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Clathrin-Associated Endocytosis as a Route of Entry into Cells for Parvoviruses

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1. Introduction

For successful infection, viruses must solve several problems and overcome barriers that would impede or prevent infection. Some of these barriers include finding a suitable host, circumventing the immune system, interacting with cells within their host range, attaching to the cell surface, penetrating to the interior of the cell and finding ways for transcription, synthesis of their gene products, genome replication, assembly of the particle components, and escape from the infected cell. As a whole, viruses are paradoxically, fascinatingly simple yet complex. Some viruses, such as those that are enveloped, enter the target cell by attacking the cell membrane with a fusion protein, or a fusion domain of an attachment protein, that invades the cell membrane and promotes fusion of the viral envelope with the cell membrane. This fusion event may occur either at the plasma membrane level or at a membrane site in the endocytic vesicle. The viral genome then is free of its envelope and can enter the cell interior. Nonenveloped viruses generally do not enter by fusion at the plasma membrane level but are commonly taken into the cell by endocytosis, then, by some mechanism, are able to invade the cell cytoplasm by escaping from confinement in the endosome. The pathways for virus entry along with the entry subtleties are nearly as diverse as the virus families themselves. Many of the parvoviruses are known to employ the clathrin-associated acid mediated endocytotic pathway for penetration into their host cell and this chapter deals with parvovirus entry.

The virus family *Parvoviridae* is so named because of the small size of the virus particles. As a group, they are approximately 22-26 nm in diameter, have T=1 capsid structure, and contain a single stranded DNA genome. Six genera have been named. They infect a variety of mammalian, avian, non-mammalian vertebrate, and invertebrate hosts (Table 1). Some of these viruses will be described in the discussion of endocytic entry pathways that follow. Parvovirus host range and entry was reviewed by Cotmore and Tattersall (2007)

emphasizing viruses in the genus *Parvovirus*. For purposes of illustration of parvovirus entry, bovine parvovirus 1 (BPV-1) will be emphasized in the current review. In subsequent descriptions this virus is referred to simply as BPV. BPV is a member of the genus *Bocavirus*. Other Bocaviruses are canine minute virus (CnMV) and human bocavirus (HBoV). BPV-1 is the prototype virus of this genus. It is a small (22 nm), non-enveloped virus with icosahedral structure. BPV is a pathogen of cattle found worldwide that causes severe gastroenteritis in calves, mild respiratory infection, and may cause reproductive failure (Manteufel & Truyen, 2008; Sandals et al., 1995). It is a contamination risk for commercially prepared bovine serum and bovine-derived products. The genome consists of single stranded DNA that is composed of about 5515 nucleotides (Chen et al., 1986; Qiu et al., 2007; Sun, et al., 2009). The genome contains non-identical palindromic sequences at the two ends. The palindromic sequences have signals that are important for genome replication and packaging (Berns, 1990; Shull et al., 1988). The genome has three open reading frames (ORFs): the left ORF encodes the nonstructural proteins NS1 and NS2; the central ORF encodes the nonstructural protein NP-1; and the right ORF encodes two or three structural proteins VP1, VP2, and VP3 (Johnson & Hoggan, 1973; Qiu et al., 2007). According to parvovirus structural protein nomenclature, the viruses with two proteins (VP1 and VP2) designate the largest protein VP1. The two proteins are co-terminal at the carboxyterminal ends but a unique sequence (called VP1u or the VP1 unique sequence) is present on the larger protein. Similar nomenclature applies to AAV except there are three structural proteins and the unique ends are the VP1/VP2u sequences. Parvoviruses do not encode polymerases. These enzymes are provided by the host cell. BPV genome replication relies on host cell DNA polymerase and replication factors found in S-phase cells (Berns, 1990). Moreover, transcription is carried out by cell RNA polymerase II which requires a double-stranded transcription template. Thus, genome replication is a necessary precursor to transcription. The genomic organization of HBoV, the second human-pathogenic parvovirus known (discovered after Parvovirus B19), closely resembles the other known bocaviruses BPV and CnMV (Allander et al., 2005; Kaplan et al., 2006; Ma et al., 2006; Sun et al., 2009). The mid-ORF product of HBoV is homologous to NP-1 of BPV and CnMV, and these proteins have 47% amino acid identity. The proteins of the two major ORFs (NS and VP) of HBoV have 42-43% homology with NS1, VP1, and VP2 proteins of BPV and CnMV (Allander et al., 2005; Bi et al., 2007).

Most parvoviruses, as understood to date, utilize several different subtle variations of a general strategy to deliver their genome to the cell nucleus, the site of virus replication. These are late penetrating viruses, in the sense that they are pH-dependent and persist longer through the endocytic transport system than earlier penetrating viruses (Greber, 2011). The rugged, mature, extracellular virion undergoes multistep conformational changes that are time- and cell compartment-dependent. In general, several discrete steps have been recognized that entering parvoviruses follow. These are interaction with a cell surface receptor, trafficking through the endosomal pathway to the late endosome or lysosome, or through macropinocytosis for porcine parvovirus (Boisvert et al., 2010), escape from the endosomal pathway using the newly deployed phospholipase 2 (PLA_2) domain of the capsid protein, and cytoskeletal-controlled transport of the modified particle to the nucleus.

