MINIREVIEW

Pathways of Cell Infection by Parvoviruses and Adeno-Associated Viruses

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Animal viruses have developed various strategies for infecting cells, and all begin with adsorption to cell surface receptors, penetration into the cytosol, uncoating or release of the viral genome, and targeting the genome and any required accessory proteins toward the correct cellular organelle or compartment for replication (26, 48, 63). Since genome delivery and release require the rearrangement of the viral structures, infection is normally a multistep process involving various viral and cellular components. Viruses that replicate in the nucleus must have mechanisms for transporting the genome and other components to the vicinity of the nuclear pore and into the nucleus (84). The endosomal pathways of the cell are used by many viruses; vesicles are transported by microtubule-dependent motors through the cytoplasm. Nucleus-replicating viruses may require cytoskeleton-driven transport of the capsid or of viral components mediated by either microtubules or actin or, in some cases, by both structures (39, 66). Nuclear transport of viral components involves signal-mediated interactions with the nuclear import machinery (84). While enveloped viruses enter the cell by glycoprotein-mediated fusion of the viral envelope with a cellular membrane, much less is known about the entry of most nonenveloped viruses, but capsid-dependent mechanisms for penetrating the cell membrane or lysing endosomes are likely to be involved.

PARVOVIRUSES

The parvoviruses are among the smallest animal DNA viruses. The family *Parvoviridae* contains two subfamilies: the *Parvovirinae*, which infect vertebrates and include the three genera *Parvovirus*, *Erythrovirus*, and *Dependovirus*, and the *Densovirinae*, which infect invertebrates (22, 41). The dependoviruses depend on a helper adenovirus or herpesvirus to supply functions needed for their replication. When added to cells alone, they still infect the host cell efficiently, but the genome undergoes an incomplete replication and may integrate into the host genome to establish latency (15, 56). Human adeno-associated viruses (AAVs) include several different serotypes, and the prototype strain (AAV2) infects many types

of cells and tissues of various species, including humans, dogs, and mice (40, 41, 60). The autonomous parvoviruses, *Parvovirus* and *Erythrovirus*, do not need a helper virus but require cellular S-phase functions for their DNA replication. The autonomous parvoviruses infect only a restricted range of host and tissues, and densoviruses infect invertebrates (10, 19).

PARVOVIRUSES AND DISEASE

The human parvovirus B19 virus causes aplastic anemia in patients with sickle cell disease, childhood fifth disease (erythema infectiosum), arthropathy in adults, and rare fetal infections (11). Canine parvovirus (CPV) and feline panleukopenia virus (FPV) are closely related parvoviruses. CPV appeared in 1978 as a new virus infecting dogs and is the result of a mutation in a strain of FPV that allowed the virus to expand its host range to canines (46). The minute virus of mice (MVM) and other rodent parvoviruses cause a variety of diseases in neonatal and older animals, and MVM shows various tissue tropisms (12, 46). The AAVs are being developed as vectors for gene therapy, with alterations in the tissue tropism of the viruses being introduced to alter the target tissues for alternative therapies.

The 26-nm-diameter parvovirus icosahedral capsid packages a single-stranded DNA genome of around 5,000 bases (74, 87). The capsid is assembled from 60 copies of the structural proteins, which come in two or more different overlapping forms (17). VP1 and VP2 of MVM and CPV are translated from alternatively spliced messages, while VP3 is formed in full capsids by the cleavage of a peptide from the N terminus of VP2 exposed outside the capsid (72, 76, 82). VP1 contains the complete sequence of VP2 and a unique 143-residue N-terminal sequence necessary for viral infectivity but not for capsid formation (76, 78). The VP1-unique regions contain a phospholipase A2 (PLA2) motif which appears active when released from the capsid (91). AAV capsids are formed from VP1, VP2, and VP3, which are formed by alternative splicing and start codons (9). The atomic structures of the capsid surfaces of the mammalian viruses CPV, FPV, MVM, and AAV have a variety of features, and the host ranges of CPV, FPV, and MVM are controlled by sequences on the capsid surfaces (1–3, 14, 30, 75, 86). An insect parvovirus has a relatively smooth surface compared to that of the vertebrate parvoviruses (65).

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TABLE 1. Parvoviral receptors

Virus	Receptor (reference)	Coreceptor (reference)
AAV2	Heparan sulfate proteoglycan (71)	αVβ5 integrin (70)
		Human fibroblast growth factor receptor (51)
AAV4	α2-3-linked sialic acid (80)	
AAV5	α2-3-linked sialic acid (34)	
	α2-6-linked sialic acid (34)	
	Platelet-derived growth factor (47)	
B19V	Erythrocyte P antigen[a] (12)	
ADV	ADV binding protein	
CPV	Transferrin receptor (46)	
	Sialic acid (5)	
FPV	Transferrin receptor (46)	

[a] Globoside.

