Review

The parvovirus capsid odyssey: from the cell surface to the nucleus

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During cellular entry and infection, the parvovirus capsid follows a complex path from the cell surface to the nucleus, where the DNA is replicated. Various receptors have been characterized that bind to different parvoviruses and mediate their entry into cells. However, the subsequent trafficking pathways within the endosomal system, cytoplasm and into the nucleus are still not well defined. Studies of viruses entering various cell types under different conditions show particles located in many different endosomal compartments, within the cytoplasm and in the nucleus with significant variations in timing and distribution. Here, we define the previously unresolved issues that are now better understood for the infection pathways of these viruses, and outline some of the areas that remain to be clarified in future studies.

Parvoviruses: infection and disease

Parvoviruses infect a wide variety of animal hosts ranging from crustaceans to man [1], and the diseases they cause range in severity from sub-clinical to severe and even fatal infections, depending on the virus and host factors such as age and susceptibility. The adeno-associated viruses (AAV), which are considered to be non-pathogenic, depend on helper adenoviruses or herpesviruses for their replication and are being developed as gene-therapy vectors.

Parvovirus infection requires successful entry into animal cells through a series of interactions that culminates in the release of viral genetic material into a compartment in which replication occurs. Many viruses use normal receptor-mediated endocytic and vesicular trafficking pathways for uptake and directed cytoskeleton-dependent transport for entry [2–4]. The choice of receptor can determine host specificity and tissue tropism, and might influence the subsequent endosomal trafficking within the cell. Here, we review the current understanding of cellular entry by parvoviruses, highlighting differences in the process between viruses within the family and the areas for future research. We focus on the five main functional stages of infection and intracellular trafficking: (i) receptor binding and uptake, (ii) vesicular trafficking, (iii) endosomal escape, (iv) cytoplasmic trafficking, and (v) nuclear entry.

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Receptor-dependent virus internalization from the cell surface

The pathways of endosomal uptake and trafficking are summarized in Figure 1. All parvoviruses use receptor-mediated endocytosis for cellular uptake, and a wide variety of glycoproteins, glycans and glycolipids might function as receptors for various viruses (Table 1). Some viruses use multiple receptors, although, in some cases, exactly how these multiple interactions allow optimal infection under different circumstances is uncertain. For example, canine parvovirus (CPV) and feline panleukopenia virus (FPV) use the transferrin receptor (TfR) as a primary receptor for uptake, but some strains of these viruses can also bind to sialic acids on the cells of some hosts. However, although neuraminidase treatment reduces cell binding by the viruses in feline cells, it does not alter virus-infection rates, and a direct role of sialic acid interaction in cell infection seems unlikely [5–7]. The TfR is endocytosed by clathrin-mediated endocytosis, which is probably the default uptake pathway for CPV and FPV entry [8]. However, altering or removing the Tyr-Thr-Arg-Phe (YTRF) internalization signal in the cytoplasmic tail of the TfR delays uptake, but does not prevent infection [10]. Furthermore, expressing dominant-negative dynamin-2 in cells reduces and delays, but does not abolish, uptake and infection by CPV and AAV, indicating that other uptake mechanisms can also be used [9–11]. Both minute virus of mice (MVM) and AAV type-2 (AAV2) capsids are taken up by clathrin-mediated endocytosis despite their use of sialic acids or heparin sulfate proteoglycan (HSPG) as primary receptors, which are not specifically associated with the clathrin pathway [12,13]. Other potential routes of uptake for these viruses include non-specific pinocytosis of capsids from the cell surface or other less-well defined clathrin-independent mechanisms [2,3].

