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Poxvirus host cell entry

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Poxviruses are characterized by their large size, complex composition, and cytoplasmic life cycle. They produce two types of infectious particles: mature virions (MVs) and extracellular virions (EVs). Both MVs and EVs of vaccinia virus, the model poxvirus, take advantage of host cell endocytosis for internalization: they activate macropinocytosis — the most suitable form of endocytosis for large particles. Although largely dependent on the same cellular machinery, MV and EV entry differs with regard to the mechanisms used to trigger macropinocytosis and to undergo fusion. While EVs have to shed an additional membrane to expose the fusion complex, MV fusion requires the inactivation of fusion inhibitory proteins absent in EVs. This review highlights recent advances in the understanding of poxvirus MV and EV cell entry.

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Introduction

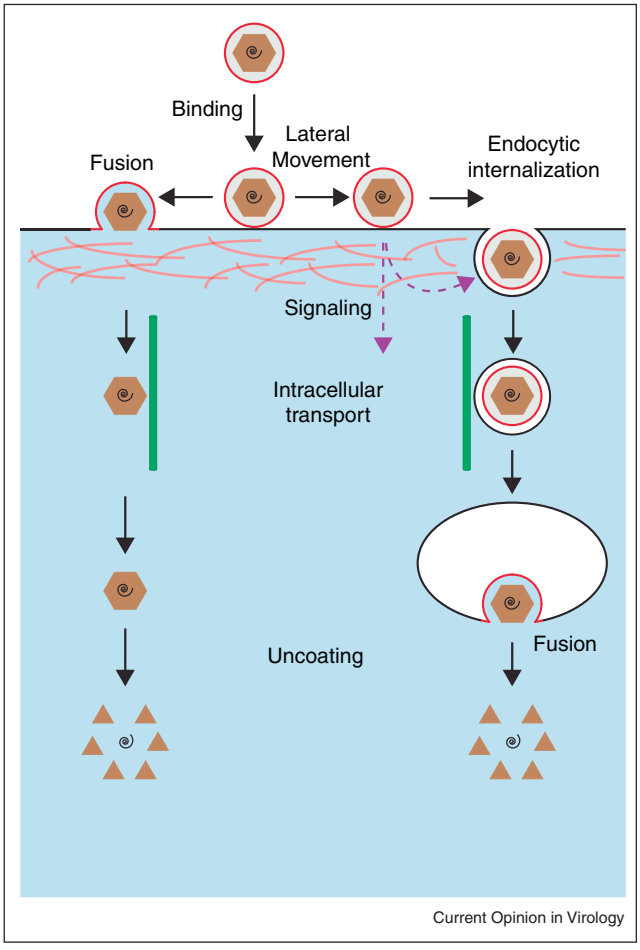
Viruses are obligate intracellular pathogens with one goal — to successfully deliver their genome and accessory proteins in a replication competent state into the host cell. Entry of enveloped viruses (outlined in Figure 1) can be divided into six general steps: binding, cell surface movement, signaling, internalization, intracellular transport, and membrane penetration [1]. Binding occurs via cellular factors in the plasma membrane that contribute to enrichment of viruses on cells (attachment factors). Binding can be followed by lateral movement of viruses on the cell surface to facilitate additional receptor interactions or transport to suitable subdomains of the plasma membrane. Interaction with cellular entry receptors promotes subsequent entry steps such as activation of fusion proteins, signaling, or endocytosis. Depending on the nature of such interactions, virus particles either fuse directly with the plasma membrane or trigger endocytic internalization. Endocytic vesicles provide the virus with a means of cytoplasmic transport and supply the cues needed to promote virus escape from endocytic organelles by membrane fusion. This entry program is typically followed by delivery of incoming viral genomes to the site of virus replication.

One family of enveloped viruses, the *Poxviridae*, are large, complex DNA viruses that replicate entirely in the host cell cytoplasm and have co-evolved with animals from insects (entomopoxviruses) to vertebrates (chordopoxviruses) [2]. Three poxviruses are human pathogens: variola virus (VARV), the etiologic agent of smallpox, monkeypox virus (MPXV), which induces morbidity similar to smallpox, and molluscum contagiosum virus, which causes benign skin lesions. The best understood poxvirus is vaccinia virus (VACV), which is closely related to VARV and was used as a live vaccine for the eradication of smallpox [3].