CELL RECEPTORS

Both carbohydrate and protein receptors have been reported for many viruses (Table 1). AAV2 and AAV3 use heparan sulfate proteoglycan (HSPG) for low-affinity attachment to the cell surface and for infection of host cells (53, 71), although AAV2 mutants that do not bind HSPG are still infectious (43, 61). Other receptors associated with cell infection by AAV2 include αVβ5 integrin and basic fibroblast growth factor receptor 1 (51, 70), although Qiu and Brown reported that αVβ5 integrin is not involved (52). AAV4 and AAV5 bind sialic acids during the infection of cells, although the type of sialic acid bound differs: AAV4 binds to α2-3-linked sialic acids, and AAV5 binds to both α2-3- and α2-6-linked sialic acids (32, 80). AAV5 can also use platelet-derived growth factor receptor for cell binding and infection (47). AAVs appear to be quite promiscuous in their use of receptors. A variety of different cross-linking ligands were able to mediate the virus infection, and AAVs could be adapted to a variety of alternative receptors with mutations or insertions within the capsid protein (6, 23, 54, 62, 85, 89).

The human parvovirus B19 virus replicates only in human erythroid progenitor cells, and cell binding and infection require the erythrocyte P antigen (globoside) (10). However, a number of cells which express globoside on their surfaces are not susceptible to infection, which may be due partly to an intracellular blocking of the transcription of viral messages in nonerythroid cells (13, 35). It is also likely that there is another protein-based receptor for the parvovirus B19 virus (83).

Both CPV and FPV bind the sialic acid on some host cells, and sialic acid binding appears to be specific for N-glycolyl neuraminic acid (NeuGC), which is found on the erythrocytes and cells of most cats, monkeys, and horses but not on the cells of most dogs. NeuGC binding is temperature and pH dependent and is controlled by residues adjacent to a depressed region (the dimple) on the virus capsid (5, 73). Mutations within a loop between VP2 residues at amino acid positions 359 to 377 or of the VP2 residue at amino acid position 323 make sialic acid binding dependent on low pH (a pH of <6.5) or stop sialic acid binding (5, 64, 73). However, sialic acid binding does not mediate infection of tissue-cultured cells, as the viruses can infect cells displaying only N-acetylneuraminic

acid and non-sialic acid binding mutants infect the same range of cells as wild-type viruses. FPV capsids bind sialic acids on feline cells most efficiently under the nonphysiological conditions of pH below 6.5 and a temperature of 4°C. CPV can bind the sialic acid on feline cells at pH values of >7.0, at least partially, and that inhibits infection, as mutants which do not bind the sialic acid are selected by passage of CPV in cat cells (5). The transferrin receptor (TfR) is used by CPV and FPV for infecting cells, and differences between the feline and canine TfR specifically control the binding of viruses to cells (31, 45). The specific contact between the CPV or FPV capsids and the TfR appears to be important for successful cell infection, as some changes in the virus or the receptor may still allow capsid binding to occur but do not mediate infection (30). The host ranges of CPV and FPV were found to be controlled by their cell binding abilities. Both CPV and FPV capsids bound and entered feline cells, but only CPV bound to the canine cells. This specificity appears to be due to TfR binding by the capsids, as the feline TfR expressed on Chinese hamster ovary-derived TRVb cells allowed efficient CPV and FPV binding and infection, while the canine TfR bound CPV but not FPV capsids and allowed infection by CPV (31).

ENDOCYTOSIS, CYTOPLASMIC TRAFFICKING, AND NUCLEAR ENTRY

All parvoviruses require receptor-mediated endocytosis for cell infection. CPV, MVM, and AAV are taken up rapidly into cells, most likely by clathrin-mediated endocytosis, as CPV and AAV2 endocytosis was inhibited by overexpression of the Lys44Ala (K44A) dominant interfering mutant of dynamin-2 (7, 21, 34, 44). Both CPV and AAV infections can be inhibited by treatment of cells with lysosomotropic agents, including NH₄Cl and bafilomycin A1 (7, 8, 27, 44), indicating that low endosomal pH is required for infection or for endosomal trafficking. Shortly after uptake, CPV capsids colocalize with transferrin in perinuclear endosomes (Fig. 1) (44, 69).