Techniques employed in the laboratory for the elucidation of viral entry pathways involve both direct observational detection of virus in cells and indirect detection of virus movement by blocking specific functions in the process with chemical inhibitors. Electron microscopy is used as a method for showing virus associated with cell structures giving "snap shot" views of virus over time intervals in the entry process. Parvoviruses, for example, can be seen associated with clathrin coated pits and in endosomes (Dudleenamjil et al., 2010; Parker & Parrish, 2000; Vendeville et al., 2009). Chemical inhibitors are assumed to block the entry pathway at certain specific points and incomplete virus replication in the presence of inhibitor is presumed to indicate viral penetration using the pathway blocked by the drug. Commonly used drugs are chlorpromazine (prevents assembly and disassembly of clathrin lattices at the cell surface and on endosomes by inhibiting clathrin polymerization which blocks assembly of coated pits and is also a phospholipase A_2 inhibitor, Blanchard et al., 2006); bafilamycin A1 (inhibits vacuolar proton ATPases inhibiting acidification of endosomes by blocking transport of protons into the vesicle, Clague et al., 1994; Drose & Altendorf, 1997; Wassmer et al., 2005); ammonium chloride (penetrates into the endosome increasing endosomal pH, Jin et al., 2002; Liebl et al., 2006); chloroquine (blocks assembly of clathrin coated pits and raises endosomal pH, Mani et al., 2006; Ros et al., 2002). Brefeldin A has no effect on the early endosome but inhibits vesicle transport and early-to-late endosome transition (Clague et al., 1994; Nebenfuhr et al., 2002).

Some drugs block virus entry through caveolae and can be used to help distinguish virus entry through caveolae or clathrin-associated endosomes. The antifungal drug nystatin complexes with and sequesters cholesterol inhibiting lipid rafts and caveolae (Chazal & Gerlier, 2003; Damm et al., 2005; Sieczkarski & Whittaker, 2002). Phorbol-12-myristate-13-acetate, a mitogen and a tumor promoter, decreases membrane caveolin-1 (Smart et al., 1994). Methyl-β-cyclodextrin disrupts detergent-resistant lipid rafts (Beer et al., 2005). Genistein blocks phosphorylation of tyrosine kinase which is involved in the formation of caveosomes (Pelkmans, 2005a). Other drugs block transport of virions (or modified virions) through the cell cytoplasm. Some viruses depend on microtubules for transport and some associate with actin filaments. Nocodazole blocks microtubule polymerization (Brandenburg & Zhuang, 2007; Pelkmans & Helenius, 2003), vanadate is a dynein inhibitor (Beckerle & Porter, 1982), erythro-9-3-(2hydroxynonyl)adenine also is a dynein inhibitor (Krietensson et al., 1986). Cytochalasin D disrupts actin microfilaments and blocks actin polymerization (Vendeville et al., 2009), and latrunculin A also inhibits actin polymerization (Damm et al., 2005; Forest et al., 2005).

There are some important concerns about the interpretation of the results obtained in studies that employ inhibitors. Concentrations of the drugs must be below the cell toxicity levels to preserve virus-dependent cell activities. In most cases complete shut-off of virus replication does not occur. So, the possibilities of pathway leakiness or the presence of more than one entrance pathway must be considered. None of the drugs possesses absolute specificity, therefore possible side effects may occur that affect interpretation. In studies like these, the use of several chemical inhibitors having different modes of action help to somewhat mitigate the problem of cloudy interpretation if the results are mutually supportive.

Genus	Species	Pathogenesis[1]
Parvovirus	*Canine parvovirus*	Gastroenteritis, cardiovascular failure
	Chicken parvovirus	Enteric disease, cerebellar hypoplasia
	Feline panleukopenia virus	Gastroenteritis, leukopenia, cerebellar hypoplasia
	Goose parvovirus	Gastroenteritis, anorexia, nasal discharge, death
	Hamster parvovirus	Osteolytic infection, facial and tooth malformation
	H-1 parvovirus	A hamster osteolytic virus
	Human PARV 4	Viremia, ?other disease
	Kilham rat virus	Mostly asymptomatic, small litters, runting
	Lapine parvovirus	Enteritis
	LUIII virus	Systemic infection in hamsters
	Mink enteritis virus	Gastroenteritis, leukopenia
	Mouse minute virus	Asymptomatic, MMVi is immunosuppressive
	Murine parvovius 1	No known disease
	Porcine parvovirus	Reproductive failure, embryonic death
	Raccoon parvovirus	Enteritis
	Rat minute virus 1a, 1b, 1c	Asymptomatic
	Rat parvovirus	(Also known as Kilham rat virus)
Erythrovirus	*Human parvovirus B19*	Erythema infectiosum (Fifth disease), exanthema, hydrops fetalis
Bocavirus	*Bovine parvovirus 1*	Gastroenteritis (calf scours), dyspnea, stillborn calves
	Bovine parvovirus 2	Same as BPV-1
	Bovine parvovirus 3	Same as BPV-1
	Canine minute virus	Diarrhea, anorexia, dyspnea
	Human bocavirus	Upper respiratory, coryza
Amdovirus	*Aleutian mink disease virus*	Immune complex disease, glomerulonephritis, death
Dependovirus	*Adeno-associated virus (types 1-11)*	None known, latency in chromosome 19
Densovirus	*Brevidensovirus*	Infects mosquito larvae
	Densovirus	GmDNV kills moth larvae
	Iteravirus	Infects silkworm larvae
	Pefudensovirus	Host is the cockroach

Table 1. Members of family *Parvoviridae*. Other presumptive parvovirus isolates are not listed. [1]In some instances virus infection in adult animals is largely asymptomatic but in neonates some pattern of symptomatic infection has been recognized.

