Tegument Assembly, Secondary Envelopment and Exocytosis

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Abstract

Alphaherpesvirus tegument assembly, secondary envelopment, and exocytosis processes are understood in broad strokes, but many of the individual steps in this pathway, and their molecular and cell biological details, remain unclear. Viral tegument and membrane proteins form an extensive and robust protein interaction network, such that essentially any structural protein can be deleted, yet particles are still assembled, enveloped, and released from infected cells. We conceptually divide the tegument proteins into three groups: conserved inner and outer teguments that participate in nucleocapsid and membrane contacts, respectively; and "middle" tegument proteins, consisting of some of the most abundant tegument proteins that serve as central hubs in the protein interaction network, yet which are unique to the alphaherpesviruses. We then discuss secondary envelopment, reviewing the tegument-membrane contacts and cellular factors that drive this process. We place this viral process in the context of cell biological processes, including the endocytic pathway, ESCRT machinery, autophagy, secretory pathway, intracellular transport, and exocytosis mechanisms. Finally, we speculate about potential relationships between

cellular defenses against oligomerizing or aggregating membrane proteins and the envelopment and egress of viruses.

Introduction

The alphaherpesviruses include important human pathogens herpes simplex viruses 1 and 2 (HSV-1 and -2), varicella-zoster virus (VZV), and various veterinary viruses. The envelopment and egress processes are understood in broad strokes, but many of the individual steps, and their molecular and cell biological details, remain unclear. A virus replication cycle begins as virus particles enter by fusing their virion envelope with cellular membranes, undergo post-entry transport to the nucleus, and release their viral DNA genomes into the nucleus. At this point, the virus may begin a lytic replication cycle, producing progeny virus particles and ultimately killing its host cell, or in peripheral nervous system neurons, alphaherpesviruses may establish latency, and persist for the lifetime of the host.

During lytic replication in non-neuronal cell types, or following reactivation from latency in neurons, DNA replication, capsid assembly, and genome packaging occurs in the host cell nucleus. Nucleocapsids then exit the nucleus via the process of primary envelopment, whereby nucleocapsids transiently acquire an envelope to shuttle across the nuclear membranes.

Meanwhile, newly-translated viral tegument proteins begin to assemble in the cytoplasm, and newly-translated viral transmembrane proteins traffic within the host endomembrane system. Nucleocapsids, tegument complexes, and viral membrane proteins then converge on intracellular membranes, thought to be trans-Golgi or endosomal organelles, and interactions between these sub-assemblies drive the process of virion envelopment - called "secondary envelopment" within the herpesvirus field to distinguish it from the process of primary envelopment/nuclear egress. Secondary envelopment, and possibly subsequent membrane trafficking steps, produces an enveloped infectious virion in the lumen of a secretory vesicle. This virion-in-a-vesicle then traffics to the cell periphery, and the surrounding secretory vesicle fuses with the plasma membrane, a process called exocytosis. This deposits the virion outside of the

infected cell where it can infect another host cell, or the virion can be shed to the environment to infect another host organism.

A multitude of viral mechanisms, host cell biological mechanisms, and virus-host interactions drive tegument assembly, secondary envelopment, intracellular trafficking, and exocytosis of progeny virus particles from the infected cell. This review will focus on the viral tegument and membrane proteins, and what is known of the molecular and cell biology of these final steps in the virus replication cycle.

Tegument

The herpesvirus tegument is analogous to the matrix proteins of many other enveloped viruses in that they link the viral nucleocapsid to the viral envelope. Accordingly, the herpesvirus tegument, as a whole, plays a central role in the process of secondary envelopment. But, in contrast to typical viral matrix proteins, the herpesvirus tegument layer is much larger and more complex, consisting of around 24 different viral proteins (Kramer et al., 2011; Loret et al., 2008), forming a highly robust and redundant protein interaction network.

Identification of proteins incorporated into the tegument is not always straightforward. Whether detected by radiolabeling, immuno-assays, or mass spectrometry, contaminating infected cell material can present false positives, and proteins with very low abundance may not be detected (Engel et al., 2015; Leroy et al., 2016; Loret et al., 2008). Many host proteins are also incorporated into the tegument and envelope, but whether they are incorporated specifically and what functional roles they may play, if any, is not known (Kramer et al., 2011; Leroy et al., 2016; Loret et al., 2008).

Most of the roughly 24 viral tegument proteins do not appear to affect tegument assembly and secondary envelopment processes (Crump, 2018; Owen et al., 2015). Instead, many of these proteins have other well-studied enzymatic or regulatory roles, such as protein kinases (UL13 and US3), thymidine kinase (UL23), RNA endonuclease responsible for viral host shutoff (vhs/UL41), dUTPase (UL50), countermeasures against intrinsic cell defenses (ICP34.5, and

US11), E3 ubiquitin ligase (ICP0), and transcription regulator (ICP4). While these proteins are not required for assembly and secondary envelopment, they may be specifically incorporated into the tegument because they are delivered to the cytoplasm during entry and serve to modulate viral and host functions during the earliest stages of infection.

The functions of several other tegument proteins, namely UL55 (Nash and Spivack, 1994), US2 (Kang et al., 2013), and US10 (Nishiyama et al., 1993; Yamada et al., 1997) warrant further study, but these proteins are generally dispensable for virus replication *in vitro*, and so they are not thought to play major roles in tegument assembly or envelopment (Crump, 2018; Owen et al., 2015).

A large body of literature, discussed and cited in detail below, has identified at least 12 viral tegument proteins that form a protein interaction network (Figure 1) that drives tegument assembly and secondary envelopment. This network is highly robust with functional redundancy, so most individual tegument proteins and protein-protein contacts are not necessary for tegument assembly, secondary envelopment, or even production of infectious virions. In addition to this complexity and functional redundancy, the study of tegument proteins that drive assembly and secondary envelopment is complicated by the fact that many of these proteins also have enzymatic or regulatory roles during other stages of infection. As one example, in addition to its role in tegument assembly, VP16 (UL48) serves as a transactivator of viral gene transcription and interacts with vhs (UL41) to modulate its mRNA degradation activity. Deletion of VP16 (UL48) therefore impacts virus production in multiple ways: a direct effect on tegument assembly and indirect effects due to changes in viral transcription and unconstrained vhs (UL41) activity blocking viral and host gene expression (Mossman et al., 2000). Loss-of-function approaches, such as deletions in viral genes, are frequently difficult to interpret due to the multifunctional nature of most viral proteins, the ability of cloned virus strains to rapidly regenerate genetic diversity (Parsons et al., 2015), and therefore the tendency of mutant viruses to rapidly acquire compensatory mutations in other viral genes (Haugo et al., 2011).