The poxviruses are unique in that they produce two types of infectious particles: mature virions (MVs) and extracellular virions (EVs) [4]. When intact, both viral particles do not share common viral surface epitopes. VACV MVs consist of the viral core containing the dsDNA genome, two proteinaceous lateral bodies, and one lipid bilayer containing at least 25 viral proteins. EVs consist of an MV-like particle surrounded by a second viral membrane containing cellular and at least six unique viral proteins. MVs are the more abundantly produced infectious form and are thought to mediate host-to-host transmission, while EVs mediate virus spread within an infected host [5]. The majority of EVs remain associated with the producer cell. These particles can induce the formation of actin tails that push them away from the producer cell mediating local virus dissemination. EVs released into body fluids are thought to mediate virus transmission from tissue to tissue. With two infectious virus forms wrapped with different numbers of membranes with divergent lipid and protein compositions, poxviruses offer a challenging and unique opportunity to investigate entry of enveloped viruses.

Here we review the host cell entry pathways of poxvirus MVs and EVs. Focus is placed on the most recently published findings involving the cellular aspects of poxvirus endocytosis and regulation of fusion. Most details on cellular and mechanistic aspects of poxvirus entry presented in this review have been learned through the study of different VACV strains in immortalized epithelial cell

Figure 1



Entry pathway of an enveloped animal virus. Enveloped animal viruses enter host cells in a stepwise process: viral particles bind to attachment factors in the host cell plasma membrane and may undergo lateral movement. Virions may employ two entry routes: fusion at the plasma membrane or endocytosis. The latter may be triggered by virus–receptor interactions, followed by vesicular transport. Maturation of endosomes provides the cues for membrane fusion. Capsid release, whether at the plasma membrane or from an intracellular vesicle, is followed by intracellular transport of capsids and genome uncoating at the site of viral replication.

lines. Where available, data relating to other host cell types are included.

Binding

As poxvirus MVs and EVs do not share any common viral epitopes, it is not surprising that they use different attachment factors [6]. VACV MVs employ several, partly redundant binding mechanisms that involve glycosaminglycans (GAGs) in addition to other surface molecules. Although the dependence on GAGs for binding and infection varies with cell type, virus strain, and experimental conditions [7–9], binding of viral A27 [10,11] and H3 [12] to heparan sulfate, and D8 [13] to chondroitin sulfate has been described.

GAG-independent binding may be mediated by interaction between A26 and the extracellular matrix glycoprotein laminin [14], or by the viral L1 protein via an unidentified cellular factor [15]. The finding that MVs move along filopodia towards the cell body suggests that at least one attachment factor can undergo retrograde flow along with the actin cytoskeleton [16,17]. Direct binding of MVs to liposomes has also been observed, suggesting that cellular proteins may be partly dispensable for attachment [18,19].

For free EVs, no cellular attachment factors have been defined. The phosphatidylserine (PS)-binding serum protein Gas6 boosts EV infection with little impact on MV infection. Gas6 may be involved in recruitment of EVs to the TAM receptor tyrosine kinase Axl [20], although the functional significance of this interaction has not been defined.

Although the full repertoire of host cell attachment factors exploited by MVs and EVs remains to be determined, it is evident that redundant attachment modes have evolved. This may contribute to the ability of poxviruses to infect a broad range of cell types within an infected organism [3]. Whereas host tropism of many viruses is determined by the attachment and entry receptors used, poxvirus tropism is usually determined by post entry steps [21].

Endocytosis vs. plasma membrane fusion

Fusion of enveloped viruses with cellular membranes can occur either at the plasma membrane or after endocytic uptake of viral particles. While fusion at the plasma membrane would depend less on cellular machinery, fusion after endocytosis would have several striking advantages for both MVs and EVs [1]: first, cellular transport mechanisms ferry virus particles to the point of fusion, avoiding cytoskeleton barriers such as cortical actin. Second, spatially/temporally controlled endosomal cues, including acidic pH and proteases, among others, may activate viral fusion at the appropriate cellular location. Third, no virus components that could be recognized by the immune system are left at the plasma membrane. Fourth, endocytic vesicles may protect activated viral fusion proteins, often sensitive to neutralizing antibodies, from immune recognition [22].