AUTONOMOUS PARVOVIRUSES

After endocytosis, penetration of CPV capsids into the cytosol is a slow process. Antibodies against the TfR cytoplasmic tail reduced virus infection when injected into cells 4 h after inoculation, showing that many infecting capsids remain associated with the TfR in endocytic compartments for several hours after uptake (44). Antibodies to the CPV capsid injected into the cytoplasm also prevented virus infection when injected 4 or more hours after inoculation of the virus, indicating that the capsids pass slowly out of the endosomal vesicle (78, 79). After uptake, capsids were detected in perinuclear compartments for several hours (44, 69). After fluorescence in situ hybridization (FISH), CPV DNA colocalized with the capsids in perinuclear compartments for at least 8 h (69). MVM capsids also require uptake through the endosomal system, where infection could be blocked by bafilomycin A₁ or chloroquin for several hours after uptake from the cell surface (55). The mechanism of escape from endocytic vesicles into the cytosol is still unknown, although there does not appear to be a wholesale lysis of the endosomal vesicle, as there is little transport of alpha-sarcin into the cytoplasm (44, 68). A PLA2 homologous
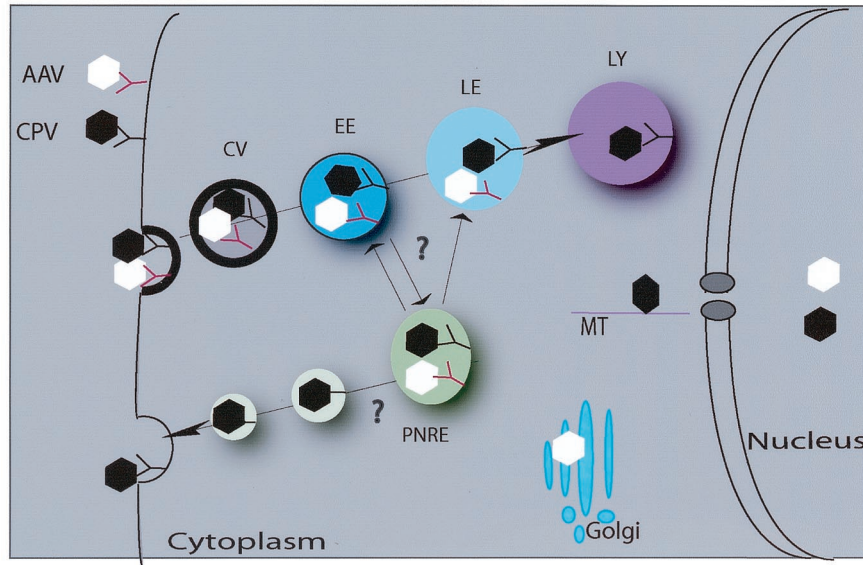
FIG. 1. A schematic representation of the entry mechanisms utilized by parvoviruses within the host cell. Cell entry of autonomous parvovirus CPV and AAV is shown. After binding to their cell surface receptors, both viruses are internalized into clathrin-coated vesicles (CV), followed by transport to early (EE), late (LE), or perinuclear recycling endosomes (PNRE). Later in entry, in the case of AAV, capsids are found in Golgi compartments, whereas CPV can be found in lysosomes (LY). The site of the capsid escape from endocytic vesicles into the cytosol is still unclear. CPVs make use of microtubules (MT) during the traffic through the cytosol toward the nucleus. Viral capsids are able to enter the nucleus in intact form without apparent deformation.

sequence is present in the VP1-unique region of most parvoviruses and has been shown by mutation of its predicted active site and by inhibitor studies to be active (Fig. 2) (19, 68, 91).

The capsids are most likely released into the cytoplasm from a vesicle in a perinuclear location, and there is likely further processing in the cytoplasm and transport to the nucleus. MVM capsids are affected by the activity of the proteasome,

