if these structures are preserved after exocytosis. Changes in electrophysiology are predicted to cause the caustic itching (pruritis) exhibited during PRV infection, and a related phenomenon could underlie the severe pain (postherpetic neuralgia) often suffered following VZV reactivation.

Spread Between Neurons and to Distant Sites

As previously noted, herpes virions can egress from the cell bodies of neurons as well as from axons. The biological consequence of virion release from the neuron cell body in peripheral ganglia has broad implications in the pathogenesis of the neurotropic herpesviruses. In the simplest scenario, HSV or PRV would only transmit between neurons and the cells they innervate at the periphery by repeated cycles of anterograde and retrograde axonal transport (Figure 2). The tendency for HSV-1 lesions (herpes labialis, or cold sores) to creep from one reactivation episode to the next would be explained by lateral spread of infection in the mucosal epithelium, allowing for seeding of new neurons that innervate cells adjacent to the active lesion. This view is consistent with the presentation of cold sore lesions, which tend to be focal and exhibit limited dissemination in the innervated dermatome. This is in contrast to reactivated VZV infections, which are discussed below.

Spread of HSV-1 within sensory ganglia may be limited by satellite cells that surround each neuron. Although these support cells are susceptible to PRV and HSV-1, they generally do not appear to be productively infected (22, 32, 63, 87). Perhaps the best evidence for the absence of lateral spread within peripheral ganglia comes from an in vivo study of PRV infection (158). PRV inoculated into the anterior chamber of the rat eye transits by retrograde axon transport to neuron cell bodies in the superior cervical ganglion. Subsequent labeling of the subset of superior cervical ganglion neurons that project to the anterior chamber by retrograde labeling with wheat germ agglutinin conjugated to horseradish peroxidase demonstrated that PRV remained confined to the neurons projecting axons to the anterior chamber of the eye and did not spread to neighboring neurons projecting to other sites. These results provided compelling evidence that viral dissemination occurred only by circuit-specific transmission. However, the PRV strain used in this seminal study, PRV Bartha, is an attenuated vaccine strain. Whether these results hold true for wild-type isolates of PRV or HSV-1 has not, to the best of my knowledge, been examined. But available evidence argues that wild-type virus strains are not always subject to this restriction. HSV inoculated on the mouse flank results in a zosteriform spread throughout the innervated dermatome, which suggests HSV spreads between neurons in dorsal root sensory ganglia (DRG) before returning back to the skin (173). Applying PRV to the flank model does not

produce zoster-like lesions in the skin, but nevertheless the virus transmits through the dermatome, indicating that PRV, like HSV, disseminates laterally within the peripheral ganglia (19). In contrast, the attenuated Bartha strain remains confined to the inoculation site in the skin and does not spread through the dermatome.

In contrast to HSV and PRV, VZV reactivation often produces infection throughout a dermatome, which is the prototypic shingles manifestation (herpes zoster). Observations of human DRG infected with VZV either from human patients or in a mouse xenograft model are consistent with productive infections of neurons and satellite cells (48, 142). Furthermore, satellite cell and neural syncytia in human DRG are also evident. Lateral spread in sensory ganglia would likely result in infection of sensory neurons that project to internal organs. These general visceral afferent neurons are similar to somatosensory pseudounipolar neurons and are coresident in sensory ganglia. Because visceral infections are not associated with cold sore eruptions, this would argue that HSV-1 does not randomly spread between neurons in sensory ganglia in adult humans. However, neonatal transmission of HSV-2 can produce life-threatening infections that involve disseminated infections in internal organs (72). VZV spread to internal organs can also occur, although this outcome is more common in immunocompromised individuals (11).

Disseminated infections can occur with HSV, PRV, and VZV by means of cell-associated viremia. While this is most prevalent in VZV infections, PRV transplacental spread occurs by monocytes that transmit the infection (119). HSV infections are generally not associated with cell-associated viremia, but in the absence of an interferon response, infection transmits efficiently to the liver and other organs (95, 133). Neonatal disseminated HSV-2 infections are also associated with cell-associated viremia (42).