Electron microscopy (EM) is a commonly used method to study viral entry pathways. However, the use of high numbers of viruses for EM experiments entails the risk of observing minor or artificial entry pathways that do not correspond to the infectious entry mechanism. In most cases these experiments also defy thorough quantification. The reliance on EM to study MV entry has produced conflicting results wherein either fusion or endocytosis has been proposed as the major infectious entry pathway for diverse poxviruses [23–27].

For free EVs, EM studies suggested that entry into host cells occurs directly at the plasma membrane [28]. According to this model, the outer EV membrane is disrupted upon contact with GAGs, exposing the underlying MV-like particle which fuses with the plasma membrane. Entry of EVs from the tips of actin tails has not been studied.

Improved understanding of endocytic mechanisms has allowed for tailored perturbation studies. Using a series of inhibitors, distinct endocytic mechanisms can be downregulated, allowing for careful assessment of the contribution of each endocytic pathway to infection [16,29••]. More importantly, productive infection can be quantified. In addition, the use of fluorescent particles and MV- or EV-specific antibodies has provided an unprecedented opportunity to follow the fate of virion membranes and cores simultaneously by fluorescence microscopy. This allows the visualization of VACV MV and EV binding, internalization, and core release by fusion (see Figure 2). Recently, using a combination of these methodologies, it has been demonstrated that the main entry route of VACV MVs and EVs in HeLa and other cell types is endocytosis [16,17,29••,30•,31].

Endocytosis

For internalization, both MVs and EVs exploit macropinocytosis [16,32••]. This form of endocytosis involves dramatic actin rearrangements resulting in engulfment of large amounts of fluid. Macropinocytosis is signaling-induced and involves many cellular factors. The primary endocytic vesicles, macropinosomes, are of heterogeneous size and morphology. They can undergo homotypic and heterotypic fusion and acidification. The exact details of macropinosome maturation and their cross-talk to canonical endosomes and lysosomes, however, remain elusive. A detailed review on the cellular factors and mechanisms of macropinocytosis has been published recently [33].

Infection with either VACV MVs or EVs induces actin rearrangements manifesting in systemic plasma membrane blebs [16,32••]. They are hypothesized to fold back and enclose bulk fluid by a fusion event resulting in macropinosome formation [33]. MV-induced macropinocytosis is dependent on PS in the viral membrane and requires epidermal growth factor receptor (EGFR) signaling [16,29••]. Any potential relationship between these factors remains undefined. With the exposure of PS, MVs resemble apoptotic bodies. As apoptotic clearance can occur by macropinocytosis [34], it has been postulated that VACV MVs employ apoptotic mimicry [16]. Uptake of apoptotic bodies elicits anti-inflammatory signals [35] and it is intriguing to speculate that VACV infection could benefit from such modifications of the host immune system. Despite several known PS receptors, those used by MVs remain elusive. It will be of interest whether potential VACV PS receptor(s) bind phosphatidylglycerol or the D-stereoisomer of PS, two lipids absent from the MV membrane which can functionally substitute the naturally occurring PS [36]. Another cellular factor required for MV entry is VPEF/FAM21. Unlike previously suggested, VPEF is a cytosolic protein shown to be involved in Arp2/3-dependent vesicle scission and may therefore be involved in macropinosome trafficking or maturation [17,37].

Macropinocytosis of VACV MVs occurs in several different cell lines including monocyte-derived dendritic cells (MDDCs) [30•], which constantly macropinocytose as part of their immunosurveillance function [38]. Macropinocytosis also serves for the entry of different VACV strains, although the activated signaling cascades differ [29••,39]. MV entry into CHO cells, in contrast, is likely independent of macropinocytosis [29••].