Immunofluresceinated virus particles have been used to show association of virus with cell receptors and clathrin-rich early endosomes (Freistadt & Eberle, 2006). Purified virus particles are directly labeled with the fluorescein fluor by treatment with N-hydroxysuccinimide-fluorescein. Attachment and uptake of virus by cells is tracked by flow cytometry, fluorescence microscopy, or confocal microscopy. Most viruses, including the very small parvoviruses, cannot be seen as individual particles by light microscopy, but clumps of viruses can be seen. Also, accumulated virus particles present in cell organelles such as late endosomes-lysosomes can be seen. Among the Rab proteins in cells, Rab5 is primarily associated with early endosomes, Rab7 with late endosomes, and Rab 11 with recycling endosomes. Co-localization of virus with specific Rab proteins provides evidence of virus trafficking through certain endosomal compartments.

A recent report provided cryoelectron microscopic images of the HBoV particle (Gurda et al., 2010). Other reports show the small, T=1 capsid of parvoviruses displays amino acid side chains to the inner capsid surface which bond to the bases of the single stranded genome (Agbandje-McKenna & Chapman, 2006; Chapman & Agbandje-McKenna, 2006). Such interactions occur as the genome is packaged into the preformed shell. Several features including the small size, compact capsid, interior molecular interactions, and lack of an envelope combine to make this virus remarkably environmentally stable. These viruses undergo several essential protein conformation shifts during the trafficking process but are able to remain essentially intact after attachment, engulfment, endosomal transit, travel through the cytoplasmic environment, association with the nuclear membrane, and travel across the nuclear membrane. Although there are some capsid alterations during this process (Cotmore & Tattersall, 2007), the viral genome remains capsid-associated as it enters the nuclear compartment.

2. Attachment of virus to the cell

The term *receptor* to indicate the cell surface structure to which the virus attaches and the term *antireceptor* to indicate the virus-associated component that binds to the cell receptor are used to describe the virus-cell interaction that leads to sticking of virus to cell. Several studies have been reported that show the orientations of the capsid proteins and the capsid surface topography (Agbandje-McKenna & Chapman, 2006; Agbandje et al., 1998; Chapman & Agbandje-McKenna, 2006; Chapman & Rossmann, 1993; Tsao et al., 1991) and show the major structural protein forming ridges and valleys on the faces and edges with a pore at each vertex. In the parvoviruses studied for surface structure, there is a surface "spike" positioned on the icosahedral face or axis of three-fold rotational symmetry. The antireceptor is located with this icosahedral face spike (Cotmore & Tattersall, 2007).

Parvoviruses are known to utilize a variety of cell surface molecules as their receptors including glycoproteins, glycolipids, and glycans (Cotmore & Tattersall, 2007; Harbison et al., 2008). Members of the genus *Parvovirus* in the FPV serotype (Feline and canine parvoviruses) can use transferrin receptors (TfR) for attachment and entry (Cotmore &

Tattersall, 2007; Hueffer et al., 2004; Parker et al., 2001; Suikkanen, 2003). These FPV viruses also bind to neuraminidase-sensitive N-glycolyl neuraminic acid side chains present on some cells, but these probably only act as attachment receptors and not entry receptors because infectious entry is insensitive to neuraminidase and infectious entry occurs on TfR. The block to FPV infection in canine cells is largely due to the lack of a functional cell surface receptor. Feline panleukopenia virus (FPV) and canine parvovirus (CPV) both bind feline TfR and use it to infect cat cells, but CPV preferentially binds canine TfR and infects dog cells (Palermo et al., 2006). In contrast to the FPV entry program, MVM binds to sialoglycoprotein receptors and both binding and entry are neuraminidase sensitive suggesting these receptors provide two functions, attachment and entry. Porcine parvovirus (PPV) is a major etiologic agent of reproductive failure in swine. PPV binds to sialic acid receptors on surface glycoproteins (Boisvert et al., 2010). These authors found pre-treatment of cells with neuraminidase prevented infection. Resialation on sialidase-treated cells with either α-2,3-O-sialyltransferase or α-2,3-N-sialyltransferase partially restored infectivity suggesting both O-linked and N-linked forms of carbohydrate moieties may act as receptors, but leaves open the possibility of other sialic acids functioning in the attachment process.