An unresolved question for most parvoviruses is the specific roles that receptor binding and/or clustering have in initiating cell-signaling pathways that affect uptake or cellular gene expression. AAV2, for example, binds and clusters αVβ5 integrins, which signal through Notch1 and Rac to enhance internalization by clathrin-mediated endocytosis [14]. Receptor clustering and cross-linking might also affect the intracellular trafficking of the receptor–virus complex. Clustering of TfR with oligomeric transferrin changes the intracellular trafficking pattern when compared with the monomeric transferrin, a phenomenon

0966-842X/$ – see front matter doi:10.1016/j.tim.2008.01.012 Available online 9 April 2008

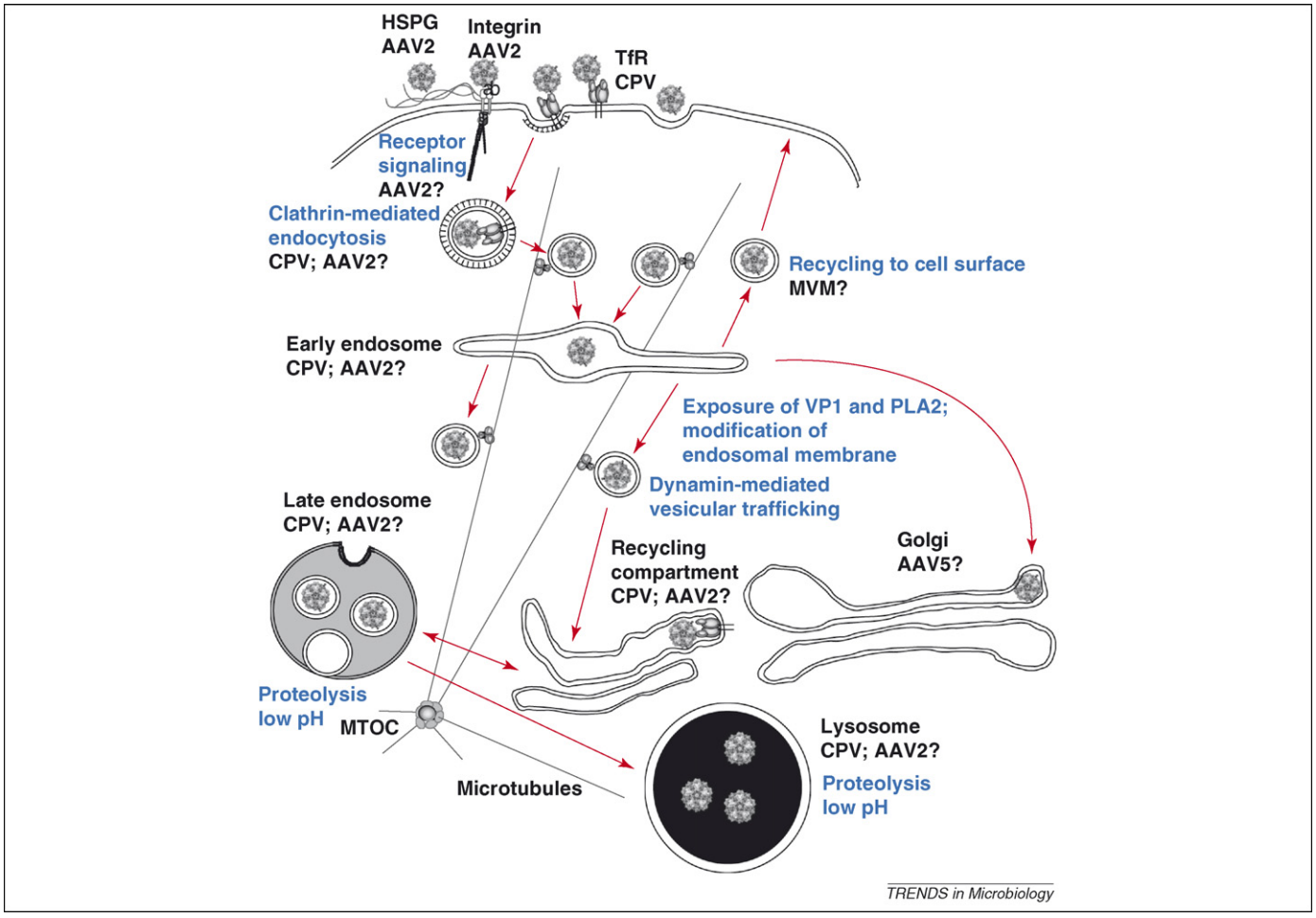

Figure 1. The processes of cell uptake and endosomal trafficking by parvoviruses, outlining the known pathways and the various steps that seem to differ between parvoviruses. Capsids are shown in association with TfR (as for CPV), αVβ5 integrin (AAV2) and HSPG (AAV2), but many others are possible. In most cases, uptake from the cell surface seems to be clathrin-mediated, but other uptake pathways are also possible. The activation of signaling pathways and actin polymerization during AAV2 entry is shown. Red arrows indicate intracellular pathways that have been shown or suggested for various viruses, but they probably differ depending on cell type, the conditions and the methods used to examine for virus trafficking. Abbreviations: HSPG, heparin sulfate proteoglycan; MTOC, microtubule organizing center; TfR, transferrin receptor.

which might also affect the entry pathways of multivalent viral capsids [15].

Interestingly, in some cases, viruses bind to cells and are internalized but infection does not occur, possibly owing to an alteration in trafficking that does not allow viral release or that delivers the particle to the wrong compartment. For example, transduction of recombinant AAV2 capsids is more efficient from the basolateral surface of polarized human airway epithelia compared with the apical, despite similar numbers of particles entering from each surface [16]. Similarly, chimeric TfRs with the cytoplasmic and transmembrane sequences replaced with those of the influenza neuraminidase, or the extracellular domain replaced with an anti-viral antibody fragment, are both able to bind and take up CPV but do not allow efficient infection [6]. However, the specific structural changes or intracellular blocks to infection in these cases have not been determined.

Trafficking within the endosomal system

The rapid dynamics and complexity of viral movement within and between endosomal compartments are

Table 1. Receptors defined as binding to parvoviruses, which, in most cases, also mediate the process of cell infection

Virus	Cell-surface receptors and binding molecules	Host(s)