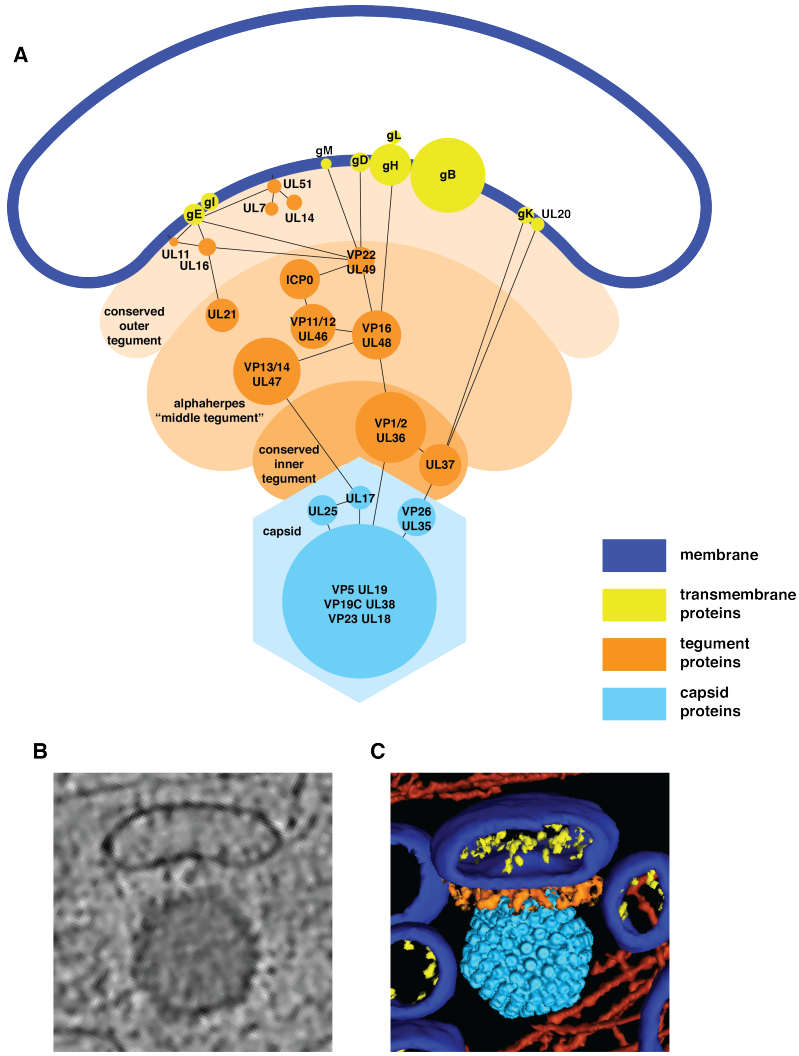

Figure 1. Alphaherpesvirus tegument and membrane protein interaction network drives secondary envelopment. (A) Tegument proteins (orange) interact with capsid proteins (light blue), viral membrane proteins (yellow), and directly with membranes (dark blue) during secondary envelopment. We conceptually divide the tegument proteins into 3 groups: Conserved inner tegument proteins, which are the major capsid-interacting proteins; "Middle" tegument proteins, which are among the most abundant tegument proteins, but are unique to the *alphaherpesvirinae*; Conserved outer tegument proteins, which participate in many membrane contacts. Each circle, representing a structural protein, is scaled by the molecular weight and estimated abundance (Loret et al., 2008) of the indicated protein, to represent the approximate relative mass in a typical HSV-1 virion. (B) CryoEM tomography slice showing an HSV-1 nucleocapsid interacting with an intracellular vesicle, possibly undergoing secondary envelopment. (C) Surface rendering depicting the nucleocapsid (light blue), tegument (orange), membrane protein densities (yellow) in the lumen of the vesicle (dark blue). Data in panels B-C kindly provided by Kay Grünewald (Ibiricu et al., 2011) and reproduced under the terms of the Creative Commons Attribution License.

Inner Versus Outer Tegument

In much of the literature, tegument proteins are conceptually divided into "inner tegument proteins", which are more tightly associated with the capsid, and "outer tegument proteins", which form the bulk of the tegument. The largest tegument protein, VP1/2 (UL36), its binding partner UL37, and protein kinase US3 are typically considered inner tegument proteins. This classification is mainly supported by biochemical fractionation of virions and studies showing that outer tegument proteins dissociate from the capsid during entry (Radtke et al., 2010; Wolfstein et al., 2006). However, this classification between inner and outer tegument proteins has been challenged by several notable studies using fluorescence-based methods to estimate the stoichiometry and radial distribution of select tegument proteins within individual virus particles (Bilali et al., 2017; Bohannon et al., 2013; Laine et al., 2015). While canonical inner tegument proteins VP1/2 (UL36) (Bohannon et al., 2013) and UL37 (Bilali et al., 2017; Bohannon et al., 2013) appeared to be incorporated with little variance, similarly to the stoichiometric incorporation of capsid proteins, US3 was highly variable (Bohannon et al., 2013). Canonical outer tegument proteins exhibited a wide range of variation, from stoichiometric incorporation to seemingly random incorporation (Bilali et al., 2017; Bohannon et al., 2013). Furthermore, super-resolution fluorescence microscopy showed that both the inner tegument protein UL37 and outer tegument protein VP16 (UL48) were located at nearly the same average radius from the capsid (Laine et al., 2015). Thus, the historical distinction between "inner" and "outer" tegument proteins does not necessarily reflect how individual tegument proteins are incorporated or distributed within the virion.

Given our collective knowledge of the alphaherpesvirus tegument, we find it more useful to think about tegument proteins in terms of their connectivity within the protein interaction network, the requirement for assembly and secondary envelopment, and degree of conservation among herpesvirus subfamilies. As detailed below, we conceptually distinguish between a conserved "inner tegument" that makes contacts with the nucleocapsid, a conserved "outer tegument" that mediates many membrane contacts, and a "middle tegument"

that links the two. Paradoxically, while these "middle tegument" proteins are among the most abundant in the virion and form central hubs within the tegument protein interaction network, they are unique to the *alphaherpesvirinae* and do not appear to be absolutely necessary for production of infectious virus particles. Therefore, this review will also consider, from an evolutionary perspective, what conserved viral protein interactions and host cell biological mechanisms may be minimally required for tegument assembly, secondary envelopment, and production of infectious virions.

Conserved Inner Tegument Protein VP1/2 (UL36) is the Lynchpin of Capsid-Tegument Interactions

VP1/2 (UL36), the largest tegument protein, directly links the capsid to the tegument. Previous studies identified capsid-binding sites within the C-terminal third of VP1/2 (UL36), and more recently, cryoEM studies have shown that VP1/2 (UL36), together with capsid-associated proteins UL17 and UL25, form the capsid vertex specific component (CVSC), also called the capsid-associated tegument complex (CATC) (Dai and Zhou, 2018; Fan et al., 2015; McElwee et al., 2018). The CVSC is a pentameric protein complex composed of two VP1/2 (UL36) molecules, two UL25 molecules, and one UL17 molecule. Five CVSC complexes bind with 5-fold symmetry around each of the 11 penton vertices. However, while UL17 and UL25 are required for primary envelopment and nuclear egress, VP1/2 (UL36) is not. This contradiction has been clarified by a study (Fan et al., 2015) showing that while VP1/2 (UL36) is required for formation of the CVSC density in cryoEM reconstructions, in the absence of VP1/2 (UL36), UL17 and UL25 can still bind to capsids, likely in a more disordered fashion, and promote nuclear egress. In addition to its role at the penton vertices, the CVSC, including VP1/2 (UL36), contributes to formation of the portal vertex-associated tegument (PVAT) (McElwee et al., 2018). However, additional densities within the PVAT remain unaccounted for, and whether there are additional contributions from VP1/2 (UL36) or other tegument proteins remains to be determined (McElwee et al., 2018; Schmid et al., 2012).

The N-terminal third of VP1/2 (UL36) projects away from the capsid (Laine et al., 2015; Newcomb and Brown, 2010; Scrima et al., 2015) and binds other

tegument proteins, namely the inner tegument protein UL37 (Bucks et al., 2007; Klupp et al., 2002; Vittone et al., 2005) and the outer tegument protein VP16 (UL48) (Ko et al., 2010; Svobodova et al., 2012), linking capsids to the rest of the tegument. UL37, in turn, also appears to bind the small capsid protein VP26 (UL35) to provide another capsid-tegument interaction (Lee et al., 2008), and may also interact with viral envelope proteins (see "*Tegument/Membrane Interactions in Secondary Envelopment"* below). In the absence of VP1/2 (UL36), capsids still associate with cellular membranes to some degree (Kharkwal et al., 2015), arguing for the existence of other capsid-tegument-membrane interactions; however, these capsids accumulate in the cytoplasm, fail to recruit a substantial, electron-dense tegument layer, and do not undergo secondary envelopment (Desai, 2000; Schipke et al., 2012).