The mammalian adeno-associated viruses (AAVs) have been extensively studied for use as gene vectors for therapy of diseases resulting from genetic defects. Cellular receptors and coreceptors are an area of crucial interest in viral vector-mediated gene therapy because receptor preference and receptor tissue distribution may dictate vector choice for a given organ. By understanding the mechanisms of viral entry into target cells it may be possible to manipulate the gene vector in order to target a cell type of interest. Heparan sulfate proteoglycan mediates attachment of AAV-2 to susceptible cell lines (Summerford & Samulski, 1998), and other cell entry receptors or co-receptors for AAV-2 include human fibroblast growth factor receptor-1 (FGFR-1) (Qing et al., 1999), αVβ5 integrin (Sanlioglu et al., 2000; Summerford et al., 1999), and hepatocyte growth factor receptor (c-Met) (Kashiwakura et al., 2005). It has been suggested that heparin sulfate may play a role in AAV-2 infection as a low affinity attachment molecule (Qiu et al., 2000). Reports indicate that a group of basic amino acids that contribute to heparin binding are clustered in three positions on the three-fold spike of the AAV-2 capsid (Kern et al., 2003). Additionally, AAV-5 reportedly binds to cell surface 2,3-linked sialic acids (Walters et al., 2001) and the AAV-5 receptor for hemagglutination and transduction is α2,3-N-linked sialic acid and that for AAV-4 is α2,3-O-linked sialic acid (Kaludov et al., 2001). AAV-5 also binds to the platelet derived growth factor receptor (Di Pasquale et al., 2003). AAV type 3 can use fibroblast growth factor receptor 1 for binding (Blackburn et al., 2006).

A characteristic feature of BPV is its ability to hemagglutinate erythrocytes, hence its original name, hemadsorbing enteric virus (HADEN) (Abinanti & Warfield, 1961). This aspect of virus-cell interaction is possible because this virus attaches to receptors on the red cell membrane. Glycophorin A is an abundant transmembrane glycoprotein found in the erythrocyte membrane (Tomita et al., 1978). The glycophorin A monomer is composed of a 131 amino acid sequence found in three domains that form the hydrophilic

cytoplasmic domain, the hydrophobic transmembrane domain, and the aminoterminal glycosylated external domain. Glycophorin A naturally exists as a homodimer with extensive O-linked oligosaccharide glycosylation on the external domain. Exposure of the red cell surface to proteolytic enzymes or to neuraminidase destroyed the erythrocyte receptors for BPV indicating the receptor consists of a sialylglycoprotein (Thacker & Johnson, 1998). Virus probes on western blots and virus attachment to purified glycophorin A on dot blots confirmed virus attachment to this glycoprotein. Moreover, purified glycophorin A completely competed out virus attachment to the natural receptor. Further, Blackburn et al. (2005) showed BPV binding to α-2,3-linked sialic acid located on the O-linked oligosaccharides of the glycophorin A molecule. Treatment of glycophorin A with α2,3,-6,-8 neuraminidase eliminated binding of virus to this receptor. Beta-elimination of O-linked sialic acids on glycophorin A also eliminated binding while removal of N-linked carbohydrates using the N-glycosidase PNGase failed to eliminate virus binding. After enzymatic removal of the receptors, virus binding could be restored by reconstitution of the O-linked α2,3 neuraminic acids. On nucleated bovine host cells BPV attachment occurs on both α-2,3-O-linked and α-2,3-N-linked sialic acids (Johnson et al., 2004).

In studies on PPV infection, prior treatment of cells with neuraminidase prevented infection by eliminating PPV receptors (Boisvert et al., 2010). Resialation with α-2,3-O-sialyltransferase or α-2,3-N-sialyltransferase, or with a combination of the two enzymes partially restored infectivity. Therefore, the sialylglycoprotein receptors on the cell surface for PPV appeared to consist of both O- and N-linked sialic acids. Possibly other sialic acid receptor moieties exist in addition to these because total reconstitution did not occur but it was unclear whether completion of the reconstitution reaction could occur under the experimental conditions.

Host range is a property of the virus-host interaction that provides a suitable environment for complete virus replication resulting in the production of virus progeny. Only certain organs, tissues, and cells are within the host range of viruses. Commonly, host range is thought of relating to the availability of cell surface receptors. However, determination of host range and its resultant tissue tropism can occur at many levels including attachment to compatible receptors, the entry pathway, uncoating, the transcriptional environment, genome duplication, translational processes, assembly of virus particles, and mechanisms of escape from the host cell. At one level, host range for autonomous parvoviruses is determined by the viral requirement for S-phase cells. Frequently, in examples of parvovirus disease, the tissues involved are those which have an abundance of mitotic cells. In the case of FPV and CPV, related parvoviruses, attachment then entry by endocytosis can occur in many different kinds of non-permissive cells, indicating that their host range can be determined by events after cell entry. Amino acid residues 359 to 375 found in a flexible capsid protein loop were found to exhibit differential conformation when exposed to various concentrations of protons and Ca^{++} (Simpson et al., 2000). It was found that this region was functionally associated with both hemagglutinating activity and host range determinants providing continuance of successful replication.

