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ESCRT-III-dependent and -independent egress of herpesviruses

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Enveloped viruses complete their replication cycle by forming virions that bud from infected cells through membrane scission. The mechanisms by which this is achieved are less well-understood than the well-characterized membrane scission of vesicles budding inwards into the cytosol. The scission of vesicles that bud away from the cytosol is mediated by machinery of the endosomal sorting complexes required for transport (ESCRT)-III, which is highjacked by viruses of several different families. Other groups of viruses can bud independently of ESCRT-III activity. It has not been fully elucidated how the latter achieve this in the absence of host ESCRT-III, but it is known that some viral proteins directly mediate membrane scission. The *Herpesviridae* constitute a family of highly diverse viruses that bud at the inner nuclear membrane and cytoplasmic membranes in infected cells. Many investigators have attempted to determine the mechanism of membrane scission during herpesvirus budding, and have found this to be complex, not exactly conforming to either of the two methods. The present review attempts to synthesize the disparate findings into a model of herpesvirus egress based on both ESCRT-mediated and viral protein-mediated mechanisms.

KEYWORDS

viral egress, budding, ESCRT, herpesvirus, membrane scission

Introduction

Enveloped viruses must produce viral particles by deforming the host cell membrane and then pinching off from the membrane in a scission step. In eukaryotic cells, vesicles can bud inwards into the cytosol or outward away from the cell (Figure 1A) (1). Types of vesicles budding into the cytosol include clathrin-, coat protein I (COPI)- and COPII-coated vesicles (2). In this case, the bud neck is surrounded by cytoplasm and the BAR and dynamin family fission machineries bind outside the bud to constrict and pinch off the membrane necks. This process has been well characterized (Figure 1A) (2).

Vesicles budding outwards away from the cytosol include intraluminal vesicles (ILVs) and extracellular vesicles as well as enveloped viruses (1). It is less clear how this "reverse-topology scission" is directed from the inner surface of the membrane itself. The machinery