Minute virus of mice	Sialic acids	Rodents
Human B19 virus	Globotriaosylceramide or globoside erythrocyte P antigen	Humans (primates)
FPV and CPV	Transferrin receptor-1, Sialic acid in some breeds	Cats, dogs, related carnivores (host ranges may differ)
AAV2	Heparan sulfate proteoglycan αVβ5 integrin, fibroblast growth factor receptor 1 [60]	Human
AAV4	*O*-linked α2–3 sialic acid	Human
AAV5	*N*-linked α2–3 sialic acid, platelet-derived growth factor receptor	Human
AAV6	*N*-linked α2–3 and α2–6 sialic acid	Human
AAV8	37/67-kDa laminin receptor	Human
Bovine AAV	Gangliosides [61]	Bovine

becoming increasingly appreciated. Somewhat different pictures are seen when capsid distribution in cells is examined by live-cell studies versus analysis of capsids in cells that are formaldehyde fixed before microscopic analysis. The lack of dynamic and information, and the difficulty of determining the overlap in fixed cells also make live-cell microscopy an important method for analysis. For CPV, cells fixed after viral uptake and stained with antibodies show capsid accumulation in perinuclear vesicles within 30 min [8]. This pattern can be disrupted by depolymerization of the microtubule network with nocodazole, low temperature or by expression of a dynamin-2 K44A dominant-negative mutant. These treatments reduce infection, although it is difficult to distinguish the effects of the drugs on cell viability and permissiveness for replication versus direct effects on infectivity [17,18].

After uptake, capsids are found in several intracellular locations, but determining which endosomal compartments the viruses must pass through before escaping into the cytoplasm has proven a difficult task [19]. Although not always well documented, the particle-to-infection ratio of most parvoviruses seems to be high (100:1 to >1000:1), meaning that most particles entering the cell do not replicate. In the case of CPV, the entering virions infect slowly and capsids can stay associated with TfR in the endosomal system of the cell for up to ~4 h because infection can be blocked even at that time by injecting an antibody against the cytoplasmic tail of the TfR into cells. After fixing the cells at different times after uptake, CPV and AAV capsids could be co-localized with markers of the early endosome, late endosome, recycling endosome and lysosome within the first hours of infection [8,13,20,21]. Live-cell analysis with fluorescently labeled particles, which more accurately represents dynamic processes than analysis of fixed cells does, has indicated that there are several simultaneous and overlapping types and rates of particle movement within the vesicular system, some of which correspond to trafficking on the cytoskeleton versus random movement of vesicles through the cytoplasm [22] (C.E. Harbison, unpublished).