3. Engulfment

Several possible pathways of receptor-mediated endocytosis are recognized: clathrin- and caveolae-mediated endocytosis, macropinocytosis, and novel nonclathrin/noncaveolae pathways (Brindley & Maury, 2008; Damm et al., 2005; Dimitrov, 2004; Kee et al., 2004; Marsh & Helenius, 2006; Meier & Greber, 2004; Meier et al., 2002; Mercer & Helenius, 2009; Pelkmans & Helenius, 2003; Pelkmans et al. 2004; Sieczkarski & Whittaker, 2002; Stuart & Brown, 2006). To date, for viral infections that have been studied, viruses mostly take advantage of clathrin-mediated endocytosis for internalization (Marsh & Helenius, 2006; Pelkmans & Helenius, 2003; Sieczkarski & Whittaker, 2002). During clathrin-mediated endocytosis, transport vesicles are surrounded by a clathrin coat, which is a three-dimensional array of triskelia. A triskelion is composed of three clathrin heavy chains (CHCs, approximately 190-kDa) and three light chains (CLCs, about 25-29 kDa), and has three-fold rotational symmetry (Edeling et al., 2006; Merrifield et al., 2005). Ligands that are to be transported to the cytosol, including viruses, are concentrated on the cell surface, and the concentrated ligands, as a patch, trigger recruitment of clathrin-adaptor proteins to the cytoplasmic side of the plasma membrane. Clathrin-adaptor complexes (APs) include the main distinct complexes, AP1 and AP2. A third protein, AP180, is used mainly for synaptic vesicles. The AP-2 complex consistes of α-adaptin, $\beta2$-adaptin, $\mu2$-chain, and $\sigma2$-chain. APs bind to membranes by recognizing phosphoinositides and link clathrin to the membrane. Therefore, clathrin coated vesicles (CCVs) are three-layered: 1) the inner, membrane layer with its embedded receptor/ligand complex, 2) the middle layer that is composed of APs and other regulatory proteins for clathrin assembly, and 3) the outer clathrin shell (Edling et al., 2006). CCVs comprise one of the most common and well defined coated transport vesicles. The internal pH in the CCV is around 6.5. Once fission of the pit has occurred through the action of accessory proteins including dynamin and other proteins forming CCVs, the clathrin coat must be rapidly shed to allow fusion of the vesicle with its target membrane. Uncoating of clathrin is resolved by auxilin and the molecular chaperone Hsc70 (heat shock protein 70). Auxilin interacts with assembled clathrin and binds to Hsc70 via its carboxyl-terminal J domain triggering Hsc70's ATPase activity. Hsc 70 then interrupts clathrin-clathrin interactions, causing shedding of the clathrin coat. Disassembled clathrin, accessory proteins and the endocytic recycling compartment (internal pH about 6.5) are recycled and promote the clathrin-coated vesicle cycle (Brandenburg & Zhuang, 2007; Dawsen et al., 2006; DeTulleo & Kirhchausen, 1998; Doxsey et al., 1987; Edling et al., 2006; Heuser & Anderson, 1989; Huang et al., 2004; Lemmon, 2001; Pu & Zhang, 2008; Sun et al., 2002). The cargo-containing vesicle then matures to the early endosome with an acidic environment (pH 6.5 to 6.0). In addition to the pH, markers for the early endosome include Rab5-GDP and Early Endosome Associated Protein-1. Virus-engaged receptors are uncoupled from their ligands at this mildly acidic environment of the early endosomes and ligand molecules are recycled back to the plasma membrane (Van der Goot & Gruenberg, 2006). The early endosomes are major sorting stations where the endosome is recycled back to the cell membrane, or where endocytosed cargo, including some viruses, can be released to the cytoplasm or can progress farther into the endosomal pathway to more acidic late

endosomes (pH 6.0 to 5.0) and lysosomes. The acidification of endosomes is required for release of virus into the cytoplasm (Damm & Pelkmans, 2006; Gagescu et al., 2000; Lakadamyali et al., 2006; Marsh & Helenius, 2006; Pelkmans & Helenius, 2003; Russell et al., 2006; Sieczkarski & Whittaker, 2002; Smith & Helenius, 2004; Van der Goot & Gruenberg, 2006). The late endosome becomes a degradative body with lower pH. It acquires the marker for mannose-6-phosphate receptor (MPR+) and the Rab7-GDP marker. They also acquire the unusual lipid lysobisphosphatidic acid (LBPA).

After engulfment the genome or nucleocapsids of viruses are released into the cytosol by fusion of the viral envelope with the endosomal membrane for enveloped viruses or, for non-enveloped viruses, capsid disassembly occurs in the endosome for some viruses with subsequent genomic escape to the cytosol (Brandenburg et al., 2007; Stidwill & Greber, 2000). The acidic pH of endosomes plays an essential role to trigger these events. Further, some non-enveloped viruses begin the uncoating process in the late endosome, but complete uncoating is delayed and it is a nuclear event. For example, in some instances such as adenovirus, AAV and canine parvovirus infections, the genome together with modified capsid components translocate to the nuclear membrane (Meier & Greber, 2004; Sonntag et al., 2006; Vihinen-Ranta et al., 2002) where final uncoating occurs for adenovirus but the small parvovirus capsid crosses the nuclear membrane before final uncoating within the nucleus. Digestion of material enclosed in the late endosome may not be complete and fusion with lysosomes may occur forming a hybrid organelle with an internal pH of about 5.0 and are MPR-negative. Viruses that exploit clathrin-dependent acid-mediated entry are sensitive to the inhibitors of endosomal acidification. Thus, inhibition of virus replication by endosomal pH inhibitors is taken as evidence for virus tracking through an acid-mediated endocytosis pathway.

The transport of endosomes is mediated by microtubules and proceeds toward the microtubule organization center which is found in the perinuclear area of the cell. Thus, transport to the late endosomes/lysosomes is beneficial for both virion conformation adjustment and for transport to the nucleus. Once the virus has escaped from the endosomal compartment the virus particle itself may interact with microtubule motors or with actin filaments to complete the journey to the nuclear membrane. In the case of parvoviruses, proteosomal digestion can help or hinder virus infection. AAV particles are degraded by the proteosome causing an aborted infection (Douar et al., 2001) while proteosome processing is required for MVM infection (Ros et al., 2002).

