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Viral Weapons of Membrane Destruction: Variable Modes of Membrane Penetration by Non-Enveloped Viruses

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Abstract

Significant progress has recently been obtained in our understanding of cellular entry by nonenveloped viruses (NEVs). A key step in the entry process involves the disruption or remodeling of the limiting cell membrane allowing the virus to gain access to the cellular replication machinery. Biochemical, genetic and structural data from diverse virus groups have shed light on the process of membrane penetration thereby revealing both the conservation and divergence of the mechanisms and principles governing this process. In general, membrane breach is achieved via the highly regulated spatiotemporal exposure of a virally-encoded membrane lytic factor, resulting in the transfer of the viral genome or nucleocapsid into the cytosol.

Introduction

Cellular membranes present a formidable barrier to extracellular viruses aiming to hijack host cell machinery required for their replication. Enveloped viruses achieve cell entry via direct fusion of the outer viral membrane with a cellular membrane, resulting in transfer of the nucleocapsid to the cytoplasm. In contrast, NEVs, which by definition lack an outer membrane, mediate membrane rupture via deployment of a previously sequestered viral lytic factor. Although the specific details of membrane penetration vary between different NEVs, there are several shared features. Following assembly of nascent virions, additional maturation steps, often involving proteolysis and conformational alterations, yield mature particles structurally primed for host cell invasion. Once a target cell is encountered, various signals trigger capsid rearrangements that culminate in membrane lytic factor exposure. Triggers for uncoating vary widely among NEVs, and include low pH, receptor engagement, protease cleavage, chaperone-assisted morphological changes or divalent cation chelation. In general, a specific signal drives uncoating and ensures that viruses target their membrane lytic factors to an appropriate subcellular membrane at the most opportune time during infection.

In this review, we provide an overview of the field by comparing and contrasting the details of membrane penetration for several NEVs. We briefly highlight both the structural prerequisites for membrane penetration, cellular triggers for uncoating and lytic factor release, and the membrane disruption event itself. These key characteristics of nonenveloped

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virus membrane penetration are summarized in Table 1, which the reader might find helpful to refer to throughout the text. In addition, we discuss several areas for which additional information is needed.

Proteolytic processing primes virus particles for cell entry

A mature virus must successfully withstand the stresses of the extracellular environment while maintaining the ability to disassemble and release its genome at the right time and correct location within the host cell. This balance is struck by locking the capsid in a metastable conformation, primed to disassemble only when the appropriate signals from a host cell are received [1].

During assembly and maturation, immature particles frequently require proteolytic processing of capsid proteins to generate mature, infection-competent virions. Infection by Flock house virus (FHV) is dependent upon the post-assembly, autocatalytic cleavage of the major capsid protein, α, to generate two fragments, β and γ, the latter of which possesses membrane lytic activity [2–4]. For adenovirus (AdV), proteolytic processing of a subset of capsid proteins is absolutely required for infectivity. A temperature sensitive mutant (Ad2*ts1*) fails to package wild-type levels of the viral protease. The *ts1* mutation results in non-infectious particles that are blocked for endosome escape due to a failure to undergo disassembly and release of the membrane lytic factor, protein VI [5–8]. Poliovirus (PV) capsid proteins are initially expressed as a single polyprotein and subsequently undergo post-translational processing to generate the mature capsid proteins. Cleavage of VP0 to VP2 and VP4 is critical, as mutant PV particles that do not undergo this step are non-infectious [9].

Cellular triggers cause conformational changes in virions and allow release of lytic proteins

Early host cell interactions often elicit rearrangements of the viral capsid to allow lytic factor release. In addition, most NEVs do not penetrate the plasma membrane, but instead must transit through endosomal, golgi or endoplasmic reticulum (ER) compartments to promote uncoating and genome release.

Receptor engagement is one of several entry steps that often induce structural alterations of the capsid. HAdV5 cell attachment and internalization is mediated by two proteins, fiber and penton base, located at the five-fold vertices of the particle. Primary recognition of the Coxsackie and Adenovirus Receptor (CAR) by the HAdV5 fiber results in fiber dissociation at or near the cell surface, thus triggering the first of a series of sequential capsid disassembly steps [10, 11]. While low pH is generally thought to facilitate AdV uncoating, there is also evidence arguing against a pH-dependent mechanism [12]. Secondary interactions of the penton base with integrin co-receptors may also prime AdV particles for efficient entry [13]. Structural studies have indicated a possible conformational change in pentons bound to integrin [14] required for subsequent endosome penetration via protein VI [15].