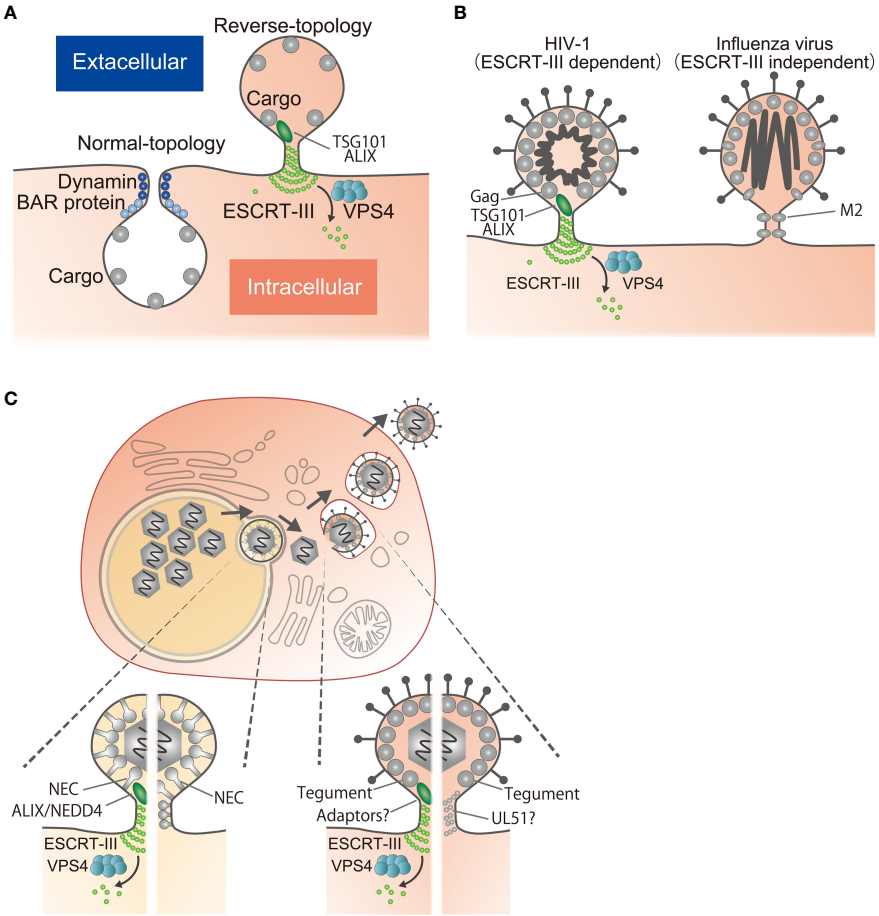

FIGURE 1
Reverse-topology scission involved in viral egress. **(A)** Normal-topology scission (left), carried out by dynamin and BAR proteins, for the biogenesis of vesicles directed inwards into the cytosol. Reverse-topology scission (right), carried out by ESCRT-III, functions in vesicle budding away from the cytosol. Note that only the cytosolic side of the membrane neck is accessible for the protein scaffolding and scission machinery. **(B)** HIV-1 Gag recruits ESCRT-III via TSG101 or ALIX to mediate scission (left). Influenza virus M2 mediates scission by virtue of its own activity through lipid re-ordering (right). **(C)** Egress of herpesviruses. After genome replication in the nucleus, viral capsids in the nucleus bud through the INM to form vesicles in the perinuclear space that fuse with the ONM to release the capsids into the cytoplasm. Capsids bud again into cytoplasmic vesicles to produce infectious virions, followed by release to the extracellular space. During budding at the INM, the NEC mediates scission by recruitment of ESCRT-III via ALIX/NEDD4 or by itself through its polymerization. During budding at the cytoplasm, tegument proteins may mediate scission by recruitment of ESCRT-III via multiple interactions or by polymerization as is the case for proteins such as UL51.

of the endosomal sorting complexes required for transport (ESCRT)-III molecules is the sole mechanism thus far identified that is responsible for such reverse-topology scission (Figure 1A) (1, 3, 4). Because of the particular topology of viral budding (Figure 1B), studies of viral egress have been focused on whether or not the process is ESCRT-III dependent (5, 6). Nevertheless, for some viruses including herpesviruses, there is no consensus regarding the dependence of viral egress on ESCRT-III.

Herpesviruses are enveloped double-stranded DNA viruses, with a mature virion consisting of three elements: an icosahedral capsid with a linear double-stranded DNA genome, a host-membrane-derived envelope spiked with viral glycoproteins and the tegument, a proteinaceous layer between the capsid and envelope (7). The *Herpesviridae* family is subdivided into the *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* subfamilies, based on their molecular and biological properties (7). Herpes simplex virus 1 (HSV-1) is the prototype of the alphaherpesvirus subfamily and causes a variety of conditions such as mucocutaneous disease, keratitis, skin disease and encephalitis in humans (8). Herpesviruses share a common virion morphology and approximately 40 conserved genes (7). Tegument proteins form a group of structural components playing an important role in virion assembly (9), similar to the matrix proteins of other viruses.

Herpesviruses replicate their genomes and package nascent viral progeny genomes into capsids in the host cell nucleus. These capsids must first acquire envelopes by budding through the inner nuclear membrane (INM) into the perinuclear space between the INM and the outer nuclear membrane (ONM). This is followed by fusion with the ONM to release capsids into the cytoplasm, which then bud again into cytoplasmic vesicles to produce infectious virions (10) (Figure 1C). This variety of viral factors and the complexity of the budding processes make it difficult to understand their relationship to ESCRT-III. Here, I review

mechanisms of membrane scission for viral egress, focusing on herpesviruses, which have unique properties.