CONCLUSIONS

Whereas many viruses transport retrogradely in axons of neurons, neuroinvasive herpesviruses are notable for their efficient entry into the nervous system and coordination of retrograde and anterograde trafficking at opposing stages of the infectious cycle. These properties make viruses such as HSV-1 candidates for the development of gene delivery vectors that target the nervous system, but also underlie the potential for these viruses to become highly virulent following dissemination into the central nervous system. Ongoing research will resolve how herpesvirus assembly and egress are coupled to neuroinvasion and pathogenesis, and may yield the tools to produce both novel gene vectors and antiviral treatments to combat severe forms of neuroinvasive disease.

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Glossary

PNS	peripheral nervous system
PRV	pseudorabies virus
HSV	herpes simplex virus
CNS	central nervous system
VZV	varicella zoster virus

MTOC	microtubule organizing center
NPC	nuclear pore complex
TGN	*trans*-Golgi network

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SUMMARY POINTS

1. Herpesviruses overcome multiple barriers to establish latent infections in the nervous system.
2. Tegument proteins retained on capsids likely recruit microtubule motors necessary for axon transport.
3. Several viral and cellular factors have been identified that contribute to capsid delivery to the nucleus and to our understanding of how and when these functions delineate steps in neuronal infection.
4. Removal of VP16 from capsids upon axon entry may promote establishment of latency in the infected neuron.
5. HSV and PRV effectively transmit between synapse-linked neurons, but productive recurrent infections in natural hosts do not involve synaptic transmission.
6. Neurologic responses such as pain and pruritis may indicate productive infection and spread within ganglia.
7. In addition to their characteristic neurotropism, HSV, PRV, and VZV share broad-tissue tropism, which accounts for replication in the mucosa, visceral organs, and cell-associated viremia.

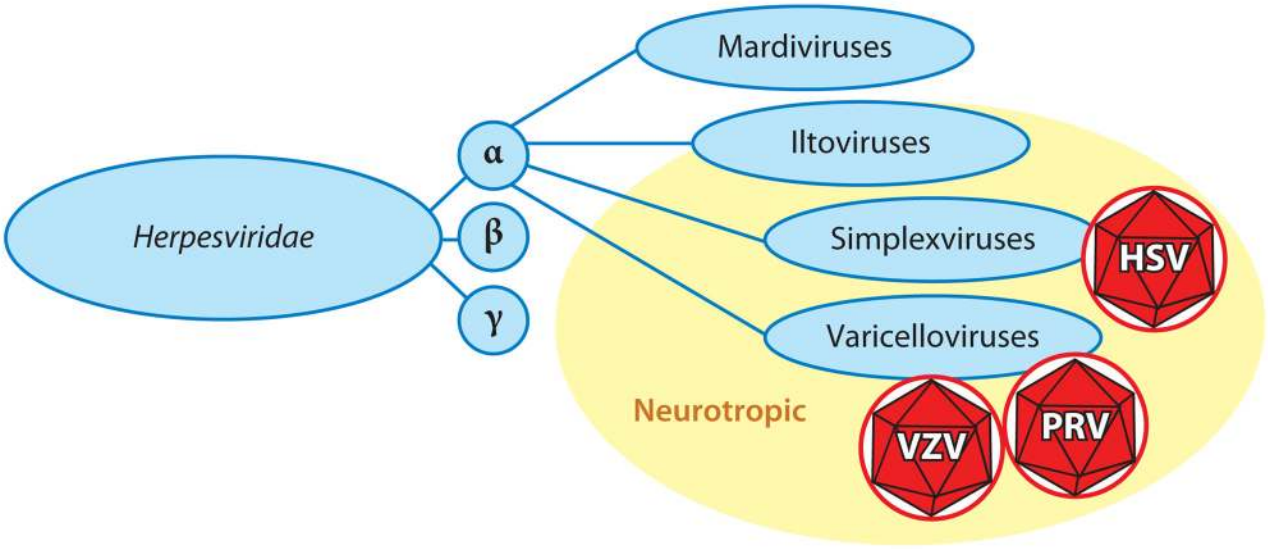

Figure 1.
The *Alphaherpesvirinae* subfamily consists of four genera. Two of the genera, the *Simplexvirus* and *Varicellovirus*, consist of human and veterinary pathogens that establish latency in peripheral neurons. Herpes simplex virus (HSV) is a simplexvirus, while varicella zoster virus (VZV) and pseudorabies virus (PRV) are varicelloviruses. At least one member of the *Iltovirus* genus is also proposed to establish latent infections in peripheral neurons (177), but the *Mardivirus* genus lacks neurotropism.