Structural changes upon receptor binding also mediate the conversion of PV from a metastable state into a structure capable of undergoing membrane penetration. The poliovirus receptor (PVR/CD155) binds within the “canyon” region, a depression surrounding the five-fold axis of the virus particle [16]. Binding results in the conversion of mature virions to A (135S) particles, characterized by externalization of the N-terminus of VP1 and release of VP4, both of which play a role in membrane penetration [17–20].

Following receptor binding, NEVs are frequently transported into the endolysosomal pathway. The concomitant decrease in pH within these vesicles is thought to signal conformational changes in NEVs, as agents that block this event often inhibit infection. For example, bafilomycin and NH_4Cl reduce infectivity of adeno-associated virus (AAV) [21] and canine parvovirus (CPV), two members of the Parvovirus family [22]. For CPV, exposure to low pH correlates with divalent cation release, and may allow exposure of the N-terminus of VP1 [23–25]. In addition, low pH-induced conformational changes within the capsid of murine polyomavirus cause VP1 to become more susceptible to protease cleavage *in vitro* [26].

Interestingly, the γ peptide of FHV, located on the interior of the particle post-assembly, becomes transiently exposed to the cellular environment following maturation. Following particle internalization, the acidic environment of the endocytic pathway is postulated to cause an irreversible structural change in the capsid that leads to release of γ from the particle [27, 28]. Optimal membrane penetration is thought to require full release of γ from the capsid rather than transient exposure, as cleavage-defective particles have decreased membrane lytic activity compared to cleavage-competent particles upon heat-mediated disassembly [28].

Low pH is also indirectly responsible for structural changes in viral particles, as several NEVs depend on processing by acid-dependent proteases within endocytic vesicles to expose a membrane lytic factor [27–31]. In the mature reovirus particle, the outer capsid protein σ3 functions to sequester the membrane penetration protein, μ1 [29]. Removal of σ3 by endosomal cathepsins B and L, along with additional conformational changes, generates a discrete intermediate particle, the ISVP (intermediate subviral particle) [30, 31]. This particle is further processed to generate a second distinct intermediate particle (ISVP*), characterized by the autocatalytic cleavage of μ1 to generate μ1N (the membrane lytic agent) and μ1C fragments [32].

Cellular chaperones can also mediate lytic factor release for certain NEVs. For example, polyomavirus depends on ERp29, an ER-localized member of the protein disulfide isomerase family of proteins, to destabilize the capsid and expose the C-terminus of VP1 [33]. This structural change drives externalization of myristoylated VP2, which is postulated to mediate the membrane penetration event, and produces virions that are significantly more hydrophobic [34].

Exposed lytic factors mediate membrane disruption

The actual membrane penetration event is the least understood stage of the NEV entry pathway. However, new information has emerged revealing how relatively small viral peptides or their hydrophobic domains facilitate the transfer of viral genetic material and/or nucleocapsid into the cytoplasm. The molecular details of membrane disruption and the structural characteristics of the domains involved vary for different viruses (Table 1). Structurally, there are three major classes of membrane lytic factors: amphipathic α-helices, myristoyl groups, and lipid-remodeling enzymatic domains. Mechanistically, membrane disruption can be divided into three categories: transient modification of the cellular membrane, pore formation, and total disruption of the limiting membrane (Figure 1). Not surprisingly, in most cases there is a direct link between the scale of membrane disruption and the viral payload (i.e. genome or nucleocapsid) that must be delivered to the cytoplasm.

Several NEVs encode proteins that possess an amphipathic α-helical domain. The membrane lytic factor for AdV, protein VI, encodes an N-terminal amphipathic α-helix that is critical for efficient membrane disruption [35]. Previous studies showed that VI membrane lytic activity is pH-independent [35]. A mutant virus that harbors a single substitution (L40Q)