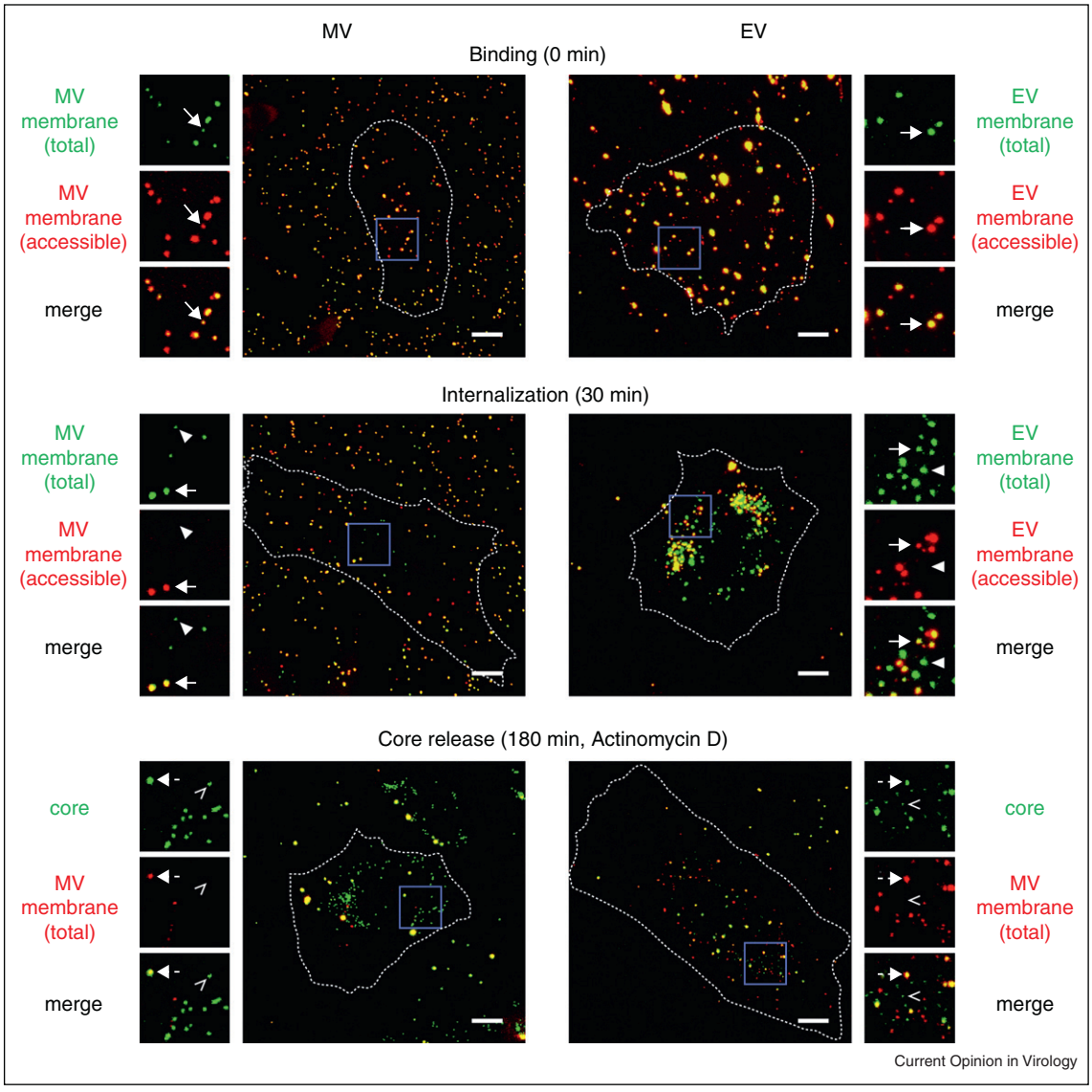
Using fluorescence microscopy, internalization assays, and inhibitor studies, it was demonstrated that macropinocytosis is a productive route of intact EV entry into HeLa cells [32••]. Unlike MVs, intact EVs do not rely on exposed PS to trigger macropinocytosis, although they share the need for EGFR signaling. As reported by Sandgren and coworkers, macropinocytosis of EVs is also needed for early viral gene expression in MDDCs [30•].