Importantly, VP1/2 (UL36) and its binding partners, UL37 and VP16 (UL48), are the only three tegument proteins that are necessary for production of infectious virions by HSV-1 (Desai et al., 2001; Weinheimer et al., 1992). In contrast, pseudorabies virus (PRV) containing mutations disrupting VP16 (UL48) or UL37 can be propagated, although these viruses are severely attenuated (Fuchs et al., 2002a; Leege et al., 2009). These observations underscore the critical role of VP1/2 (UL36) as the lynchpin of capsid-tegument interactions. Since VP1/2 (UL36) and UL37 are core genes conserved among the herpesviruses, they likely represent an ancestral inner tegument that links nucleocapsids to the rest of the tegument.

"Middle" Tegument Proteins

VP11/12 (UL46), VP13/14 (UL47), VP16 (UL48), and VP22 (UL49), the most abundant tegument proteins in viral particles, are unique to the *alphaherpesvirinae*, suggesting that there is significant divergence in tegument structure and function between herpesvirus subfamilies (Mocarski, 2007). VP16 (UL48), described above as a VP1/2 (UL36) binding partner, also interacts with each of the other middle tegument proteins, VP11/12 (UL46), VP13/14 (UL47), and VP22 (UL49), making it a central hub of tegument interactions (Elliott et al., 1995; Kato et al., 2000; Svobodova et al., 2012; Vittone et al., 2005). VP22 (UL49) is also a hub of protein-protein interactions, as it is reported to interact

with VP16 (UL48) and UL16 (Maringer et al., 2012; Starkey et al., 2014). VP22 (UL49) also interacts with several viral membrane proteins and may directly bind to the membrane bilayer via charged amino acids (see "*Tegument/Membrane Interactions in Secondary Envelopment"* below). VP11/12 (UL46) and VP13/14 (UL47) both bind VP16 (UL48), and VP13/14 (UL47) is reported to bind the capsid protein UL17, which may provide another link between capsid and tegument (Scholtes et al., 2010). VP22 (UL49) and VP11/12 (UL46) may also interact indirectly via their common binding partner ICP0 (Lin et al., 2013; Maringer and Elliott, 2010; Maringer et al., 2012). Numerous other potential interactions have been detected using yeast two-hybrid, protein cross-linking, and co-immunoprecipitation/mass spectrometry approaches, but most of these have not yet been validated using other methods.

In HSV-1 and PRV, three of these middle tegument proteins, VP11/12 (UL46), VP13/14 (UL47), and VP22 (UL49) are not necessary for virus replication (del Rio et al., 2002; Elliott et al., 2005; Kopp et al., 2002; Mossman et al., 2000; Pomeranz and Blaho, 2000; Weinheimer et al., 1992; Zhang and McKnight, 1993; Zhang et al., 1991). VP16 (UL48) is necessary for HSV-1 replication, but it is not necessary for replication of VZV and PRV (Cohen and Seidel, 1994; Fuchs et al., 2002a). Strikingly, simultaneous deletion of all four middle tegument proteins does not fully block production of infectious virions in PRV, although these viruses are severely attenuated (Fuchs et al., 2003). Thus, while these tegument proteins are present at high abundance and form central hubs in the tegument protein interaction network, they are not absolutely required for tegument assembly and secondary envelopment. Since these proteins are also not conserved between the herpesvirus subfamilies, it seems unlikely that they represent the ancestral herpesvirus tegument. Probably other tegument protein interactions exist between the conserved inner and outer tegument proteins that are capable driving tegument assembly and secondary envelopment in the absence of these "middle" tegument proteins, but few of these putative interactions have been discovered.

Conserved Outer Tegument Proteins

In addition to conserved inner tegument proteins VP1/2 (UL36) and UL37, several outer tegument proteins are conserved among the herpesvirus subfamilies and participate in tegument assembly and secondary envelopment. Conserved proteins UL11 and UL16, together with the alphaherpesvirus-specific protein UL21, are reported to form a tripartite complex (Han et al., 2012; Harper et al., 2010; Lee et al., 2008; Loomis et al., 2003; Sadaoka et al., 2014; Vittone et al., 2005; Yeh et al., 2008), and deletion of UL11 and UL16 reduces, but does not fully eliminate secondary envelopment (Baines and Roizman, 1992; Fulmer et al., 2007; Kopp et al., 2003; Starkey et al., 2014). UL16 interacts with VP22 (UL49), one of the central hubs of the tegument protein interaction network (Starkey et al., 2014). UL11 and UL16 provide multiple links between tegument and membrane (see "*Tegument/Membrane Interactions in Secondary Envelopment",* below), and UL16 and UL21 are also reported to interact with nucleocapsids (de Wind et al., 1992; Meckes and Wills, 2007; Meckes et al., 2010; Oshima et al., 1998), possibly providing direct links between nucleocapsids and membranes during secondary envelopment.

UL51, UL7, and UL14 are also conserved among the herpes subfamilies, and deletion in any of these genes causes impaired secondary envelopment (Albecka et al., 2017; Fuchs et al., 2005; Klupp et al., 2005; Nozawa et al., 2002; 2005; Oda et al., 2016). Both UL7 and UL14 bind UL51 (Albecka et al., 2017; Oda et al., 2016; Roller and Fetters, 2015), so these proteins may also form a tripartite complex. Like UL11, UL51 provides multiple membrane contacts (see "*Tegument/Membrane Interactions in Secondary Envelopment",* below).

Notably, although these tegument proteins are conserved among the herpesvirus subfamilies, and many of them are considered necessary for replication of the betaherpesvirus HCMV, these proteins are not strictly necessary for tegument assembly and secondary envelopment in HSV-1 and PRV (Mocarski, 2007). These proteins may represent an ancestral herpesvirus outer tegument that may drive tegument assembly and secondary envelopment in the absence of the middle tegument proteins, so further research into the

molecular and structural details of these tegument interactions may be particularly valuable.

Tegument/Membrane Interactions in Secondary Envelopment

Just as the alphaherpesvirus tegument is far more complex than the matrix of other viruses, so too are the alphaherpesvirus membrane proteins. HSV-1 incorporates 9-12 glycoproteins into virions: gB, gC, gD, gE, gG, gH, gI, gL, and gM, and it is ambiguous whether gJ, gK and gN are incorporated into virions (Loret et al., 2008). In addition, HSV-1 incorporates at least 4 other non-glycosylated transmembrane proteins into mature virions: UL20, UL45, UL56, and US9, and it is ambiguous whether UL43 is incorporated (Loret et al., 2008). In addition, several tegument proteins, such as UL11, UL51, and possibly VP22 (UL49) (detailed below), contain membrane-binding motifs and are peripherally associated with the membrane. Like the tegument protein interaction network, which is robust with redundancy, the interactions between tegument and intracellular membranes during secondary envelopment is also robust and redundant.

The conserved tegument proteins UL11 and UL51 (see "*Conserved Outer Tegument Proteins*", above) are acylated - UL51 is palmitoylated, and UL11 is both myristoylated and palmitoylated - allowing them to directly interact with cellular membranes (Loomis et al., 2001; MacLean et al., 1989; Nozawa et al., 2003). UL11 and its binding partner UL16 are both reported to also bind the cytosolic tail of gE (Farnsworth et al., 2007; Han et al., 2012; 2011; Yeh et al., 2011), and UL51 is also reported to interact with gE (Roller et al., 2014). More recent proteomics approaches have identified many other viral proteins that may also be palmitoylated, including tegument proteins UL24, US2, and US3, and membrane proteins gE, gI, gK, gG, gH, and UL56 (Serwa et al., 2015).