Electron microscopic images of BPV-infected cells show vacuoles consistent with CCVs containing virus particles (Dudleenamjil et al., 2010) at 15 minutes post-infection. In a separate report on canine parvovirus (Parker & Parrish, 2000), virus particles were found in endosomes at 5 minutes and 15 minutes post-infection. Densonucleosis virus was shown by EM in CCVs of *Lepidoptera* cells at 5 minutes post infection (Vendeville et al., 2009). These images in three different host cells were very similar and characteristic of CCVs bound with membrane-linked clathrin. An electron micrograph (Fig. 1) illustrates the possible invagination and pinching off process and shows a CCV that contains a virus-like particle.

In contrast to clathrin-mediated pH-dependent endocytosis, caveolae-mediated entry is an event triggered by binding of, for example, virus particles to receptor molecules on the cell surface that induces the clustering of lipid rafts with a high content of cholesterol and sphingolipids (Chazal & Gerlier, 2003; Hommelgaard et al., 2005; Pelkmans, 2005a). The area of the plasma membrane with the clusters invaginates to the cytosol, and the vesicle is surrounded by caveolins, the most characterized proteins of caveolae-mediated entry. These caveolin coated vesicles formed at the cholesterol-rich microdomains at the plasma membrane are the caveolae. Caveolins stabilize caveolae, and they are remarkably static in caveosomes (Marsh & Helenius, 2006; Pelkmans, 2005b). Accessory proteins involved in caveolae-mediated entry are dynamin and actin, and they are recruited by tyrosine kinase activites (Dimitrov, 2004; Marsh & Helenius, 2006; Pelkmans, 2005a; Pelkmans & Helenius, 2003; Pelkmans et al., 2005; Smith & Helenius, 2004). The caveolae containing the virus/receptor complex, close, pinch off from the cell membrane, and fuse together forming caveosomes. Caveosomes are part of the endocytic organelles with a neutral pH and the absence of markers for early, recycling, and late endosomes (Pelkmans, 2005a; Pelkmans & Helenius, 2003). Nevertheless, caveosomes connect with the smooth endoplasmic reticulum (ER), early and late endosomes, and the cell membrane. Release of virus taken up by the caveolar-raft system can occur from caveosomes (Echo 1), the ER (SV40), and endosomes (polyomaviruses and BK virus) (Eash et al., 2004; Marsh & Helenius, 2006; Pelkmans & Helenius, 2003). The interaction with endosomes may be crucial for some viruses that are taken up by caveolae-mediated endocytosis but require a low-pH environment for escape to the cytoplasm. Chemical inhibitors, targeted for a certain part of interconnected organelles of caveolae entry, are extensively exploited for examination of caveolae pathways used for virus entry. Results of studies utilizing inhibitors targeting caveolae found no evidence for BPV entry through these vesicles (Dudleenamjil et al., 2010) nor for PPV (Boisvert et al., 2010).

Active cell entry routes of some members of the Parvoviridae family have been described. Parvoviruses are known to utilize a variety of cell surface molecules as their receptor including glycoproteins (Blackburn et al., 2005; Thacker & Johnson, 1998), glycolipids, and glycans (Cotmore & Tattersall, 2007). It has been reported that parvoviruses with known cell entry routes enter into CCVs and establish successful infections (Basak & Compans, 1989; Cotmore & Tattersall, 2007; Harbison et al., 2008; Op De Beeck & Caillet-Fauquet, 1997; Parker & Parrish, 2000; Ros et al., 2002; Vendeville et al., 2009). Most studied for illumination of this process are MVM, CPV, PPV, DNV and AAV. Adeno-associated virus (AAV)'s entry into the host cell is mediated by clathrin coated pits and then routes to the late endosomes (Bartlett et al., 2000). The virus particles then escape to the cytoplasm where they are partially degraded by the proteasome and delivered to the nucleus for replication (Douar et al., 2001). The canine parvovirus, having used the transferrin receptor (TfR) for attachment (Parker et al., 2001), enters through the CCVs, and localizes in endosomes (Hueffer et al., 2004; Parker & Parrish, 2000; Vihinen-Ranta et al., 2002). Both CPV and FPV bind to TfR but species-specific binding controls host range. CPV binds to the filopodia of canine cells while FPV infects cats binding to the TfR on feline cells. FPV does not bind the canine TfR, does not infect dogs, or infect cultured canine cells (Harbison et al., 2009). Conversely, CPV can infect feline cells by binding to TfR on the cell body. Minute virus of