It may seem surprising that both infectious particles produced during VACV infection employ the same internalization mechanism. However, the size limitations that, for example, coat structures imposed on many endocytic pathways may make macropinocytosis the only internalization strategy possible for uptake of large particles into non-phagocytic cells. For MVs, endocytosis may prevent early immune recognition of viral factors left at the plasma membrane. Free EVs are less efficiently neutralized with antibodies and contain complement control proteins [40,41]. Endocytosis of intact EVs sustains this protection until particles are inaccessible to the humoral immune system. Epitopes on the inner membrane, many of which are targets of neutralizing antibodies, are also shielded from the immune system [42,43]. Endocytic entry of cell-associated EVs on actin tails has not been shown, but would be compatible with the recently proposed repulsion of superinfecting EVs [44]. If mere contact of EVs with infected cells caused disruption of the outer membrane and subsequent fusion of the underlying MV with the plasma membrane, EV entry and EV repulsion mechanisms would be mutually exclusive.

Membrane penetration

The final step of poxvirus entry is fusion of the viral membrane with the cellular membrane of the endocytic organelle, resulting in deposition of the viral core into the host cell cytosol.

Figure 2



Visualization of VACV entry into HeLa cells by fluorescence microscopy. Binding, internalization, and core release of MVs (left) and EVs (right) of VACV IHD-J can be detected by fluorescence microscopy. Binding/internalization: MVs and EVs are visualized as green punctae (see below for details). Bound virions (arrows) can be discriminated from internalized virions (arrowheads) by their accessibility to antibody staining under non-permeabilizing conditions (red). Thus, bound MVs and EVs appear green and red (yellow), while internalized virions are just green. Core release: all virus cores are visible as green punctae and antibodies were used to stain the MV/MV-like membrane (red). Virus particles that still contain this membrane (arrow with broken line) therefore appear green and red (yellow), whereas released cores (empty arrowhead) are green only. Scale bars: 10 μm. Core release was detected in cells treated with actinomycin D to prevent rounding of cells expressing early viral genes. Details of staining: binding/internalization of MVs: IHD-J wt, anti-L1 (unpermeabilized), and anti-A27 (permeabilized); binding/internalization of EVs: IHD-J F13-GFP, anti-B5 (unpermeabilized); core release of MVs/EVs: IHD EGFP-A5, anti-L1 (permeabilized).

For VACV, fusion is thought to be mediated or catalyzed by a large macromolecular assembly of viral proteins in the MV or MV-like membrane, the entry/fusion complex (EFC) [45]. The EFC consists of eight transmembrane proteins: A16, A21, A28, G3, G9, H2, J5, and L5 [45–52,53••,54] and is associated with three additional viral membrane proteins: F9, L1, and O3 [55–57]. A twelfth MV membrane protein, I2, is also required for fusion [58]. The exact architecture of the EFC and the molecular details of the fusion reaction remain elusive.

Two heterodimeric protein complexes negatively regulate EFC function. Dimers of A25 and A26 in the MV membrane are thought to inhibit premature EFC-mediated fusion. MVs of VACV strains lacking either of the two fuse with the plasma membrane at neutral pH

[59••], while plasma membrane fusion of MVs with full length A25 and A26 can only be artificially induced by treating bound virions with acidic pH [31]. Thus, one might speculate that inactivation of the dimer and thus activation of the EFC only requires acidification of particles. However, while pretreatment of unbound MVs with low pH or proteases accelerates viral entry, it does not circumvent the need to passage through acidic endocytic compartments [25]. These results imply that low pH removal of A25/A26 is partly reversible or insufficient to initiate poxvirus membrane fusion activity. Since acidification of MVs bound to the plasma membrane [31] or liposomes (F.I. Schmidt and J. Mercer, unpublished data) is sufficient to induce fusion (pH optimum 4.5–5.0), the additional cue may be interaction with membranes.

MVs of several VACV strains do not need endocytic acidification for infection and fusion with the plasma membrane cannot be induced by low pH [7,59••]. This may be explained by the lack of A25 or A26, although alternative mechanisms of activation cannot be ruled out. Of note, MVs of VACV strain IHD-J are independent of low pH, but still enter cells through macropinocytosis [29••]. All sequenced VARV and MPXV strains encode full-length homologs of VACV A25 and A26 [59••,60,61], suggesting that they undergo acid-induced fusion. Myxoma virus, the causative agent of rabbitpox, does not encode homologues of A25 and A26 [2]. Interestingly, myxoma virus MVs cannot be forced to fuse at the plasma membrane by low pH treatment, but require passage through acidified endocytic compartments for infection [62•].