Many of the different serotypes of AAV are quite distinct, the amino acid sequence of their capsid proteins being between ~55% and >90% homologous, and they can also exhibit diverse intracellular-trafficking patterns. For example, capsids of AAV5, but not other serotypes, accumulate in the Golgi compartment [23]. Cell type and capsid concentration also affect both the distribution of AAV2 particles in endosomes and the efficiency of transduction [20,24]. When cells are fixed after viral uptake at low multiplicity of added particles, AAV2 capsids localize primarily in Rab7-positive vesicles (late endosomes), whereas, at high multiplicities, they are more often found in Rab11 vesicles (recycling endosomes). Studies in which Rab7 or Rab11 are overexpressed or inhibited by RNAi treatments indicate that the Rab11 pathway enables more efficient transduction of AAV2 compared with the Rab7 pathway [20]. However, other studies indicate that AAV2 escapes from an early endosomal compartment and that trafficking to later compartments is dispensable for entry [13,25]. The reasons for these differences are unclear but are likely to be owing to different experimental approaches or analytical methods and to the complex nature of the trafficking, and the pathways might be difficult to define when cells are examined after fixation.

Acidification of endosomes is essential for infection by all parvoviruses examined to date, although the specific functions are not yet resolved. Bafilomycin A_1 inhibits the ATPase responsible for endosomal acidification, whereas NH_4Cl neutralizes the endosomal pH, and both block infection if added within 30 min of AAV inoculation [13] or within 90 min for CPV [8]. Prior low pH incubation of capsids does not substitute for the cellular block *in vivo* [8,26]. The effect of pH neutralization might indicate that low pH triggers a required conformational change in the capsid. Some reversible changes occur in the capsid structures when incubated at low pH, and internal components of the capsids such as the VP1 unique region might be more easily released under these conditions [25,27,28]. Alternatively, the viruses might require the activity of an acid-dependent host factor present in specific endosomal compartments (such as acid-dependent proteases), or the endosomal trafficking and vesicular fusion pathways might be directly affected because some of these processes are also dependent on low pH [17,29–31]. In the latter case, the choice of which trafficking pathway is followed and the stage at which the particles leave the endosome would determine the effects of experimentally modifying endosomal pathways and post-entry capsid-processing steps. Both factors are emerging as crucial to the transduction activity of AAV particles [31].

Capsid structural changes and endosomal escape

Major conformational changes in capsid structures have not been detected during the early stages of entry. For a recent review that examines the structural aspects of infection, see Ref. [32]. The details of the responses to low pH and proteases vary between different parvoviruses, indicating that there are virus-specific processing requirements. Parvovirus B19 is sensitive to inactivation at low pH and exposes both the VP1-unique N terminus and the genome under these conditions, whereas CPV and MVM capsids remain largely intact and infectious [33–35]. CPV replicates in, and is shed from, the intestine; therefore, it is probably required to be more stable than B19, which seems to use mainly respiratory routes for infection and spread. AAV capsids, although structurally more closely related to B19, seem to be more similar in stability and exposure of structures to CPV and MVM. The full capsids of autonomous parvoviruses such as MVM and CPV expose a proportion of the virus protein (VP)2 protein N termini, and 15–22 residues of that sequence (depending on the sequence and the protease used) can be cleaved off to form VP3 [36,37]. In the case of MVM, the sequence might also be cleaved after endocytosis, enhancing the release of the VP1 N-terminal sequences and recycling to the cell surface [38–40]. Whether this happens in the case of CPV capsids has not been determined, and inhibitor studies have not identified the protease(s) responsible for the cleavage of VP2 to VP3 [36].