mice (MVM)'s cell entry and following events in the cytoplasm were investigated analyzing the effects of drugs that interfere with the endosomal acidification and ubiquitin-proteasome activities. Results suggested that MVM's entry is pH-dependent, and the interaction with the ubiquitin-proteasome system is required for MVM replication (Ros et al., 2002). The relatively rapid endocytic uptake of parvoviruses appears to be followed by slower traffic along the endocytic compartments toward the nucleus. The endosomal pathway undertaken by parvoviruses appears to be complex and depends on the virus, its concentration, and likely the cell type (Dorsch et al., 2002; Mani et al., 2006; Sonntag et al., 2006; Suikkanen et al., 2003; Yuan & Parrish, 2001). Conformational alterations in capsid structure probably occur in the endosomal compartment facilitating uncoating and transport to the nucleus.
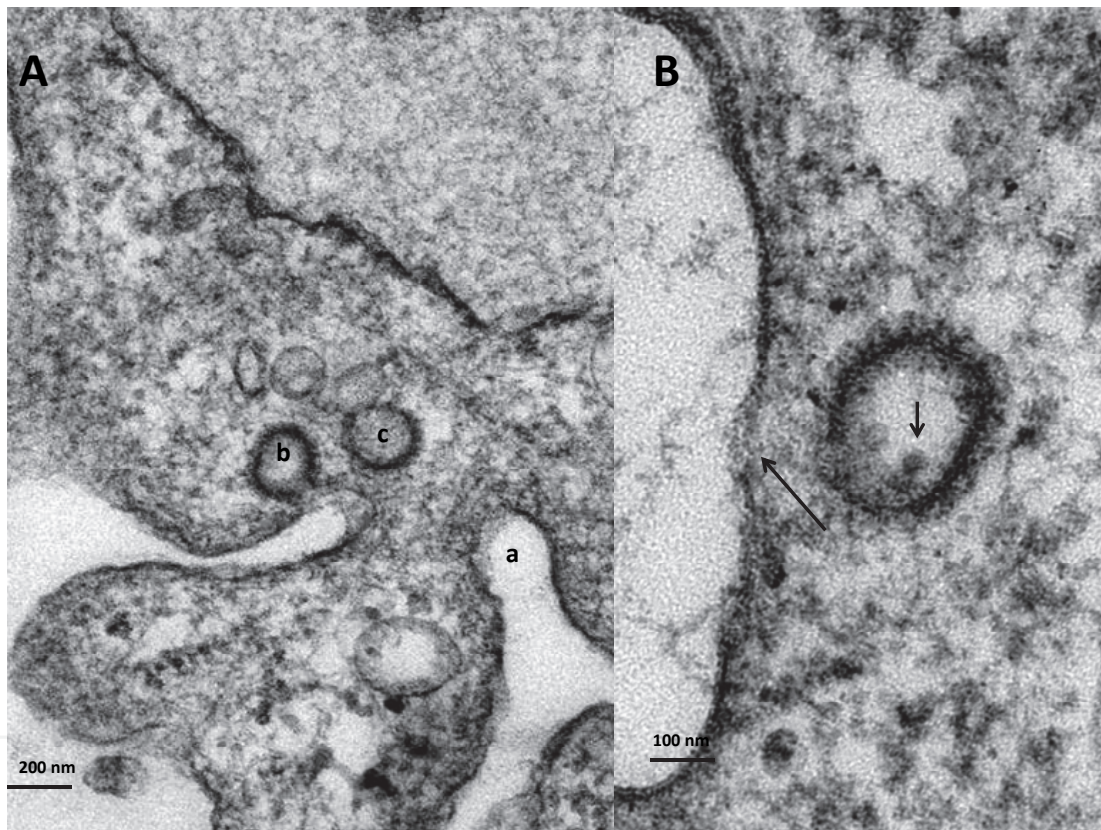
Figure 1. Electron micrograph of a clathrin coated vesicle-associated parvovirus-like particle. A) Negative-stained uninfected EBTr cell thin section showing invagination of membrane (a); (b) a nearly closed clathrin coated vesicle (CCV); (c) an enclosed CCV. B) A bovine parvovirus-infected cell (at 15 minutes post-infection) showing the cytoplasmic membrane site of CCV formation (long arrow), and a CCV containing unidentified material and a parvovirus-like particle (short arrow). A puddle of negative stain lies next to the particle slightly obscuring the capsid edge.

The vacuolar proton ATPase (V-ATPase) is a multisubunit enzyme complex, and it is responsible for the acidification of membrane-bounded organelles like endosomes. V-ATPase transports H^+ over membranes against an electrochemical potential under ATP hydrolysis, and H^+ ions acidify endosomal environments. Baf A1 blocks the V-ATPase activity causing neutralization of the acidic environment of endosomes. Baf A1 is routinely

used as a suppressor of V-ATPases, and it is used to test whether specific viral entry is reliant on endosomal acidification (Clague et al., 1994; Drose & Altendorf, 1997; Jin et al., 2005; Wassmer et al., 2005). It has been widely used as a probe to study acid-mediated viral trafficking using a variety of virus models. Entry pathway studies of mouse polyomavirus (Liebl et al., 2006), feline calicivirus (Stuart & Brown, 2006), bovine viral diarrhea virus (Lecot et al., 2005), AAV (Bartlett et al., 2000), MVM (Mani et al., 2006; Ros et al., 2002), mouse hepatitis virus type 2 (Pu & Zhang, 2008), poliovirus (Brandenburg et al., 2007), baculovirus (Long et al., 2006), hepatitis C virus (Blanchard et al., 2006), influenza virus (Guinea & Carrasco, 1995; Sieczkarski et al., 2003), HIV-1 (Fredericksen et al., 2002), and human rhinovirus 14 (Bayer et al., 1999) have analyzed the effects of Baf A1 on virus trafficking and examined the role of endosomal acidification in a route that leads to viral replication. For studies of parvovirus entry Baf A1 may be an inhibitor preferred over chlorpromazine because chlorpromazine is not only an inhibitor of clathrin lattice processing, but is also an inhibitor of phospholipase A2. Because parvoviruses use their own version of PLA2 for entry, confusion may arise regarding the point of inhibition.