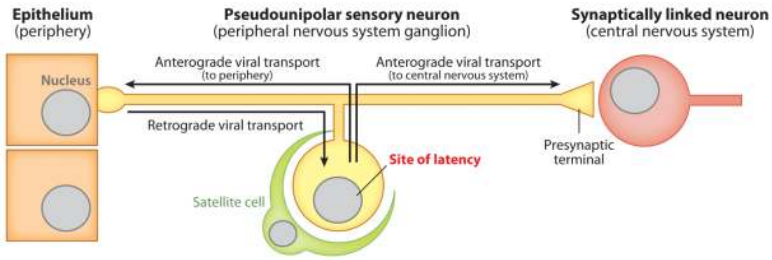

Figure 2.

Fundamentals of a neurotropic herpesvirus niche. Although the herpesviruses discussed in this article are referred to as neurotropic, these agents are more accurately described as dual tropic or multitropic. Primary infections begin in exposed tissues such as mucosal epithelia (*left*). Subsequent spread is normally restricted to innervating neurons resident in peripheral ganglia, where latent infections are maintained, following a single round of retrograde axon transport (*middle*). Reactivation results in the production of new viruses and the return to peripheral tissues (anterograde axon transport to epithelia). Severe disease associated with invasion of second-order neurons (anterograde axon transport to CNS) occurs only rarely in the natural host but may be frequent in secondary hosts (*right*). Anterograde and retrograde spread is dictated by the orientation of axonal microtubules and should not be confused with the direction of action potential propagation in the pseudounipolar neuron. Neurotropic herpesviruses can also infect cells of the circulatory system to varying degrees (not illustrated).

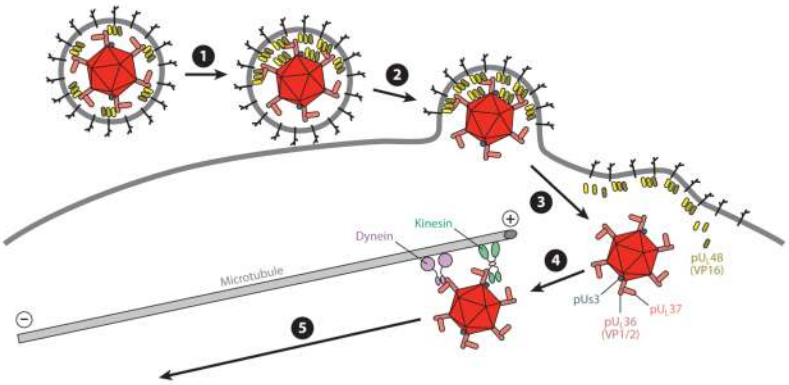

Figure 3.
Early events in herpesvirus infection. ❶ Virion contacts plasma membrane of a somatic cell or the axon membrane of a neuron. Tegument proteins redistribute and the virion orients with the bulk of its mass away from the cell. ❷ Fusion between the virion envelope and the cell membrane deposits the capsid and tegument proteins into the cytosol. ❸ The capsid releases from the majority of tegument and envelope proteins (including VP16), but a subset of inner tegument proteins (including pUs3, VP1/2, pUL37) remain capsid bound and together compose the retrograde transport complex. VP16, which enters the nucleus in somatic cells and promotes productive infection, may be lost upon entering a neuron owing to an inability to efficiently participate in retrograde axon transport. ❹ The retrograde transport complex traverses cortical actin (not illustrated) and associates with dynein and kinesin motors that in turn bind microtubules. Microtubules in axons are almost uniformly oriented, with plus-ends facing the axon terminals. ❺ Dynein dominates over kinesin activity, resulting in transport toward the minus-ends of microtubules and trafficking to the neural soma (retrograde transport).