The N termini of VP1 proteins contain both phospholipase A2 (PLA2) sequences and basic nuclear localization

signals (NLSs), and both activities are required during infection [41,42]. The VP1 N-terminal sequence seems to be extruded through a pore that passes through the capsid shell at the fivefold axis of symmetry of CPV and MVM without capsid disassembly [39,43], probably while still in the endosome, and this might form a capsid site for genome release later in entry. Acidification might not be necessary for this conformational change by all viruses because CPV capsids show VP1 release even in the presence of endosomal neutralizing drugs [19,25]. PLA2 activity seems to be either directly or indirectly responsible for endosomal escape of MVM because non-infectious point mutants lacking the PLA2 activity can infect when endosomal lysis is stimulated by addition of polyethyleneimine or adenovirus capsids [27]. Exposure of AAV to acidic conditions also results in conformational changes that activate or expose the PLA2 domain of AAV2 capsids. PLA2 active-site mutations have no influence on capsid assembly, packaging of viral genomes into particles, or binding to and entry into HeLa cells, but early gene expression is delayed, indicating an important role for the PLA2 sequence in viral entry [44]. Furthermore, capsid mutations that block exposure of the PLA2 domain dramatically decrease infectivity, and mutations of residues surrounding the pore at the icosahedral fivefold axis of symmetry can restore transduction activity in mutant particles lacking VP1 [45,46].

PLA2 sequences alter and induce curvature of membranes by modifying the lipid head groups to change their packing within the membrane, but the connection to viral membrane penetration has not yet been specifically determined. Furthermore, both the contribution of other viral or cellular factors and the details of the mechanism of escape are unknown. Transient or limited pore formation in the endosomal membrane is more likely than complete endosomal lysis as a means of CPV escape because neither α-sarcin nor large dextrans enter the cytoplasm with incoming viral capsids [8,19].

Viral trafficking in the cytoplasm and access to the nucleus

The capsid processes that operate in the cytoplasm and that are associated with nuclear entry are summarized in Figure 2. Although some conformational changes begin in the endosome, there is evidence for further processing in the cytoplasm. Treating cells with protease inhibitors that block proteasome activity reduces MVM infectivity if added 3 h after infection, theoretically late enough to affect a cytoplasmic processing event [37]. The protease might cleave the capsid VP2 at specific sites, initiating disassembly by a 'bite and chew' model, but proteasomal processes do not seem to be involved in specific cleavage of VP2 to VP3 or in the externalization of the VP1 N terminus. Viruses infecting cells in the presence of protease inhibitors accumulate at the cytoplasmic side of the nuclear membrane but do not enter the nucleus [38,47]. However, protease inhibitors can affect a variety of proteases,