ESCRT-III-dependent viral egress

ESCRT proteins were originally identified by virtue of their essential role in the formation of ILVs in budding yeast (11). Members of the ESCRT family can be classified into five groups: ESCRT-0, -I, -II -III and VPS4 complex (3, 12). Of these, ESCRT-III catalyzes membrane fission and the VPS4 complex mediates ESCRT disassembly (3, 12). ESCRT-III proteins are recruited onto the membrane by ESCRT-II or adaptors such as ALIX. This is followed by the formation of membrane-binding spirals that mediate membrane deformation and scission, in cooperation with the ATPase VPS4 (1, 3). VPS4 ATPases disassemble ESCRT-III filaments into their constituent subunits using the energy of ATP hydrolysis (13, 14). The activity of VPS4 is crucial for the ESCRT-III assembly cycle, as shown by severe defects in ESCRT-III-mediated membrane scission on expression of a dominant negative VPS4 allele. ESCRT-III appears to mediate reverse-topology scission events in a wide range of cellular processes, including vesicle budding from the cytoplasmic membranes, autophagy, membrane repair and cytokinesis (1, 4).

A unique mechanism of scission during viral egress was originally revealed by the finding that disruption of a short tetrapartite motif (the L domain) found in retroviral proteins typically results in defects during the late stages of budding, particularly at the final step of vesicle fission (15). Similar motifs have been identified in a variety of different viruses (5, 15). These motifs mediate the recruitment and interaction of the ESCRT proteins to facilitate virus egress. Briefly, the PTAP motif binds the ESCRT-I protein TSG101 and the YPXL motif binds the ESCRT-III adaptor ALIX, whereas the PPXY motif binds NEDD4 ubiquitin ligase family proteins (16–21). The ESCRT proteins are rich in ubiquitin-binding domains, reflecting their prominent role in sorting ubiquitinated proteins into ILVs (3, 15). In the case of NEDD4-dependent budding, ESCRT-III is thought to be recruited via ubiquitin on the viral protein complex (15).

Different viral species exhibit a wide variety of functional L domains and hence different ESCRT proteins are required for budding. For example, human immunodeficiency virus 1 (HIV-1) Gag protein recruits TSG101 and ALIX through its PTAP and YPXL motifs, respectively (16, 17, 19, 20) (Figure 1B). Equine infectious anemia virus recruits ALIX via the YPXL motif (19–21) and Rous sarcoma virus uses NEDD4 through the PPXY motif (18).

There are also other ways to recruit ESCRTs in addition to the three traditional L domains. The matrix (M) protein of the paramyxovirus parainfluenza virus 5 (PIV5) lacks the well-defined PTAP, YPXL and PPXY motifs but mediates budding through ubiquitination and the ESCRT-III pathway (22). An FPIV motif within the PIV5 M protein is essential for viral budding and can functionally compensate for the absence of the L domain during HIV-1 budding. Similarly, NEDD4L ubiquitin ligase allows the release of HIV-1 mutants lacking the PTAP and YPXL motif (23–25). HIV-1 Gag does not interact with NEDD4L directly but the cellular protein Angiomotin links HIV-1 Gag and NEDD4L to facilitate budding (26, 27). ESCRT-III appears to mediate egress of viral progeny in many different families of viruses, including retroviruses, filoviruses, rhabdoviruses, arenaviruses, paramyxoviruses, flaviviruses, bunyaviruses, poxviruses and hepadnaviruses as well as herpesviruses (5, 28–30).

ESCRT-III-dependent egress of herpesviruses

It was reported early on that the expression of a VPS4 dominant negative allele in HSV-1-infected cells suppressed envelopment in the cytoplasm (31–34). The canonical motifs of the L domains are present in various tegument proteins, but siRNA for TSG101 or ALIX had no effect on HSV-1 reproduction (35). This led to the conclusion that HSV-1 recruits ESCRT-III machinery via complex interactions between multiple viral proteins and ESCRT proteins at the cytoplasm in a redundant fashion (Figure 1C). Another group showed that one of the ESCRT-III components, CHMP4C, has a predominant role in HSV-1 envelopment in the cytoplasm (36). A role for ESCRT-III in the life cycle of the betaherpesvirus human cytomegalovirus (HCMV) has also been reported; expression of dominant negative mutants of VPS4 or ESCRT-III protein severely impaired HCMV replication (37).