since infection can be reduced by some proteasome inhibitors, including inhibitors of chymotrypsin-like activity (*N*-tosyl-L-phenylalanine chloromethyl ketone and aclarubin), but not by inhibitors of trypsin-like activity (55). It is not clear what mechanisms are involved in that activity, but a direct proteolytic activity or a role in controlling the ubiquitination of the capsid may be involved. Active transport mechanisms are also likely
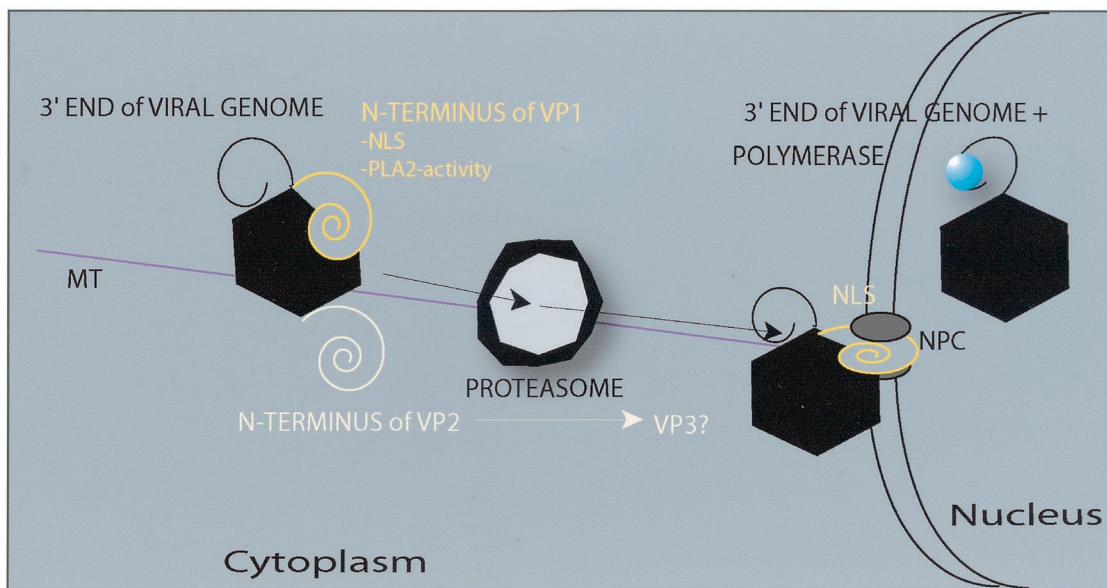

FIG. 2. Intracellular trafficking and modifications of autonomous parvovirus capsids. Once inside the cell, capsids undergo modifications, exposing on their surfaces not only the N-terminal end of the VP1 capsid protein but also the N-terminal sequence of the VP2 capsid protein and 3′ end of the viral genome. In the cytosol, capsids are also affected by the activity of proteasomes, possibly causing the intracellular formation of the VP3 capsid protein. Later in entry, viral capsids are imported into the nucleus through the NPC with the help of the NLS. MT, microtubules.

to be required for the particles or the viral DNA to reach the nuclear pore. The cytoplasm contains a lattice-like mesh of microtubules, actin microfilaments, and intermediate filaments which restrict the diffusion of macromolecular complexes (38, 59). CPV capsids injected into the cytoplasm rapidly became localized in a perinuclear location, and the capsids mostly entered the nucleus only 3 to 6 h later (79). Transport to the perinuclear region and nucleus were prevented by nocodazole treatment to depolymerize microtubules and by an antibody against the intermediate chain of the microtubule-based, minus-end-directed motor protein dynein (67, 69, 79). By electron microscopy, capsids were associated with tubulin and with apparent dynein structures in vitro. Viral capsids could be precipitated from infected cells along with intermediate chains of dynein. These results suggest that microtubules and dynein move CPV capsids within the cytoplasm, facilitating their transport to the nucleus and infection of cells (67).

Transport of the capsid and/or viral DNA into the nucleus is an important step in infection of cells with an intact nucleus and occurs principally through the nuclear pore complex (NPC). Although small macromolecules can diffuse freely through the pore, transport of larger molecules is specific, requiring ATP and soluble cytosolic factors, including Ran-1, and is mediated by nuclear localization sequences (NLSs) (25, 42). Parvovirus capsids appear to be able to pass through the NPC intact, and over a period of 2 or more h, microinjected CPV capsids enter the nucleus, where they are recognized by antibodies that bind only intact capsids (79). Modification of viral capsids may allow exposure of NLSs needed for nuclear targeting and transport of viral particles (Fig. 2). Only two basic sequences that might be classical NLSs are present in the VP2 capsid protein sequence of CPV, while the VP1-unique region contains several such sequences. A VP1 sequence (PAKRARRGYK) between residues at amino acid positions 4 to 13 functions for nuclear transport when conjugated to bovine serum albumin (77). That N-terminal unique sequence was detected on capsids between 1 and 4 h after injection of the capsids into cells, and it was also exposed on incoming infectious virions, since antibodies specific for the VP1-unique region blocked infection when injected into cells before inoculation of the virus (78). In addition, some specific changes in the VP1 N-terminal basic sequence reduced the relative infectivity of the capsids (78). MVM capsids have NLSs in both VP1 and VP2, with two NLSs mapped near the VP1-specific N terminus, while VP2 appears to make use of an internal basic sequence (KGKLTMRAKLR) in a conformation-dependent manner (36, 37). An intact VP1-unique region that could mediate efficient nuclear transport was also required for efficient infection of cells by MVM capsids (Fig. 2) (37).