Cellular enzymes methionine aminopeptidase 2 and N-myristoyltransferase 1/2 add myristoyl moieties during protein translation, and this modification is irreversible (Udenwobele et al., 2017). Palmitoylation is driven by a large number of cellular protein acyl-transferases with different substrate affinities. Unlike myristoylation, palmitoylation is reversible, and de-palmitoylation is

mediated by cellular acylprotein thioesterase 1/2 (Ko and Dixon, 2018). Some cellular proteins cycle between palmitoylated and de-palmitoylated states on a timescale of seconds, allowing dynamic modulation of their membrane association, biophysical properties in the membrane, and intracellular trafficking and localization (Ko and Dixon, 2018). Therefore, dynamic palmitoylation/de-palmitoylation may serve as a molecular switch that regulates the function of these proteins during tegument assembly and secondary envelopment.

VP22 (UL49) is a central hub of both the tegument protein interaction network (see "'*Middle' Tegument Proteins"*, above) and also of tegument-membrane interactions. VP22 (UL49) is reported to bind the cytosolic tails of gE, gM, and gD (Farnsworth et al., 2007; Fuchs et al., 2002b; Maringer et al., 2012). VP22 (UL49) also binds to UL16, which in turn binds UL11 and gE, providing another contact to membranes (Starkey et al., 2014). Furthermore, VP22 (UL49) may also directly associate with membranes via basic residues binding to acidic cellular phospholipids (Brignati et al., 2003). Triple deletions of gE, gI, and either gM or gD blocks secondary envelopment (Brack et al., 1999; Farnsworth et al., 2003), whereas individual deletions of these glycoproteins or VP22 (UL49) have minimal effects on secondary envelopment, indicating that these protein interactions function in a redundant manner. VP16 (UL48), another central hub of tegument protein interactions, is reported to bind gH (Gross et al., 2003; Kamen et al., 2005), although this interaction may not be conserved with PRV (Omar et al., 2013).

Finally, the capsid protein UL37 may also participate directly in secondary envelopment as it may share some structural homology with cellular proteins that regulate intracellular membrane trafficking (Pitts et al., 2014) and is reported to interact with viral envelope proteins gK and UL20 (Jambunathan et al., 2014). gK and UL20 form a complex, and deletion of either protein impairs secondary envelopment (Foster et al., 2004; Jayachandra et al., 1997; Melancon et al., 2005). However, the secondary envelopment defects of these mutants may not be solely due to loss of particular tegument-envelope contacts because gK and UL20 (similarly to gM) influence the intracellular trafficking of

viral envelope glycoproteins gD, gH, and gL, as well as host membrane proteins (Crump et al., 2004; Lau and Crump, 2015; Ren et al., 2012).

Interestingly, the only conserved tegument-membrane interactions reported so far are the conserved outer tegument proteins UL11 and UL51 binding directly to the membrane via acyl modifications. All of the other reported interactions between tegument proteins and viral transmembrane proteins are unique to the *alphaherpesvirinae* because many membrane contacts are either mediated by the "middle" tegument proteins, which are not conserved, or involve the cytosolic domains of gD, gE, gK, and UL20, which are all unique to the alphaherpesvirus subfamily (Mocarski, 2007). Therefore, just as there appear to be significant differences in the "middle" tegument, there also appears to be significant divergence between the herpesvirus subfamilies in the tegument-membrane interactions that drive secondary envelopment.

L-Particle Envelopment

In addition to assembly of infectious virions, the alphaherpesviruses produce a great number of noninfectious "L-particles", or "light particles" (Rixon et al., 1992; Szilágyi and Cunningham, 1991). The functions of L-particles in viral infection and pathogenesis are not entirely clear, but have been discussed in recent reviews (Bello-Morales and López-Guerrero, 2018; Heilingloh and Krawczyk, 2017; Kalamvoki and Deschamps, 2016). L-particles are similar in size to virions and contain viral tegument and envelope proteins, but do not contain viral capsids or genomes. Dense tegument structures, which closely resemble the contents of extracellular L-particles, can be observed budding into cytoplasmic membranes in the absence of capsids (Alemañ et al., 2003; Granzow et al., 2001). Therefore, L-particle assembly and envelopment appear to share mechanistic similarities with assembly and secondary envelopment of infectious virions. Moreover, following envelopment, it has also been reported that L-particles traffic to the plasma membrane using the same secretory pathway Rab GTPases as virions (Hogue et al., 2016).

Viruses lacking tegument proteins VP1/2 (UL36), UL37, and VP16 (UL48) still produce copious L-particles (Desai et al., 2001; Fuchs et al., 2002a; 2004).

Deletion of VP16 (UL48) blocks incorporation of the conserved inner tegument proteins VP1/2 (UL36) and UL37 into L-particles (Fuchs et al., 2002a), supporting the conclusion that VP16 (UL48) is an important "middle" tegument link between inner and outer tegument. L-particles are assembled even when viral DNA replication is blocked, indicating that viral proteins expressed with true-late (γ2) kinetics (e.g. VP1/2 (UL36), VP13/14 (UL47), and gC) are not required (Dargan et al., 1995).

Only simultaneously disrupting multiple membrane-tegument interactions blocks L-particle envelopment. In particular, combinations of deletions that include the conserved viral membrane protein gM or the conserved outer tegument protein UL11 are reported to block L-particle envelopment (Brack et al., 1999; Kopp et al., 2004; Mettenleiter, 2006), suggesting that some conserved membrane-tegument interactions may be minimally required for envelopment of tegument complexes into L-particles. However, the subsets of tegument-membrane interactions necessary for L-particle envelopment have not been investigated systematically.

It is tempting to speculate that production of L-particles is related to the evolutionary path these viruses followed as they diverged from an ancestral herpesvirus. Over the course of alphaherpesvirus evolution, the ancestral tegument and tegument-membrane interactions were gradually replaced by the alphaherpesvirus-specific "middle" tegument and membrane proteins. Perhaps in this course of this process, conserved outer tegument proteins, such as UL11 and its interacting partners, and conserved viral membrane proteins, such as gM, acquired the ability to drive envelopment more promiscuously, providing the flexibility to gradually evolve new alphaherpesvirus-specific "middle" tegument and membrane protein interactions.

Regardless of what role they play in viral infection and pathogenesis, L-particles may be useful, even if only as a tool to better understand secondary envelopment. Further work to identify the minimal requirements for envelopment of tegument complexes into L-particles may provide a reductionist system to clarify the mechanisms of membrane curvature during budding/wrapping, and

recruitment of cellular factors that contribute to secondary envelopment and subsequent membrane trafficking steps.

Which cellular membranes/organelles serve as sites of envelopment?

There is strong evidence that secondary envelopment occurs at the trans-Golgi network, based for the most part on colocalization with cellular trans-Golgi markers (Henaff et al., 2012). Moreover, a variety of cellular factors involved in trafficking secretory vesicles from the trans-Golgi to the plasma membrane have been implicated in HSV-1 and PRV egress. For example, in uninfected cells, protein kinase D (PKD) is recruited to the trans-Golgi network and initiates a cascade of protein and lipid-based signaling that culminates in the fission of secretory vesicles from the trans-Golgi membranes. Inhibiting PKD and downstream factors reduces HSV-1 egress, and results in the accumulation of virus particles at the trans-Golgi (Rémillard-Labrosse et al., 2009; Roussel and Lippé, 2018). Several studies indicate that HSV-1 and PRV use Rab6 and Rab8-positive cellular organelles for viral glycoprotein trafficking prior to secondary envelopment and trafficking of enveloped particles to the plasma membrane after secondary envelopment (Hogue et al., 2016; 2014; Johns et al., 2014). In uninfected cells, these Rab GTPases mediate trafficking of secretory vesicles from the trans-Golgi to plasma membrane (Fourriere et al., 2019; Grigoriev et al., 2011; Miserey-Lenkei et al., 2010; Noordstra and Akhmanova, 2017).