In contrast to the redundant role of tegument proteins in the cytoplasm, the nuclear egress complex (NEC) is essential for the envelopment of capsids at the INM (38, 39). Herpesvirus NEC, which consists of nuclear matrix and nuclear membrane proteins, forms a complex on the intranuclear side of the INM (38). Ectopic expression of the NEC results in the generation of characteristic vesicles without capsids located between the INM and ONM, suggesting that the NEC itself can induce membrane deformation and scission in the absence of any other viral factors (40). Our group showed that the NEC of HSV-1 interacts with ALIX to recruit ESCRT-III to the INM (41, 42). Furthermore, depletion of ESCRT-III components severely impaired envelopment at the INM and impaired nucleocytoplasmic transport (41). Similarly, another group showed that the NEC of the gammaherpesvirus Epstein-Barr virus (EBV) interacts with ALIX and the NEDD4 protein to recruit ESCRT-III to the INM (43, 44). These experiments reveal that budding at the INM requires ESCRT-III function in herpesvirus-infected cells (Figure 1C) (39, 45).

During viral infection, different types of extracellular vesicles (EVs) appear to be released by host cells, including so-called L-particles composed of virus envelope and tegument proteins but lacking the viral genome and viral capsid proteins. Although L-particles are themselves non-infectious, they were shown to facilitate HSV-1 infection, at least in cell cultures, most likely by delivering viral and/or cellular proteins to the target cells that are needed for virus replication and suppression of antiviral responses (46). In contrast, HSV-1 infection influences the cargo and functions of EVs released by infected cells; these EVs then negatively impact a subsequent HSV-1 infection (47–49). Because ESCRT-III mediates scission of EVs and is incorporated into them (1, 4), it is conceivable that it affects viral replication via EV biogenesis.

The effects of compounds that target the ESCRT machinery

Recently, compounds binding the ESCRT-I protein TSG101 were analyzed in the context of viral infection. These compounds target the N-terminal domain of TSG101, disrupting its ubiquitin binding and inhibiting HIV-1 replication. Electron microscopy revealed a defect in HIV-1 Gag assembly in the cytoplasm. The authors concluded that Tsg101 acts as a chaperone for HIV-1 Gag that is independent of its interaction with the PTAP motif (50). These compounds also impaired the replication of members of the filo-, alpha- and herpesvirus families but not the flaviviruses (51). In agreement with the results of experiments depleting ESCRT proteins, these compounds impair HSV-1, HSV-2 and EBV nuclear egress (52, 53). Thus, the ESCRT machinery supports maturation of herpesvirus virions at the INM in addition to its role in scission. Studies on nucleocytoplasmic transport of capsids contributes anti-viral therapies.

Mechanisms of ESCRT-III-independent egress

ESCRT-III-independent egress has been reported for several members of the groups of orthomyxoviruses, paramyxoviruses, coronaviruses, togaviruses, and herpesviruses (31, 54–58), although the responsible mechanisms have mostly not been well-studied. The best investigated ESCRT-III-independent egress is that of influenza viruses, which can still replicate in cells with a dominant negative allele of VPS4 (55). The influenza M2 protein is a proton-selective ion channel protein, crucial for the scission step during viral egress (59). *In vitro*, purified M2 protein alters the membrane curvature and generates vesicles inside giant unilamellar vesicles (GUVs), a process dependent on its cytoplasmic amphipathic helix (60). Thus, upon binding, the M2 amphipathic helix forms clusters and induces membrane curvature and lipid ordering, constricting and destabilizing the membrane neck, causing fission of liposomes (60–62). Based on these observations, it has been concluded that influenza virus M2 protein directly mediates membrane scission (Figure 1B). This model is attractive as the M2 protein at the neck of the bud will be released from the cells together with the virions and recycling of M2 proteins is not required for the next round of budding.

ESCRT-III-independent egress of herpesviruses

In the past, viral egress was mainly considered in terms of its dependency on ESCRT-III or VPS4 activity. However, the distinction between ESCRT-III-dependent and -independent budding is not clear for some viruses, including herpesviruses. Following the discovery of the intrinsic functions of influenza virus M2 proteins for membrane deformation/scission, similar assays were adapted to investigate the NEC. This documented the ability of NEC to produce vesicles inside the GUV *in vitro* (63). Purified NECs form hexagonal lattices that might reflect the driving force for particle formation because mutation at the contact site of these lattices severely impaired vesicle formation both at the GUV and in infected cells (63–67). Similar to the situation with influenza M2 proteins, it could be concluded that NEC mediates membrane scission at the bud during egress from the INM (Figure 1C). At the present time, it is unclear whether NECs can trigger INM scission in infected cells in the absence of ESCRT-III, or whether NEC scission activity and ESCRT-III machinery act independently in parallel.