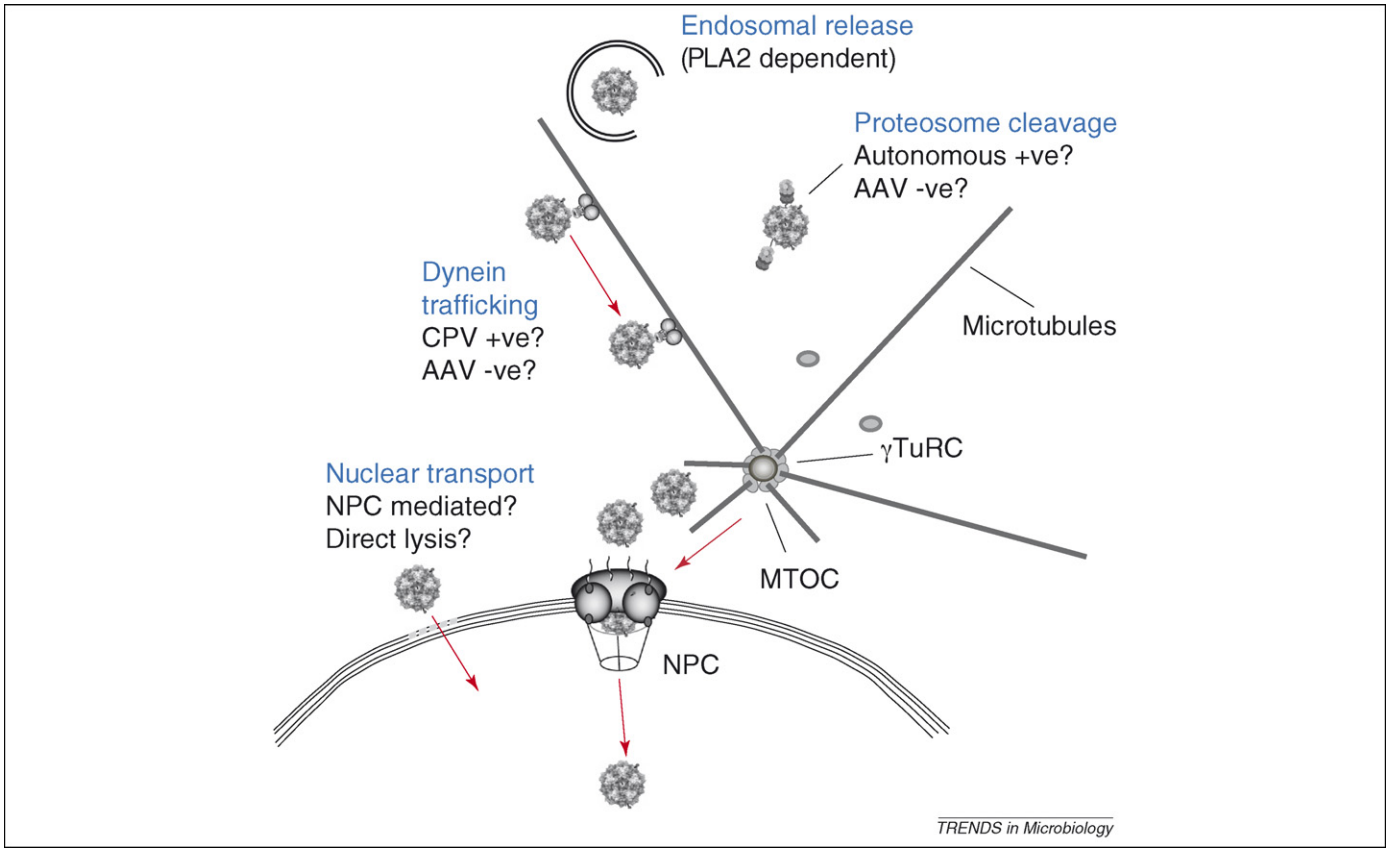

Figure 2. The cytoplasmic and nuclear trafficking of parvovirus capsids. Viral release occurs from the endosome after PLA2 modification, although the process is not completely understood. In the cytoplasm, capsids might be susceptible to digestion by proteasomes, with enhancing or inhibitory effects depending on the virus. The involvement of microtubules and microtubular motors (particularly dynein) can vary. The process of nuclear entry might involve direct transport through the nuclear pore by the more-or-less intact capsid or might directly or indirectly affect the nuclear-membrane structure. Abbreviations: MTOC, microtubule-organizing center; NPC, nuclear pore complex; PLA2, phospholipase A2; +ve, infection involves this mechanism; −ve, infection does not involve this mechanism.

including those in the endosomes, and they are also usually toxic to the cells, which could result in non-specific reduction of viral replication.