However, several lines of evidence suggest that endocytic membranes also contribute to secondary envelopment. Many viral glycoproteins contain endocytic sorting motifs (Favoreel, 2006), indicating that glycoproteins may traffic to the site of secondary envelopment via the plasma membrane. Studies using surface biotinylation (Maresova et al., 2005), antibodies against viral glycoproteins (Albecka et al., 2016), or horseradish peroxidase added to the extracellular medium (Hollinshead et al., 2012) efficiently labeled cellular membranes involved in secondary envelopment or the resulting progeny virions. Loss-of-function experiments disrupting the endocytic pathway, by disrupting dynamin and endocytic Rab GTPases, Rab5, Rab7, and Rab11, showed that the endocytic trafficking of viral glycoproteins is required for efficient secondary envelopment of HSV-1 (Albecka et al., 2016; Hollinshead et al., 2012; Johns et

al., 2014). Following secondary envelopment, PRV particle exocytosis colocalizes with the canonical recycling endosomal Rab protein, Rab11 in non-neuronal cells (Hogue et al., 2014; 2016), although Rab11 also participates in many other intracellular trafficking events. Altogether, these data indicate that at least some fraction of the viral membrane proteins and cellular membranes traffic via the plasma membrane and endocytic pathway prior to their involvement in secondary envelopment. It is well established that uninfected cells possess retrograde endomembrane trafficking mechanisms that sort endocytic cargoes into the trans-Golgi. This process is mediated by cellular retromer and sorting nexin proteins (Gallon and Cullen, 2015; Wang et al., 2018), and is exploited by several viruses and toxins for entry into cells; e.g. (Campos, 2017; Williams and Tsai, 2016). If the trans-Golgi is the site of secondary envelopment, this trafficking pathway may explain how endocytosed membrane proteins and endocytic tracers are routed to the trans-Golgi.

Alternatively, by analogy to the betaherpesviruses, it is possible that alphaherpesviruses form a unique virus-induced membrane compartment derived from both trans-Golgi and endocytic cellular membranes. HCMV causes a dramatic reorganization of cellular membranes, leading to the formation of a "virus assembly compartment" (Alwine, 2012; Henaff et al., 2012; Johnson and Baines, 2011; Tandon and Mocarski, 2012). This juxtanuclear assemblage of membranes is organized in concentric layers, with the microtubule organizing center (MTOC) and endosomal markers in the center, surrounded by trans-Golgi, Golgi, and ER markers (Das and Pellett, 2011; Das et al., 2007). Both secretory and endocytic pathway markers colocalize in the HCMV assembly compartment, and are incorporated into HCMV and HHV-6 particles (Cepeda et al., 2010; Mori et al., 2008). Although the alphaherpesviruses do not cause such an overt rearrangement of intracellular organelles, it is possible that viral infection alters intracellular membrane traffic and protein sorting to blur the distinction between trans-Golgi and endocytic organelles.

What is the role of autophagy in secondary envelopment and egress?

Another ambiguity in alphaherpesvirus secondary envelopment concerns the role of autophagy (Grose et al., 2016; 2015; Lussignol and Esclatine, 2017).

Autophagy is typically a degradative pathway in which cytosolic contents are wrapped and enclosed within cellular membranes, called autophagosomes. Subsequently, autophagosomes can fuse with endosomal organelles to form amphisomes that are capable of subsequent intracellular trafficking, and/or fuse with lysosomes to form degradative autolysosomes (Morishita and Mizushima, 2019). Uninfected cells generally exhibit a constitutive homeostatic level of autophagy, but strongly upregulate autophagy in response to cell stress signals (which may include processes of viral infection). Autophagy was originally thought to be antiviral, since it could lead to lysosomal degradation of viral proteins and particles. Accordingly, several herpesviruses encode countermeasures that downregulate autophagy in infected cells. For example, HSV-1 ICP34.5 (Tallóczy et al., 2006) and US11 (Lussignol et al., 2013) are reported to block signaling pathways leading to autophagy. In contrast, varicelloviruses, like VZV, and even some primate simplexviruses that are more closely related to HSV-1, do not encode ICP34.5 or US11 homologs (Eberle and Jones-Engel, 2018; Grose et al., 2015; Severini et al., 2013). In VZV, upregulation of autophagy and numerous autophagosomes are readily detected in infected cells *in vivo* and in culture (Takahashi et al., 2009), and inhibiting autophagy reduces viral replication (Buckingham et al., 2014). Inhibiting autophagy appears to block VZV replication at the step of secondary envelopment (Girsch et al., 2019). Autophagosome markers together with endosome marker Rab11 were associated with VZV virions and secretory vesicles, suggesting that VZV trafficking and egress may occur in amphisomes (Buckingham et al., 2016). While PRV, like VZV, does not encode ICP34.5 or US11 homologs, there are mixed reports whether autophagy is induced and hijacked by PRV (Xu et al., 2018), or has antiviral effects and is inhibited by PRV protein kinase US3 (Sun et al., 2017). In summary, there appears to be fundamental differences in how different alphaherpesviruses evade or exploit cellular autophagy mechanisms; however, in some cases, autophagy mechanisms and autophagic membranes may contribute to secondary envelopment and egress of alphaherpesviruses.

Membrane Scission by ESCRT Complexes

The cellular ESCRT (endosomal sorting complex required for transport) machinery induces membrane curvature and scission "away" from the cytoplasm, into the extracellular or lumenal space. For example, ESCRT produces intralumenal vesicles inside multivesicular bodies, but also participates in many other membrane processes throughout the cell. ESCRT functions by cascade recruitment of several multi-subunit complexes, namely ESCRT-0, -I, -II, and -III. ESCRT-0 associates with cellular membranes by binding acidic phospholipids, and can initiate the cascade by recruiting ESCRT-I complexes. ESCRT-I complexes recruit ESCRT-II complexes, which, in turn, induce protein conformational changes to activate ESCRT-III proteins. The ESCRT accessory protein ALIX is also recruited by ESCRT-I and can also activate ESCRT-III proteins. Activation then nucleates the assembly of ESCRT-III proteins to form membrane-bound spiral filaments. The ATPase Vps4 then binds, remodels, and ultimately disassembles the ESCRT-III filaments, and the coordinated activity of ESCRT-III self-assembly and Vps4-mediated remodeling and disassembly are thought to drive membrane constriction and scission (Barnes and Wilson, 2019; Christ et al., 2017).

Since this budding and membrane scission is topologically equivalent to virus particle envelopment, it is not surprising that many enveloped viruses have evolved to exploit the cellular ESCRT machinery. Several studies have shown that ESCRT-III and Vps4 are required for secondary envelopment of HSV-1 and PRV (Arii et al., 2018; Crump et al., 2007; Kharkwal et al., 2014; Pawliczek and Crump, 2009), and their disruption using knockdown or dominant-negative mutant approaches leads to the appearance of partially-enveloped virus particles that appear to be arrested at the stage of membrane scission (Crump et al., 2007; Kharkwal et al., 2016).