Confusion about the dependence of ESCRT-III has also arisen regarding envelopment in the cytoplasm. HSV-1 UL51 protein and its homologues are conserved tegument proteins which are important for envelopment in the cytoplasm (68–71). Unexpectedly, the crystal structure of the UL51 protein was found to resemble the host ESCRT-III component. As UL51 forms ESCRT-III-like filaments *in vitro*, it has been proposed that it promotes membrane scission directly at the cytoplasm (72) (Figure 1C). Whether UL51 mediates scission by itself or modifies ESCRT-III assembly in infected cells has not yet been clarified.

Reports on HCMV imply an even greater degree of complexity. In contrast to the previous report (37), others reported that expression of dominant negative mutants of VPS4 or ESCRT-III protein had no effect on the envelopment of HCMV capsids but reduced the efficacy of viral spread, perhaps due to effects on exosome-mediated signaling (73).

Thus far, there is no explanation for these discrepancies regarding the role of ESCRT-III in the life cycle of herpesviruses. Inhibition of ESCRT-III severely inhibits cell division, resistance to cell death, biogenesis of EVs and membrane repair (1, 4). Hence, the true contribution of ESCRT-III for budding per se is difficult to establish in experiments performed under conditions that avoid cytotoxicity. Cytotoxic effects of ESCRT-III deficiency need to be analyzed more carefully, especially in the case of relatively slowly replicating viruses such as herpesviruses.

Updated model of reverse-topology scission

The model where a single viral factor cleaves the membrane by clustering of viral protein and/or lipid structures might be based on *in vitro* analyses showing that the ESCRT-III protein CHMP4 forms a helical polymer on the GUVs which act as the executor of membrane scission (74, 75). This approach has documented that the other ESCRT-III components CHMP2 and CHMP3 are located at the termination site of the CHMP4 polymer to recruit VPS4 ATPase (74, 76). Based on these observations, it was proposed that the polymerization of CHMP4 protein itself cleaves the membrane in a reverse-topology manner and that the enzymatic activity of VPS4 recycles the ESCRT-III proteins from the complex (74, 75) (Figure 2A). On the other hand, imaging experiments have shown that VPS4 recruitment precedes membrane scission (77–79).

Therefore, there has been a persistent belief that the GUV assay does not adequately reflect reverse-topology membrane scission in the cell. As GUVs are less stiff than cellular membranes, it might be easier to achieve scission in the GUV system than in cells, and thus generate artifacts (80). Likewise, it is uncertain whether viral proteins alone can mediate reverse-topology scission from the cytoplasmic or nuclear space independent of any cellular machinery in infected cells.

In an effort to resolve this disparity, GUV assays were modified to create membrane nanotubes under controlled tension and force (80). ESCRT-III/VPS4 assembly was reconstituted using these membrane nanotubules, with the result that it appeared to be the case that VPS4 activity and polymerization of ESCRT-III subunits could generate forces within the nanotubes that led to their constriction and to membrane scission (81, 82). Accordingly, a model was proposed in which sequential polymerization of ESCRT-III subunits, driven by a recruitment cascade and by continuous subunit-turnover by VPS4, induces membrane deformation and fission (81–83) (Figure 2B). These modifications to the reverse-topology membrane scission model with ESCRT-III will need to be addressed for other viral factors as well. It would be important to determine whether reconstitution of viral protein complexes at the bud is necessary for, and whether any ATPases are required for, ESCRT-III-independent viral egress (Figure 2C).