within the VI amphipathic helix exhibits significantly reduced infection and attenuated endosome escape [36]. Interestingly, the L40Q mutation generates a reduction in the overall α-helicity of the protein VI molecule and perturbs bilayer insertion [36]. This highlights the importance of maintaining both the hydrophobic character and α-helical nature of protein VI to allow maximal membrane disruption. Protein VI likely mediates gross fragmentation of the endosomal membrane to allow the large (90 nm) partially uncoated capsid to enter the cytoplasm (Figure 1). Recent studies also suggest that the hydrophobic face of the amphipathic helix of protein VI inserts in a parallel fashion into the lipid bilayer, with the hydrophilic face contacting the phospholipid head groups [37]. Furthermore, membrane fragmentation may occur via induction of positive curvature, as suggested by experiments assessing membrane disruption of giant unilamellar vesicles by protein VI [37, 38]. This *in vitro* evidence suggests that protein VI mediates endosome lysis by imparting stress on the luminal leaflet of the endosomal membrane (Figure 1). A model for gross fragmentation of the endosome during AdV cell entry is further supported by studies showing that AdV facilitates the cytosolic translocation of large macromolecules (70 kDa dextrans) and ~25 nM parvovirus capsids [24, 39]. It is important to note, however, that these experiments do not distinguish between full-scale membrane disruption and large (>25 nm) pore formation. Further studies are needed to determine the exact mechanism for AdV-mediated codelivery of macromolecules or phospholipase-defective parvoviruses.

The N-terminal 21 residues of the γ peptide of FHV also form an amphipathic α-helix. Interestingly, the γ peptide of FHV is relatively unstructured *in vitro*; however, when mixed with model membranes, it becomes significantly more helical, suggesting that some viral lytic factors require contact with the lipid bilayer to adopt a membrane-active conformation [40]. A synthetic version of the γ peptide is capable of mediating liposome lysis, and lies parallel to the membrane surface, with the hydrophobic face of the helix contacting the bilayer [3, 40, 41]. Despite the parallels to HAdV protein VI-membrane interactions, it is still unclear whether the γ peptide mediates membrane disruption via the formation of discrete pores, or instead causes large-scale destabilization of the membrane occurs, as appears to be the case for HAdV [42].

PV also contains an amphipathic α-helix within the N-terminal portion of the capsid protein VP1. Following receptor binding, a conversion within PV causes the previously buried hydrophobic portion of VP1 to be externalized. Interestingly, PV harbors another protein, N-myristoylated VP4, which also plays a role in membrane penetration. Studies of poliovirus and other similar members of the *Picornaviridae* indicate that pore formation is the likely mode of RNA genome transfer to the cytoplasm. However, it remains unclear whether one or both VP1 and VP4 proteins participate in pore formation. Recent evidence suggests that VP1 primarily functions to tether the particle to the limiting membrane, while VP4 participates directly in pore formation [43]. The specific site of membrane disruption remains disputed, as early electron microscopy suggested this occurs at the plasma membrane [44], while a more recent analysis utilizing live cell imaging revealed PV escape from an endocytic vesicle [45].

Myristoyl group-mediated pore formation is also a feature of reovirus membrane penetration. As described above, auto-cleavage of μ1 during reovirus entry generates μ1N peptides that are linked to an N-terminal myristoyl group. This cleavage event is required for membrane penetration, and likely indicates that full release of μ1N, is needed for pore formation [46]. Evidence for reovirus pore formation is largely derived from the ability of μ1N to generate size-selective pores in erythrocyte or liposomal membranes [47–49]. Interestingly, although the relatively large core (~75 nm) of reovirus is transported to the cytoplasm, the pores generated by μ1N (at least in model membranes) are not large enough to directly allow passage of the subviral particle [47]. It has been suggested that pore

formation might be linked to osmotic swelling and lysis of endosomal vesicles (Figure 1). Alternatively, additional cellular factors may promote pore enlargement, however direct evidence for either of these scenarios is lacking [31].

Parvoviruses encode a phospholipase type 2 (PLA2) domain at the N-terminus of VP1. During cell entry of CPV and minute virus of mice (MVM), the PLA2 domain is extruded through pores at the five-fold vertices. Although an active PLA2 domain is essential for endosome penetration, active site point mutations in PLA2 that abolish enzymatic activity can be rescued with polyethyleneimine treatment or coinfection with HAdV [24]. To allow transfer of the capsid from the endosome to the cytosol, it appears that the PLA2 domain transiently modifies the lipid bilayer on a limited scale, perhaps via the induction of membrane curvature (Figure 1) [50]. Pore formation or gross fragmentation is unlikely, as the virus is unable to mediate the transfer of molecules of varying size (sarcin, dextrans) from the endosome to the cytoplasm [22, 51].