It is not clear how alphaherpesviruses recruit ESCRT activity to sites of secondary envelopment. The structural proteins of many other viruses recruit ESCRT proteins using particular "late domain" peptide motifs, so named because they function in one of the latest steps in the production of infectious viral progeny. These motifs include P(T/S)AP motifs that recruit ESCRT-I protein

TSG101, YPXL motifs that recruit the accessory protein ALIX, and PPXY motifs that recruit ESCRT-I and ALIX via NEDD4-like E3 ubiquitin ligases (Votteler and Sundquist, 2013). Many alphaherpesvirus tegument and envelope proteins contain such peptide motifs, suggesting that ESCRT-I and ALIX may be recruited in multiple redundant ways (Pawliczek and Crump, 2009).

In addition, ESCRT can be recruited by viral structural proteins via ubiquitination. For example, genetically fusing ubiquitin to a retrovirus Gag structural protein can functionally replace its late domain motifs (Joshi et al., 2008), and hepatitis C virus, which does not encode any known late domain motifs, recruits ESCRT-0 protein HRS via ubiquitination of its viral protein NS2 (Barouch-Bentov et al., 2016). ESCRT-I, -II, and ALIX also bind and sort ubiquitinated cargo (Christ et al., 2017). Mass spectrometry proteomics studies have found several ubiquitinated alphaherpesvirus structural proteins, including VP1/2 (UL36), VP13/14 (UL47), gB, and gH (Bell et al., 2013). Moreover, alphaherpesviruses may modulate ubiquitination directly via E3 ubiquitin ligase activity of ICP0 and deubiquitinase activity of VP1/2 (UL36), respectively (Boutell et al., 2002; Kattenhorn et al., 2005). Therefore, it is possible that dynamic ubiquitination of alphaherpesvirus structural proteins may also serve to recruit ESCRT during secondary envelopment. Interestingly, HSV-1 VP1/2 (UL36) has been shown to deubiquitinate ESCRT-I protein TSG101 (Caduco et al., 2013; Calistri et al., 2015), but the role of this in secondary envelopment is unclear.

All of the aforementioned methods of recruiting ESCRT - via late domain motifs and via ubiquitination - may contribute to secondary envelopment, but are not necessary. Knockdown or dominant-negative mutants of ESCRT-I protein TSG101, ESCRT-II protein EAP20/VPS25, or Bro1 domain proteins ALIX, HD-PTP, or BROX does not inhibit HSV-1 secondary envelopment (Barnes and Wilson, 2020; Pawliczek and Crump, 2009). Thus, while ESCRT appears to be critical cellular machinery driving membrane scission during alphaherpesvirus secondary envelopment, it is not known how ESCRT-III proteins are recruited, activated, or regulated in this process. As with the robustness and redundancy

of tegument assembly and tegument-membrane contacts, it appears that ESCRT activity may be recruited in a robust and redundant manner.

Intracellular Transport and Exocytosis

The process of secondary envelopment creates enveloped virions in the lumen of intracellular organelles, but, as discussed above, the identity of these membranes is not clear. If the membrane of secondary envelopment is not itself a secretory vesicle (e.g. a post-Golgi secretory vesicle or plasma membrane-directed recycling endosome), the virion may require subsequent membrane trafficking steps to be sorted into a secretory organelle (e.g. sorting at the trans-Golgi network, or sorting through endosomal compartments). Whether there exist additional membrane sorting and trafficking steps between secondary envelopment and transport to the plasma membrane is not known. In either case, once inside a secretory organelle, this virion-in-a-vesicle transports to the plasma membrane, where it is released by exocytosis (Hogue et al., 2014).

Whether traveling relatively short distances in non-neuronal cells or very long distances in the axons and dendrites of neurons, cellular secretory vesicles, including those containing viral cargoes, are transported on microtubule tracks by cellular kinesin and dynein molecular motors (Miranda-Saksena et al., 2018). Recruitment of microtubule motors to cellular cargoes is mediated by many different cargo adaptor proteins, which can be transmembrane proteins, lipid-binding proteins, or associate with membranes via small GTPase proteins (Cross and Dodding, 2019). For example, the Rab GTPases implicated in alpha herpesvirus egress include: Rab3 (Miranda-Saksena et al., 2009), which recruits KIF1A and KIF1Bβ (kinesin-3) via cargo adaptor DENN/MADD (Niwa et al., 2008); Rab6 (Hogue et al., 2014; Johns et al., 2014; Stegen et al., 2013), which recruits dynein and KIF5A/B (kinesin-1) via cargo adaptors BICD1/2 (Grigoriev et al., 2007; Matanis et al., 2002; Short et al., 2002; Young et al., 2005), and dynein and KIF1C (kinesin-3) via cargo adaptor BICDR-1 (Schlager et al., 2010); Rab11 (Hogue et al., 2014; Hollinshead et al., 2012; Johns et al., 2014; Stegen et al., 2013), which recruits kinesin-1 via cargo adaptor FIP3 (Simon and Prekeris, 2008) and KIF3B (kinesin-2) via cargo adaptor Rip11/FIP5 (Schonteich et al., 2008); and Rab27 (Bello-Morales et al., 2012), which recruits KIF5

(kinesin-1) via cargo adaptors Slp3 or Slp1 and CRMP-2 (Arimura et al., 2009; Kurowska et al., 2012). Rab GTPases can also directly recruit microtubule motors, as in the case of Rab6, which may directly bind dynein motor complexes via their dynactin subunits (Short et al., 2002), KIF20A (kinesin-6) (Echard et al., 1998; Hill et al., 2000), and KIF1C (kinesin-3) (Lee et al., 2015). In addition to Rab GTPases, there are likely many other cellular factors present in the viral secretory vesicle that recruit cargo adaptors and microtubule motors. For example, HSV-1 particles co-traffic with amyloid precursor protein (APP) (Cheng et al., 2011), which recruits dynein and kinesin-1 via the cargo adaptor JIP1 (Fu and Holzbaur, 2013).

Viral proteins may also function as cargo adaptors to recruit microtubule motors to viral secretory vesicles. For example, conserved inner tegument proteins VP1/2 (UL36) and UL37 recruit dynein motors that mediate post-entry intracellular transport to the nucleus (Bearer et al., 2000; Döhner et al., 2002; McElwee et al., 2013; Richards et al., 2017; Sodeik et al., 1997; Zaichick et al., 2013). VP1/2 (UL36) also contains a conserved W-acidic motif that may recruit kinesin-1 (Ivanova et al., 2016). A consensus W-acidic motif, φ(D/E)W(D/E) (where φ represents a hydrophobic amino acid), present in many cargo adaptors, binds the tetratricopeptide repeat (TPR) domain of kinesin light chain 1 and 2 (KLC1 and KLC2) (Cross and Dodding, 2019). VP1/2 (UL36) and UL37 may also be present as part of tegument-glycoprotein complexes on the cytoplasmic face of viral secretory vesicles, where they may affect post-assembly transport of virus particles (Shanda and Wilson, 2008). Other simplex proteins, HSV-1 US11 (Diefenbach et al., 2002) and HSV-2 UL56 (Koshizuka et al., 2005) are reported to recruit kinesin-1 or kinesin-3, respectively. However, the significance of all of these putative motor recruitment activities specifically during secondary envelopment and egress is not clear.

Viral membrane proteins gE, gI, and US9 may also function as a kinesin motor recruitment complex. These proteins affect intracellular transport and egress in polarized cell types, including polarized epithelial cells and neurons (Johnson et al., 2001), and also in non-polarized cell types, like common transformed cell lines (Feutz et al., 2019). In particular, these membrane proteins are collectively

required for virus particles to traffic into the axons of neurons (Draper et al., 2013; Howard et al., 2013; Kratchmarov et al., 2013a; LaVail et al., 2007; Lyman et al., 2007; Snyder et al., 2008), a necessary function for alpha herpesviruses to spread from peripheral nervous system neurons to epithelial tissues following reactivation from latency (Koyuncu et al., 2013; Miranda-Saksena et al., 2018).