A second protein complex consisting of A56 and K2 serves to regulate the fusion activity of the EFC *in trans*. This complex localizes to the plasma membrane of infected cells as well as to the outermost EV membrane [63] and blocks fusion of viral membranes with membranes containing the dimer. Together, these factors prevent MV superinfection and are thought to prevent back-fusion of disrupted EVs with the plasma membrane of producer cells [64,65•].

EVs are surrounded by two membranes and fusion of the outermost membrane would result in non-productive delivery of a membrane bound particle into the host cell cytoplasm. To allow productive infection, the EV membrane is lost by nonfusogenic disruption [28] followed by EFC-mediated fusion of the underlying MV-like particle [48,49]. Disruption of the EV membrane can be triggered *in vitro* by exposure to GAGs or low pH [28,32••,43,66]. GAG-mediated EV disruption depends on acidic residues in the membrane-proximal stalk region of B5 [67••]. Although the mechanism of acid-activated EV disruption is unknown, EVs lacking the membrane protein A34, and thus containing reduced amounts of A33 and B5 [68,69], are resistant to both GAG-mediated and low pH-mediated disruption [28,32••]. This suggests that the two mechanisms may share similar requirements.

In HeLa cells, EV membrane disruption is triggered in acidified endocytic vesicles after macropinocytosis [32••]. Acidification of endocytic compartments is required for infection in several other cell lines tested, confirming that low pH is the trigger for EV disruption *in vivo* [32••,43,66]. The MV-like membrane is exposed and the EFCs in this membrane likely mediate fusion of the particle with limiting membranes [48,49]. Since MV-like particles within EVs lack A25/A26 [60], it is possible that they do not need to be activated for fusion. Consistent with this, MV-like particles released from EVs behave like proteolytically activated MVs [43].

Independent of the activating mechanism, fusion of the EFC-containing viral membranes with macropinosome membranes releases viral cores into the host cell cytosol, thus completing the entry of both MVs or EVs. Released cores are 'activated': they undergo morphological changes [23] and start to transcribe early genes, allowing for subsequent uncoating steps resulting in DNA release and viral replication [4].