Conclusions and Future Directions

Despite significant advances in our understanding of membrane penetration by NEVs, many unanswered questions remain. For most viruses, the precise molecular mechanisms for membrane lysis are largely unknown. Although pore formation or gross membrane disruption have been suggested as mechanisms for membrane perturbation for several viruses, current models are based largely on *in vitro* techniques or macromolecule co-delivery assays that indirectly measure endosome penetration during cellular infection. While these techniques may provide useful insights, experimental results derived from them must be carefully interpreted. The identity of cellular factors required for pore formation or membrane disruption has yet to be fully established. In addition, while pore formation is known to be general mechanism for membrane disruption, little is known about the oligomeric state of the proteins that form these pores. Considering the relatively small size of these peptide monomers, it is thought that multiple copies must be involved in pore formation, yet little direct evidence for this exists. For viruses that release membrane lytic factors from the particles (e.g. AdV, poliovirus, possibly FHV), it remains to be determined how many copies of these molecules are needed to breach a membrane, thus limiting our ability to construct useful models of this event. Single particle tracking experiments should also provide useful insights into the site of membrane penetration, as many nonenveloped viruses exhibit high particle:pfu ratios, causing bulk analysis of cell entry to weigh equally productive and non-productive infections. Additional structural studies of entry intermediates primed for lysis, as well as of the membrane lytic factors themselves are clearly needed to understand the membrane penetration event at the molecular level. Finally, recent reports suggest that disruption of subcellular membranes may signal proinflammatory responses and thus a mechanistic understanding of this process and its applicability *in vivo* are clearly needed [52–54].

Highlights

- Nonenveloped viruses (NEVs) encode lytic factors that mediate membrane disruption
- Lytic factors are sequestered until a target cell compartment is encountered
- Cellular cues trigger capsid structural rearrangements and lytic factor release
- The precise modes of membrane perturbation by NEVs remain unknown

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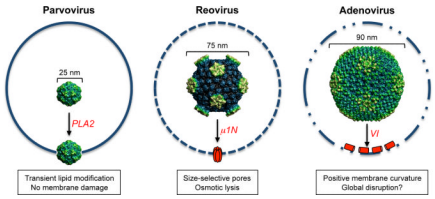

Figure 1. Proposed mechanisms of membrane disruption by NEV lytic factors

Disruption of the limiting membrane is divided into three major categories: transient lipid rearrangement, pore formation, and gross disruption. Parvoviruses deploy a phospholipase A2 domain, which mediates membrane passage via temporary modification of the endosomal bilayer. Reovirus μ1N is released from the virus, and forms size-selective pores in the endosomal membrane. Whether pore formation is linked to osmotic lysis of the endocytic vesicle is still unclear, but could explain how the large virus core translocates to the cytoplasm. AdV protein VI inserts into the lumenal leaflet of the endosomal membrane, inducing positive membrane curvature and total fragmentation of the endosome. This model is based on VI-mediated lysis of unilamellar vesicles [37, 38]. Viruses are scaled to depict the relative size of each particle. Structural models were obtained from the Virus Particle Explorer database (VIPERdb, http://viperdb.scripps.edu/index.php) [55].

Table 1

Summary of nonenveloped virus membrane penetration

Virus Family / genome	Virus	Lytic protein	Functional domain[a]	Proposed trigger for release	Site of penetration	Proposed lytic mechanism
Nodavirus / (+)ssRNA	FHV	γ-peptide	APH	Low pH	Endosome	Pore or gross fragmentation
Picornavirus / (+)ssRNA	PV	VP1/VP4	APH (VP1)/Myr (VP4)	Receptor binding	Endosome	Pore
	Minor group HRV	VP1/VP4	APH (VP1)/Myr (VP4)	Low pH	Endosome	Pore
	Major group HRV	VP1/VP4	APH (VP1)/Myr (VP4)	Low pH	Endosome	Gross fragmentation
Reovirus / dsRNA	Mammalian reovirus	μ1N	Myr	Cathepsin B & L cleavage	Endosome	Pore
	Rotavirus	VP5*[b]	Hydrophobic residues	Trypsin cleavage, calcium chelation	Plasma membrane	Membrane fusion[c]
Parvovirus / ssDNA	AAV/CPV/MVM	VP1	PLA2	Low pH/Divalent cation removal	Endosome	Transient lipid rearrangement
Polyomavirus / dsDNA	Murine polyomavirus	VP2	Myr	Low pH/Chaperone rearrangement	ER	Pore
Adenovirus / dsDNA	HAdV	VI	APH	Low pH and/or Integrin binding	Endosome	Gross fragmentation

[a] Abbreviations: APH – amphipathic α-helix, Myr – myristoyl group, PLA2 – phospholipase A2

[b] Trypsin cleavage of VP4 → VP8* and VP5*

[c] Proposed to function similarly to class II fusion proteins of enveloped viruses