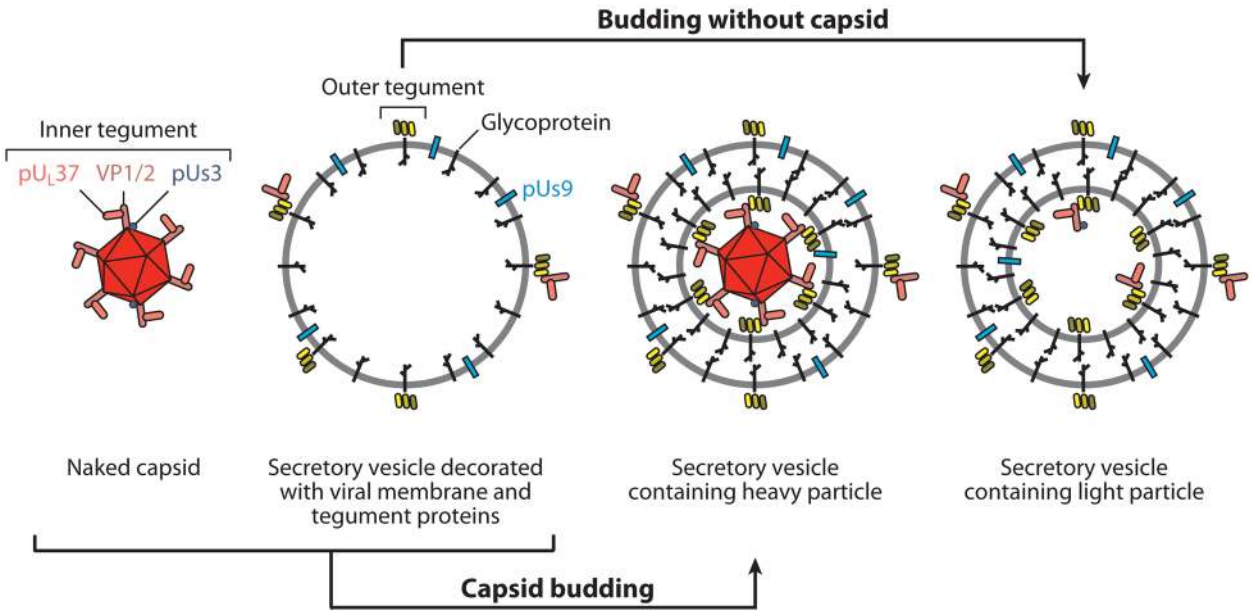

Figure 4.

Four types of cytoplasmic viral particles present during the egress stage of infection. Unenveloped (naked) capsids newly egressed from the nucleus acquire inner tegument proteins beginning with VP1/2, which binds capsids directly. The composition of these particles and the retrograde transport complex that is released into the cytosol following entry are similar, if not equivalent (see Figure 3). Outer tegument proteins associate primarily with viral membrane proteins resident in the biosynthetic pathway (secretory vesicle), which may also acquire some inner tegument proteins in the absence of capsids. Membrane proteins consist of glycoproteins and nonglycosylated membrane-associated proteins that include pUs9. Infectious virions (heavy particles) form when capsids bud into membranes decorated with viral membrane and outer tegument proteins and subsequently exocytose from the cell. Light particles are noninfectious secreted viral particles that consist of a viral envelope and tegument proteins but lack a capsid. The three membrane-associated intracellular particles expose membrane protein tails and tegument proteins to the cytosolic surface. Among the exposed proteins, pUs9 is enriched in the vesicle membrane surface and may direct trafficking of particles to the distal axon.

Table 1

Factors governing capsid delivery to the nucleus

Step	Viral proteins	Cellular proteins	References
Passage through cortical actin?	gD	ROCK1 FAK PI3K Rho GTPases	26, 28, 36, 52, 127, 129
Retrograde microtubule transport	pUL35 (VP26)?	Dynein Dynactin	7, 44–46
Nuclear delivery[a]	pUL14 pUL36 (VP1/2) pUL37 ICP0	Proteasome	33, 38, 39, 78, 143, 148, 181
Nuclear docking and genome release	pUL6 (portal) pUL25 pUL36 (VP1/2)	Importin-β Ran Nup214 Nup358hCG1	1, 2, 4, 14, 33, 68, 75, 122, 128, 131, 132, 138, 144

[a]Factors listed are required for efficient delivery of capsids to the nucleus, and maybe involved in passage through cortical actin or microtubule transport.