In HSV-1, US9 is reported to interact with KIF5 (kinesin-1) (Diefenbach et al., 2015). The alpha herpesvirus US9 proteins contain a conserved Y-acidic motif, (D/E)(A/C)YY^{po}SE^{po}S (where ^{po}S represents phosphoserine residues), followed by 2-3 additional acidic residues, and a highly basic domain. The basic domain is necessary for KIF5 (kinesin-1) binding (Diefenbach et al., 2015), and may function by recruiting the viral protein kinase US3 to phosphorylate the serine residues (Kato et al., 2005). Alternatively, the serine residues may be phosphorylated by host casein kinase 2 (Kratchmarov et al., 2013b). Mutating the tyrosine, serine, or surrounding acidic residues reduces or eliminates axonal sorting and transport of PRV, HSV-1, and BHV-1 (Brideau et al., 2000; Chowdhury et al., 2011; Draper et al., 2013; Kratchmarov et al., 2013b). A consensus Y-acidic motif, DφYφ(D/E) (where φ represents a hydrophobic amino acid), is present in many cargo adaptors and selectively binds kinesin light chain 1 (KLC1) (Cross and Dodding, 2019). Since phosphoserine is similar in size and charge to aspartic or glutamic acid, US9 phosphorylation may create a Y-acidic motif to dynamically regulate kinesin-1 recruitment to viral secretory vesicles.

In PRV, a complex of gE, gI, and US9 are also reported to recruit KIF1A (kinesin-3) (Kramer et al., 2012; Kratchmarov et al., 2013a). KIF1A (kinesin-3) is highly expressed in the nervous system, where it is the primary motor transporting synaptic vesicle precursors down the axon (Hirokawa et al., 2009). KIF1A and KIF1Bβ (kinesin-3) contain a pleckstrin homology (PH) domain that binds acidic phosphatidylinositol-phosphate lipids (PIPs) on the cytosolic face of host membranes (Klopfenstein et al., 2002). Accordingly, the association between PRV gE/gI/US9 and KIF1A (kinesin-3) appears to depend on host membranes, since it is only detected in the nonionic detergent-resistant membrane fraction, and not when membranes are fully solubilized (Kramer et al., 2012; Lyman et al., 2008). Recruitment of KIF1A (kinesin-3) to secretory

vesicles is also regulated by calmodulin and intracellular Ca^{2+} signaling (Stucchi et al., 2018), which may explain the correlation between intracellular Ca^{2+} concentration and efficient axonal transport and spread observed with PRV (Kramer and Enquist, 2012). Aside from these potential roles of PIPs and Ca^{2+}, the mechanistic and structural details of kinesin-3 motor recruitment by gE/gI/US9 are yet to be determined.

Once viral secretory vesicles arrive at the plasma membrane, actin cytoskeleton motors, such as myosinVa, promotes virion egress, likely by facilitating viral secretory vesicle transport through cortical actin to reach the plasma membrane (Roberts and Baines, 2010). Cellular SNARE proteins mediate vesicle fusion with the plasma membrane to release secretory cargoes. SNARE proteins SNAP-25 and VAMP2, which mediate exocytosis at the plasma membrane, colocalize with virus particles in the axons of neurons (Antinone et al., 2010; Miranda-Saksena et al., 2009). These SNAREs, along with two additional SNAREs, Syntaxin6 and Vti1b, which are also reported to mediate secretion of cellular cargoes (Murray et al., 2005), were also enriched in membrane fractions containing viral membrane proteins (Kramer et al., 2012). However, the cellular factors involved in virus particle exocytosis have not been thoroughly investigated. Future work is needed to identify which viral membrane and tegument proteins may be present on the cytosolic face of viral secretory vesicles, and determine how these viral proteins interact with host cell biology to modulate virus particle egress, identify additional cell biological factors involved, and demonstrate the function of these viral and cellular factors in virion exocytosis.

Conclusions

As detailed in this review, a multitude of viral and host cell biological mechanisms drive tegument assembly, secondary envelopment, intracellular trafficking, and exocytosis of progeny virus particles. Because of their redundancy and robustness, disentangling the tegument and membrane protein interactions involved in these complex multistep processes is a challenge. Further work to identify individual molecular links and minimal subsets of

tegument and membrane proteins required to drive envelopment and recruit cell biological factors may be particularly valuable.

Future Trends

Broader View: Do conserved cellular responses to aggregating and pore-forming membrane proteins contribute to the robustness of viral envelopment?

The plasma membrane is the defining feature of cellular life, which separates the cell from its environment. All cellular organisms express transmembrane proteins to be able to interact with their extracellular environment. Dimerization or oligomerization of membrane proteins is one of the common ways to transduce extracellular signals across the lipid bilayer. However, pathological oligomerization or aggregation of membrane proteins can also threaten the structural integrity of the membrane. Cells have evolved many defenses against membrane damage, including cell stress responses that are conserved between *Eukarya*, *Bacteria*, and *Archaea*, likely inherited from the last universal common ancestor of cellular organisms (Kültz, 2005). In eukaryotes, misfolding and aggregation of membrane proteins leads to a variety of interrelated defenses, including the unfolded protein response (UPR), ER-associated degradation (ERAD), autophagy, and ultimately, programmed cell death pathways (Qi et al., 2017; Ruggiano et al., 2014). Each of these stress response pathways are induced by alphaherpesvirus infection, but antagonized by viral countermeasures, and often exploited to promote viral replication (Grose et al., 2016).

This ability of multimeric membrane proteins to destabilize the membrane bilayer has been weaponized via evolutionary arms races to such an extent that pore-forming toxins represent the largest class of bacterial toxins, and are found in every kingdom of life (Dal Peraro and van der Goot, 2016). Studies of cellular responses to pore-forming toxins have described a common response to plasma membrane damage, sometimes termed "ectocytosis" or the "intrinsic repair pathway" (Romero et al., 2017). In this response, a plasma membrane lesion causes a rapid influx of Ca^{2+}, changes in cortical actin dynamics and multimerization of annexin proteins that stabilize the membrane and prevent expansion of the lesion, and recruitment of ESCRT machinery to drive blebbing

and shedding of microvesicles from the plasma membrane (Jimenez et al., 2014). Alternatively, pore-forming toxins or protein aggregates can also be endocytosed, sequestered into intralumenal vesicles by ESCRT machinery and/ or via autophagy, and subsequently exocytosed from the cell in the form of exosomes (Dal Peraro and van der Goot, 2016; Romero et al., 2017). These cellular responses rapidly remove the membrane destabilizing toxins or aggregates, allowing recovery of membrane integrity within seconds to minutes. Importantly, recent studies of mutant pore-forming toxins showed that oligomerization of the toxin protein is necessary and sufficient to induce shedding of microvesicles, even when pore formation and Ca^{2+} influx is blocked (Romero et al., 2017). This finding shows that cells possess some intrinsic mechanism to detect and respond to membrane protein oligomerization as a potential threat, even before pores or lesions are actually formed.

Studies from the retrovirus field suggest a possible link between membrane protein oligomerization, virus envelopment, and the cellular pathways producing exosomes/microvesicles (Figure 2). Many studies have noted striking similarities between retrovirus particles and cellular exosomes. Both exosomes and virus particles are capable of delivering biologically-active proteins and nucleic acids between cells, incorporate similar membrane lipids and cellular proteins, and cellular ESCRT machinery may be involved (although ESCRT-independent exosome/ microvesicle production has also been reported) (Booth et al., 2006; Nguyen et al., 2003; Nolte-'t Hoen et al., 2016; Pegtel and Gould, 2019). These similarities led several researchers to propose the "Trojan exosome" hypothesis, which posits that that viral structural proteins have evolved to direct themselves into pre-existing cellular exosome/microvesicle pathways, and retrovirus particles are essentially exosomes/microvesicles carrying infectious viral cargoes (Gould et al., 2003; Nolte-'t Hoen et al., 2016).