By contrast, different proteases seem to have both positive and negative roles in AAV infection. The proteasome plays an inhibitory part in AAV entry and treatment with proteasome inhibitors enhances AAV transduction, perhaps by altering endosomal trafficking or processing of capsids or by decreasing ubiquitination-dependent degradation of viral capsids [48]. Intact particles did not seem to be ubiquitinated in that study, and endosomal processing and partial disassembly might be required to prime AAV capsids for ubiquitination [48]. Cathepsins B and L, by contrast, have been shown to interact with AAV2 or AAV8 proteins using yeast two-hybrid screening, and inhibitors of these proteases decrease transduction *in vivo* [31]. Trypsin-mediated cleavage sites have also been identified in the AAV2 capsid surface, and might be involved in initiating structural rearrangements that increase capsid flexibility in preparation for uncoating. Although the particles remain intact *in vitro*, differences in negative staining indicate structural rearrangement or flexibility due to the cleavage event [49]. Prolonged incubation with the proteases reduces the infectivity of the particle due to loss of heparin-binding activity, but might assist in the uncoating and release of the viral genome once inside the cell [49]. Microinjection of particles treated with various proteases into cells would show whether these post-entry modifications are sufficient to activate the particles. Indeed, some processing steps are essential because AAV2 capsids injected into the cytoplasm do not lead to productive infection even in the presence of Ad5 [50]. Alternatively, cleavage of capsid proteins could lead directly to DNA uncoating or might be required to prime the capsids for ubiquitination. However, ubiquitination of uncleaved AAV2 capsids has been reported [51]. Furthermore, both ubiquitination- and proteasome-mediated degradation of AAV vectors can be modulated by epidermal growth factor receptor protein tyrosine kinase activity, and inhibition of that activity can block degradation of AAV and facilitate nuclear transport and transduction of AAV vectors [51].

From the site of endosomal release, the capsids must be transported to the nucleus. If transported to a perinuclear position within endosomes, an active mechanism for further transport of the free capsids might not be essential. Nonetheless, microtubules seem to be used by incoming CPV capsids for targeting to this location, and treating cells with nocodazole, which interferes with the polymerization of microtubules, leads to a redistribution of microinjected capsids so that they are scattered throughout the cytoplasm [52]. Genome release from the capsid occurs slowly for autonomous parvoviruses because microinjecting antibodies against the capsid into cells blocks CPV infection even when administered several hours after inoculation [52]. For AAV, the situation is not well resolved. AAV2, AAV5 and AAVrh10 (AAVrhesus10) interact with microtubule-associated proteins [53]. Different studies show various effects of nocodazole or other microtubule-directed drug treatments on virus infection or transduction. In some cases, they prevent directed motion of viral particles in the cytoplasm and nucleus, and, in others, they have little effect on transduction [14,22,54]. Taxol treatment, which stabilizes microtubules, gives a mild enhancement of transduction by AAV2 [54].

Nuclear entry and uncoating

Autonomous parvoviruses enter the nucleus and require passage of the cell through S phase for DNA replication; in the case of AAV, the helper virus also supplies the replication functions. Theoretically, the 26-nm diameter capsids of the parvoviruses should be able to pass through the nuclear pore complex (NPC) intact, and this is seen for newly synthesized capsids during viral egress [55]. However, some evidence indicates that the viruses might not enter the nucleus through the NPC, despite the NLS-targeting sequences in the N terminus of VP1 that are exposed during entry. Disruption of the outer nuclear envelope has been observed when MVM capsids are microinjected into the cytoplasm of *Xenopus* oocytes, and blocking the nuclear pore by adding wheat germ agglutinin does not inhibit nuclear entry by the injected capsids, indicating a pore-independent entry mechanism [56]. When the parvovirus MVM is added to mammalian cells, nuclear-envelope breakdown and changes in the distribution of nuclear Lamin A/C results [57]. A role for the NLS in these viruses might, therefore, be to target the capsid to the nuclear membrane and/or dock it at the nuclear pore, instead of directing transport through the pore. However, further studies are required to define the mechanisms involved in productive infection.

The uncoating mechanism of parvoviruses is also not well understood but it is clear that the particles are quite robust and complete disassembly might not be required for genome release. Instead, the viral DNA might be extruded or extracted from relatively intact capsids. Using a fluorescently labeled probe for the 5′ end of the MVM genome, partial exposure of DNA in the cytoplasm has been detected; a probe for the 3′ end (which would prime new DNA synthesis) showed that it also became exposed outside the capsid [25]. The capsid form that enters the nucleus is still unresolved, and might vary between viruses. Although MVM-capsid proteins have not been detected in the nucleus, this might simply indicate a low efficiency of transfer or that the capsids dock and release the genome at the NPC [25]. Conversely, microinjected CPV capsids seem to enter the nucleus intact, but after a delay of three to six hours [52]. For AAV, there is conflicting evidence as to whether uncoating occurs before or after nuclear entry. Fluorescently labeled AAV2 capsids have been detected within the nucleus within 2 h [13], and some particles were seen within membrane invaginations of the nuclear envelope 15 min after uptake when using single-particle-tracking technology [22]. Although two studies have indicated that adenovirus capsids have an enhancing effect on the infection of AAV and on the conversion of the AAV genome from a single-stranded DNA form to a double-stranded DNA form [58], specific roles for adenoviruses in AAV uncoating and nuclear transport are still being defined. Some studies using fluorescently labeled particles and sub-cellular fractionation followed by DNA hybridization have indicated that adenovirus