AAV CELL ENTRY AND INFECTION OR TRANSDUCTION PATHWAYS

The cell entry and infection pathways of AAV capsids appear to differ in several ways from those used by the autonomous parvoviruses (Fig. 1). AAV capsids enter cells through endosomal uptake, but a variety of different results have been reported. In a study which followed individual, labeled capsids into cells, the capsids appeared to follow different routes: some entered the cytoplasm quickly after endosomal uptake, while others remained within the endosomal system for longer periods (58). The capsids then appeared to traffic within the cell by three different pathways: microtubule-dependent transport within endosomes, free diffusion within the cytoplasm, and rapid transport in the cytoplasm in association with microtubular motors (58). In another study of AAV2 entry, capsids appeared to escape from early endosomes into the cytosol and then to become localized within the nucleus within 2 h (7). AAV2 capsids have also been reported to traffic through both early and late endosomes prior to nuclear translocation. The susceptibility of AAV2 infection to bafilomycin A$_1$ treatment differed between human 293 cells and mouse 3T3 cells (20, 27). AAV capsids have also been seen to colocalize with transferrin within perinuclear vesicles for long periods (21). Cell binding by AAV2 led to activation of Rac1, most likely through binding and clustering of αVβ5 integrins. Infection was reduced by a dominant negative inhibitor of Rac1 and by inhibitors of phosphatidylinositol (PI) 3-kinase, indicating that signaling through a Rac1 and PI 3-kinase activation cascade might be involved (57). AAV5 capsids accumulate in the Golgi compartment after uptake (4). Within the cytoplasm, the AAV2 and AAV5 capsids are ubiquitinated and may be degraded by the proteasome; proteasome inhibitors increased the transduction efficiency of virions (20, 88).

The processes of nuclear transport and second-strand synthesis during AAV infection appear to vary among different studies; this variation may reflect differences in both cells and methods. The VP2 N terminus is important in the nuclear translocation of AAV2 proteins when they are expressed as virus-like particles, although whether the N-terminal sequences are also involved in controlling infection is not clear (29) A high proportion of the viral DNA can be recovered from a nuclear fraction as double-stranded forms. The VP1 of AAV2 contains a PLA2 activity which is required for cell infection, and when that sequence was mutated, the viruses showed a delayed infectivity (24). The mechanism of AAV trafficking to the nucleus is not known, although, as with the autonomous parvoviruses, viral particles may be transported into the nucleus through the nuclear pore (57). Moreover, uptake of AAV2 capsids into the purified nuclei was examined and reported not to require transport through the nuclear pore; however, the mechanism of that alternative pathway was not defined (28).

VIRAL DNA RELEASE FROM THE CAPSID AND INITIATION OF REPLICATION

Full capsids of MVM, CPV, and AAV2 have 20 to 30 nucleotides of the 5′ end of the viral genome exposed on the outside of the capsid, and the NS1 protein is covalently attached to the 5′ end (18, 49, 81). That DNA is thought to pass through a pore at the 5-fold axis of the capsid (86, 87). The 3′ end of the viral DNA can also be exposed outside the capsid after treatments which do not cause capsid disintegration, and the 3′ terminal hairpin can act as a template for the DNA polymerase in vitro (16, 78). This finding suggests that there is a mechanism whereby this DNA exposed within the nucleus can be used as a template for initiating DNA replication by the host cell DNA polymerase, whereby the DNA can be removed without disassembly of the stable capsid.

AAV particles can efficiently transduce nondividing cells, and in the absence of a helper herpesvirus or adenovirus their genomic DNA becomes integrated into the cellular DNA (33, 90). The release of AAV DNA has not been well characterized, but in some cells the A region adjacent to the terminal sequence (the D region) may be bound by a nonphosphorylated form of a cellular protein, SKFBP, which blocks infection (50).

CONCLUSIONS

The process of cell infection by parvoviruses shows many of the features seen for other viruses which replicate in the nucleus, but the small and stable parvovirus particles must undergo more subtle changes during the various steps involved. The relatively high particle-to-infectivity ratio suggests that the virus is inefficient at infection, but this might also be a reflection of the numerous steps that the virus has to accomplish during the trafficking from the cell surface to the nucleus. Our ability to detect small changes in the virus and to follow small numbers of particles into the cell should allow us to define more specifically the principles involved in intracellular trafficking events.

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