The structural polyprotein Gag drives assembly of retrovirus particles. Gag contains membrane-binding motifs, protein-protein interaction domains, RNA binding motifs, and late domain motifs that recruit cellular ESCRT complexes. The ability of Gag to drive assembly of virus-like particles is highly robust (O'Carroll et al., 2012). Essentially any part of Gag can be replaced by

exogenous functional motifs, yet these chimeric Gag proteins still drive assembly and envelopment. The protein-protein interaction and RNA binding domains of Gag can be replaced by multimerizing leucine zipper motifs (Accola et al., 2000; Fang et al., 2007). The Gag membrane binding motifs can be replaced by exogenous acylation motifs or phospholipid binding domains (Chukkapalli et al., 2008; Jouvenet et al., 2006; Scholz et al., 2008; Urano et al., 2008). Indeed, HSV-1 UL11, the membrane-anchored conserved outer tegument protein that participates in herpes secondary envelopment, is capable of functionally replacing the membrane binding motifs of retroviral Gag, leading to the assembly and envelopment of virus-like particles on intracellular membranes (Bowzard et al., 2000).

Further studies exploring the relationship between retrovirus assembly and exosome/microvesicle pathways identified the minimal features sufficient to direct protein cargoes into exosomes/microvesicles (Fang et al., 2007; Shen et al., 2011; Yang and Gould, 2013). Antibody-mediated cross-linking was sufficient to redirect cell-surface proteins into microvesicles (Fang et al., 2007). Genetically fusing various membrane binding motifs onto oligomeric cytosolic proteins was also sufficient to redirect them into microvesicles (Fang et al., 2007; Shen et al., 2011). Even a chimeric protein consisting minimally of a membrane binding motif, a dimerizing leucine zipper domain, and a tetramerizing fluorescent protein was efficiently secreted in microvesicles (Fang et al., 2007). These studies show that membrane binding plus higher-order oligomerization are sufficient to direct proteins into exosomes/microvesicles. This explains the robustness of retrovirus assembly and envelopment: essentially any part of Gag can be replaced by exogenous functional domains, as long as membrane binding and oligomerization functions are preserved. These observations may help to explain several curious features of alphaherpesvirus tegument assembly and secondary envelopment highlighted in this review. First, these processes are highly robust, such that essentially any viral structural protein can be deleted, yet particles (virions and/or L-particles) are still enveloped and released. Second, while herpesvirus proteins that mediate most major steps in the viral replication cycle, e.g., membrane fusion and entry, post-entry transport to the nucleus, DNA replication, capsid assembly

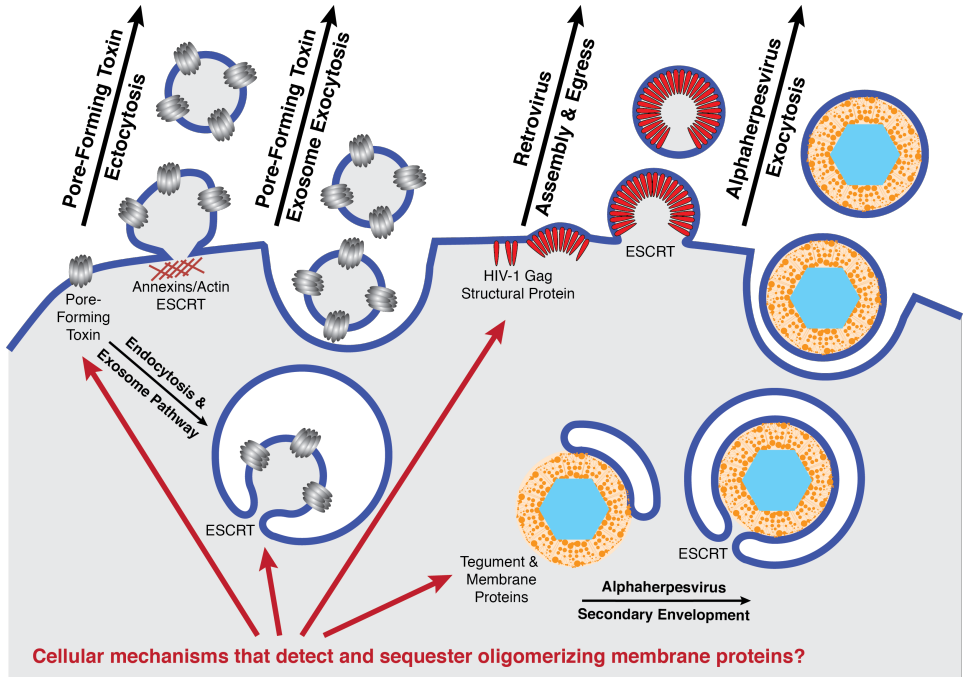

Figure 2. Common cellular responses to pore-forming toxins and other oligomerizing membrane proteins may contribute to virus envelopment. Pore-forming toxins and aggregated membrane proteins can be shed by ectocytosis, budding directly from the plasma membrane, or can be endocytosed and directed into the exosome pathway. The retrovirus Gag structural polyprotein may have evolved to mimic pathological oligomerizing membrane proteins to engage these cellular responses during assembly and egress (the "Trojan exosome" hypothesis). Does the alphaherpesvirus tegument and membrane protein interaction network engage cellular factors in this manner, and does this explain the robustness of alphaherpesvirus envelopment?

and genome encapsidation, and nuclear egress, are conserved amongst all of the herpesviruses, the most abundant middle tegument proteins and many of the tegument-membrane protein interactions are not conserved.

Since cellular organisms have evolved a broad, common, and conserved suite of responses to defend against aggregation of membrane proteins and pore-forming toxins, it is likely that enveloped viruses have evolved to exploit these

cellular pathways for assembly and egress (Figure 2). Is it possible that alphaherpesvirus tegument and envelope proteins exploit similar conserved cellular responses to drive secondary envelopment? Like retroviral Gag proteins, the alphaherpesvirus tegument and membrane protein interaction network may have evolved to use the very general properties of membrane binding plus higher-order oligomerization to engage such cellular mechanisms. Is it possible that engaging a common cellular pathway that senses and envelops membrane-bound oligomers has afforded the herpesviruses the robustness and flexibility to evolve novel subfamily-specific tegument and membrane proteins? Future work to identify cellular factors that sense the biochemical and biophysical changes in membrane dynamics caused by oligomerizing membrane proteins may shed light on these intriguing questions.

Web Resources

Alphaherpesvirinae orthologs of tegument proteins on UniProt:
VP1/2 (UL36): https://tinyurl.com/alphaherpesUL36
UL37: https://tinyurl.com/alphaherpesUL37
VP11/12 (UL46): https://tinyurl.com/alphaherpesUL46
VP13/14 (UL47): https://tinyurl.com/alphaherpesUL47
VP16 (UL48): https://tinyurl.com/alphaherpesUL48
VP22 (UL49): https://tinyurl.com/alphaherpesUL49
UL21: https://tinyurl.com/alphaherpesUL21
UL11: https://tinyurl.com/alphaherpesUL11
UL16: https://tinyurl.com/alphaherpesUL16
UL51: https://tinyurl.com/alphaherpesUL51
UL7: https://tinyurl.com/alphaherpesUL7
UL14: https://tinyurl.com/alphaherpesUL14

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