Discussion

Since the historical report that scission in HIV-1 budding is dependent on ESCRT proteins (16, 17), viral budding has been considered mainly in terms of interactions with ESCRT-III. However, there are many conflicting reports regarding the degree of ESCRT-III dependence for viral budding. Due to the wide range of ESCRT-III functions (1, 4), direct effects of ESCRT-III on viral budding may often be underestimated or overestimated. It is especially difficult to separate the roles of ESCRT-III for budding of virion and EVs, as the latter can promote or inhibit viral replication through cell-cell communication. Furthermore, some viruses mediate membrane scission through multiple mechanisms that might act in a redundant fashion. Of these, herpesviruses employ an extraordinarily complex process, making it impossible to determine the contribution of ESCRT-III at each stage, given the paucity of published reports in the field at this time. As our understanding of membrane scission by ESCRT-III advances, based on biochemical and structural analysis, it is valuable to update consensus knowledge of viral egress. In particular, there are many viruses that apparently egress independently of ESCRT-III, but the details of the responsible membrane scission processes are largely unknown, and it is unclear whether there is a common principle. Improved awareness of the different mechanisms of viral

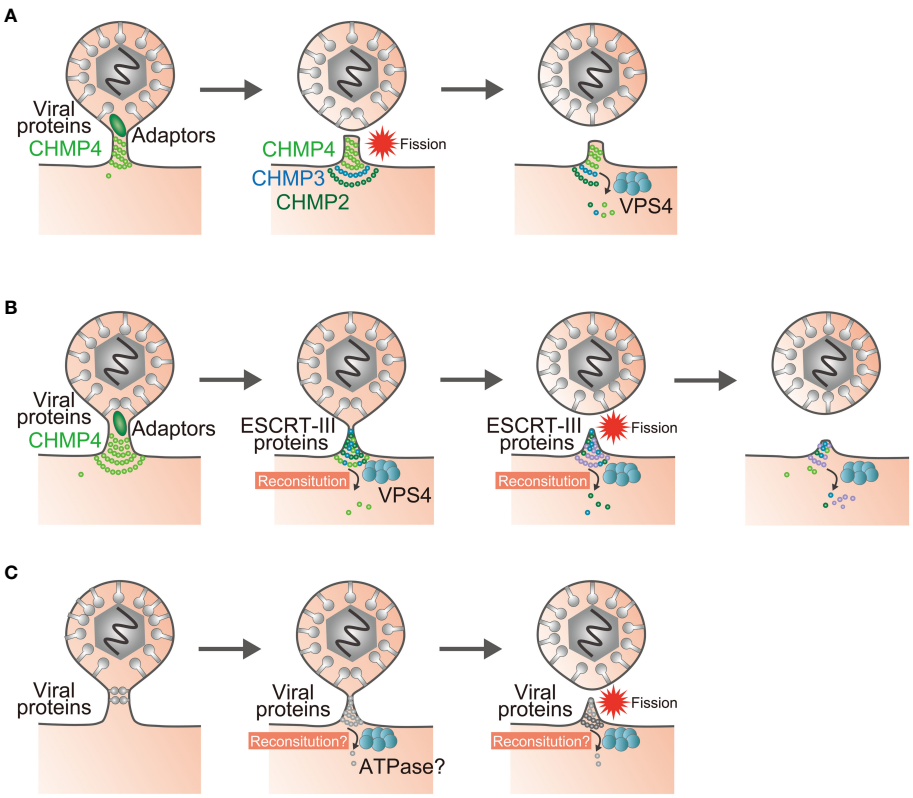

FIGURE 2
Models of reverse-topology membrane scission in infected cells. **(A)** Polymerization of the ESCRT-III protein CHMP4 mediates membrane scission, and the enzymatic activity of VPS4 recycles the ESCRT-III proteins. **(B)** The sequential recruitment of ESCRT-III components, polymerization, and replacement of different filament subunits driven by VPS4 result in constriction and scission of the membrane. **(C)** Proposed model of ESCRT-III-independent membrane scission. Viral proteins may mediate membrane scission by themselves. Question marks indicate unknown steps.

membrane scission will contribute greatly to our understanding of reverse-topology scission, which is difficult to explain in physico-chemical terms.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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