Conclusion and open questions

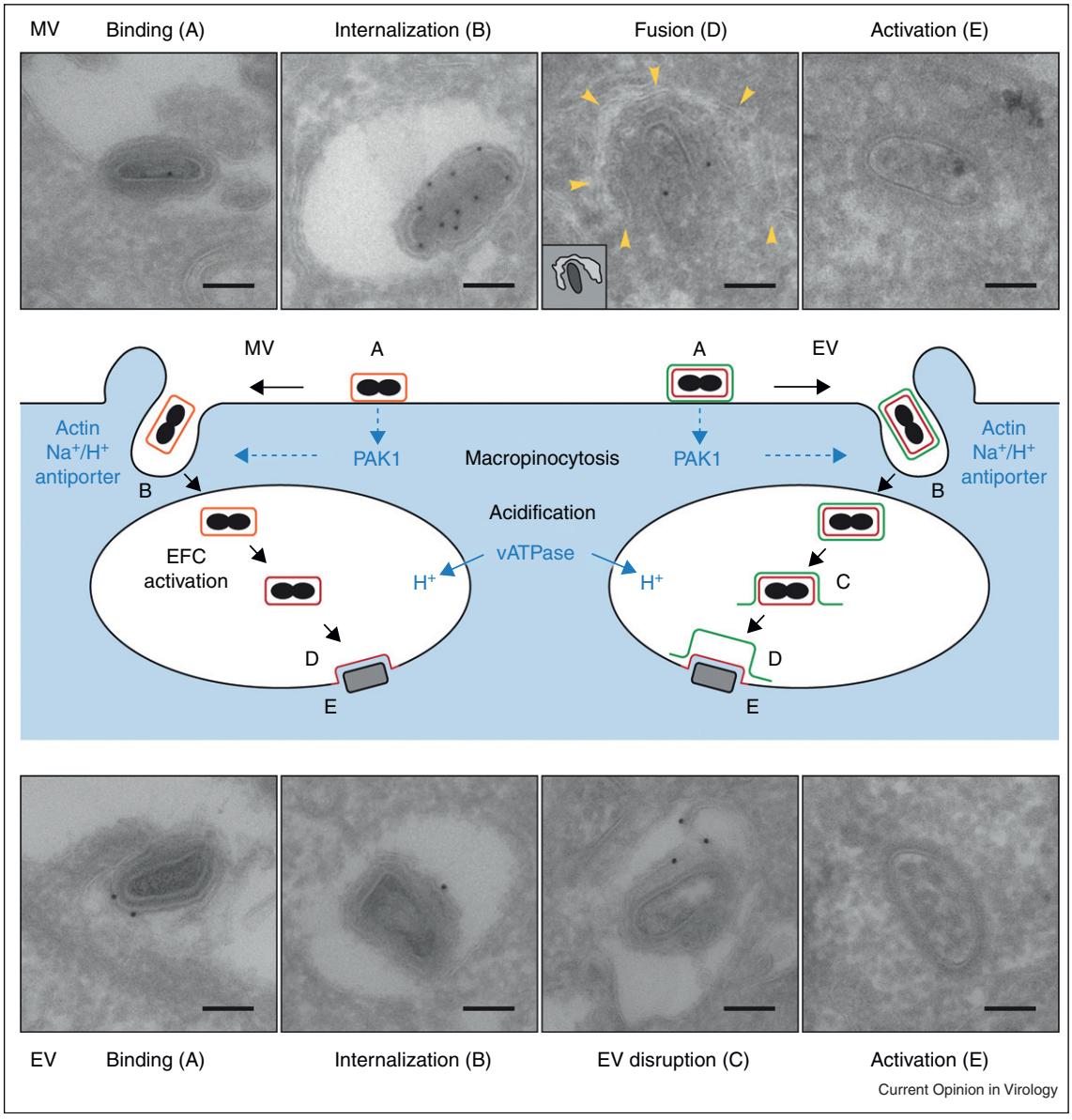
There has recently been a significant increase in the understanding of VACV MV and EV entry. The cellular mechanisms of MV and EV endocytosis have been unraveled, and many of the viral factors required for MV fusion and EV membrane disruption have been identified. A model of MV and EV entry with representative electron micrographs is shown in Figure 3.

Future studies should be aimed at further characterizing the cell biology of VACV entry including the identification of MV and EV receptors and the cellular components required for virus trafficking within cells. It will also be important to define those aspects of entry that are shared by all poxviruses, and those which are unique to VACV or the utilized cell lines.

The exact molecular mechanisms of fusion and the actual fusion peptide remain undefined. If confirmed, the poxvirus fusion machinery would represent the most sophisticated viral fusion machinery identified to date. Similarly, the molecular details of EV disruption need further investigation.

Finally, many studies of poxvirus entry have not been extended past established epithelial cell lines. These cells reflect the behavior of epithelial cells within a tissue in many, but not all, aspects. Preliminary studies on tissue explants suggest both MVs and EVs preferentially infect polarized cells from the baso-lateral side [70]. Thus, in future entry studies it will be important to use cells that more accurately reflect the *in vivo* situation encountered by

Figure 3



Entry pathway of VACV MVs and EVs. VACV MVs and EVs enter host cells in a stepwise process that includes binding **(A)**, internalization **(B)** by macropinocytosis, as well as EFC activation and fusion (**D**) in the case of MVs, or EV membrane disruption **(C)**, and fusion **(D)** in the case of EVs. Released cores are activated **(E)** and subsequently start early gene expression. Representative electron micrographs are shown for MVs (top) and EVs (bottom); scale bars: 100 nm. In top row image D, a schematic of membranes and viral cores is inset. Critical cellular components are shown in blue, arrows with solid lines represent physical movement; arrows with dashed lines depict signaling events.

these viruses, including primary epithelial cells and cells of the hematopoietic lineage. Greater understanding of the cellular mechanisms of poxvirus entry may one day help us identify new targets for the development of antivirals.

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