can facilitate AAV translocation into the nucleus [58]. Furthermore, microinjection of anti-capsid antibodies into the nucleus blocks infection, which indicates that the AAV genome is associated with the capsid after it enters the nucleus [50]. Other studies using green fluorescent protein-labeled particles indicate that, although adenovirus capsids increase the number of particles in the nucleus, the majority (>90%) of the capsids remain detectable outside the nucleus [59]. Furthermore, viral genomes have been detected by fluorescence *in situ* hybridization within the nucleus of cells within 2 h post-transduction, irrespective of Ad5 co-infection [59]. Whereas no co-localization of viral genomes and intact viral capsids has been observed within the nucleus, co-localization is detectable in the perinuclear area and within the cytoplasm [59]. These findings argue in favor of viral uncoating before or during nuclear entry.

AAV capsids enter cells efficiently in the absence of helper virus and establish a latent infection. Helper viruses enhance active infection and nuclear transfer if present in sufficient amounts to alter the trafficking processes [58]. For most of these viruses, the proportion of capsids that enter the nucleus is probably small, and the majority seems to persist in a perinuclear location for many hours, most likely in non-degradative endosomal compartments.

Concluding remarks

The studies described here are beginning to elucidate the complexity of cellular entry by parvoviruses. The differences in these processes between viruses indicate that we cannot use a single approach to generalize about even a single virus, much less the family as a whole. For example, the different receptors used by parvoviruses determine the uptake and trafficking of particles to influence the intracellular destinations of particles in the endosomal system or the cytoplasm. The rapid uptake from the surface, but delayed progression to infection, indicates that parvovirus capsids require prolonged processing or undergo slow conformational changes within endosomes. The requirement that the viruses wait for co-infection by a helper virus (for AAV) or for cellular S phase (autonomous viruses) might make slow or step-wise entry beneficial for the viruses. The nature of the interplay between the entry processes and the sites of virus–host-cell interactions is an ongoing area of investigation. Further work is necessary in several areas, including identifying the infectious endosomal pathway(s) versus any dead-end compartments, the mechanisms of endosomal penetration used by the virus and the specific role(s) of the PLA2, how and where the genome is released, and the pathways and specific capsid structures that enter the nucleus. The subtle capsid rearrangements that occur during infection, which are also preludes to DNA release, require experimental methods that can discriminate fine and probably reversible changes. It is also becoming clear that, due to differences in the infection processes, results from different parvoviruses cannot be combined and that comparisons are only possible where similar methods are used for the analysis.

The high particle-to-infectivity ratio of the parvoviruses raises concerns about how well studies of particle entry reflect the infection routes but, in most cases, it seems that the majority of particles enter pathways involved in infection. Different cell lines show different entry and infection pathways and, for many viruses, the cells that are conventionally used for doing cell biology and imaging studies (often fibroblast-like and transformed) are not the same as those infected in the animals under natural conditions (non-transformed endothelial, epithelial and lymphoid cells).

Additional technology, including live-cell microscopy for following viruses in real time, and total internal reflection fluorescence microscopy for defining viral movement on the surface of the cell, will also reveal new details. Many markers are available for different cell components, which can be used to specifically localize virions to different compartments. New methods for altering the cells, specific receptors or viruses can all be used to directly examine the infection pathways, including siRNA to knockdown expression of single genes, microinjection and dominant-negative effectors. These techniques will all enhance our ability to understand the dynamic aspects of viral-entry processes.

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