4. Virus in the early endosome

In the process of acid mediated endocytosis acidification begins in the early endosome reaching a pH of 6.5 to 6.0. Proton transport continues during the transition to the late endosome which develops a pH of about 5.0 within this vesicle. The intravesicular environment of the lysosome is also characterized by low pH. Early endosomes are considered an initial sorting station where cargos for degradation are distinguished from those for recycling and this sorting process begins in clathrin coated vesicles, depends on microtubule motility, and appears to involve endocytosis adaptors (Lakadamyali et al., 2006). Acidification of endosomes is known to be essential for viruses which internalize within CCVs. Studies on parvovirus entry have reported that BPV, MVM, AAV, and CPV internalization require endosomal acidification, and the endosomal acidic environment may induce capsid conformational changes vital for viral release from endosomes to the cytoplasm (Basak & Compans, 1989; Douar et al., 2001; Dudleenamjil et al., 2010; Mani et al., 2006; Ros et al., 2002). Because the proton concentration increases during transition from early to late endosomes and high acidity is maintained in the lysosome, it is possible that acid-dependent uncoating of viruses, specifically pH-dependent capsid protein conformational shifts, may occur at various points within the early endosome, in the late endosome, or after exposure to the harsh environment in the lysosome complex.

The mechanisms of capsid endosomal processing in a low pH environment are poorly understood but are under investigation. The N termini of AAV VP1 and VP2 (VP1/VP2u), like the N terminus of MVM, CPV, and PPV VP1 (VP1u), contain motifs homologous to PLA2 and nuclear localization signals. AAV mutants that are consistent with a nuclear localization signal-deficient phenotype, traffick through the early endosome as the recombinant AAV2 control does, but forms a more diffuse accumulation pattern in the perinuclear area consistent with an NLS defect (Johnson et al., 2010). In a recent study, AAV-8 was exposed to pHs ranging from 7.5 to 4.0 and crystal structures of empty particles

and green fluorescent protein gene-packaged particles were analyzed (Nam et al., 2011). The capsid surface topologies of particles exposed to various pHs were similar except changes located close to the two-fold depression and significant amino acid side chain conformational changes were seen on the interior surface of the capsid under the three-fold axis. The three-fold change is consistent with low pH-associated release of genomic DNA from an ordered state on the interior surface of the capsid and likely indicate capsid transitions that ultimately result in genome uncoating. The surface alteration results in disruption of VP-VP interface interactions along with a decrease in buried surface area between VP monomers. This destabilization may lead to activation of the PLA$_2$ activity for endosomal escape and the NLS for nuclear targeting. As noted, it was observed by Nam et al. (2011) that at pH 4.0 specific interactions between the capsid and the packaged DNA genome are weakened but it is insufficient for genome uncoating. Rather, the intracapsid genome may be compacted. This process could be a step toward genome release or shift of the VP1u to externalization. These authors further point out that current thinking postulates that the five-fold pore of AAV is the externalization portal for the AAV VP1 and VP2 N-termini, the other parvovirus' VP1u, and the packaged DNA. But there is only a small difference in diameter at the top of the channel between pH 7.5 and pH 4.0. Therefore, in addition to acid pH other cellular factors such as proteolytic enzymes likely operate to facilitate capsid dynamic events to externalize these VP1u and VP1/VP2 N-terminal domains and ultimately genome release after trafficking from the early endosome.

A contrast exists between the deployment times of VP1u of some autonomous parvoviruses with that of Parvovirus B19. As noted above VP1u deployment occurs during trafficking through the acidified endosomal compartments. It was found that B19 attachment to human erythrocytes caused early accessibility and activity of PLA$_2$ without entry into a nucleated cell (Bönsch et al., 2008). Thus, VP1u is displayed as the virus remains attached to the surface of erythrocytes. The phospholipase does not cause lysis of the cells but does cause increased osmotic fragility. In an earlier study on VPu of B19 virus, it was reported that the VPu motif is internally oriented but becomes exposed in heat-treated particles and in particles exposed to low pH (Ros et al., 2006).

CPV capsids labeled with fluorescent markers were seen in Rab-5 positive endosomes within minutes of uptake (Harbison et al., 2009). Capsids were also seen in Rab7- and Rab11-positive endosomal compartments by 10-15 minutes after infection. Gradually the virus accumulated near the microtubule organizing center. The CA form of Rab5 induces large, ring-like vesicles in cells and a high proportion of the CPV capsids entered these vesicles and remained there for a period of time of one hour or more. Many of the particles remained attached to the vesicle wall probably in association with the receptors.

In Lepidoptera cells densonucleosis virus (DNV) particles are rapidly internalized and are found in CCVs. They then traffic slowly within early endosomes, then to late endosomes (Vendeville et al., 2009). An alternative route from the endosome is to the multivesicular body (MVB) which contains numerous intraluminal vesicles (ILVs). Fusion of the MVB with

the plasma membrane releases small exosomes to the extracellular environment. Exosomes are microvesicles 40-100 nm in size that exhibit a cup-like morphology and appear to be released ILVs (Meckes & Raab-Traub, 2011). The size of an exosome would accomodate one or more parvovirus particles but this pathway would be a detour diverting virus from the entry process leading to nuclear penetration which is essential for virus replication. It is not clear at this time if parvovirus particles are extruded from the cell in exosomes. It would be feasible for cells outside of the viral host range to expel endocytosed particles in this manner, but such a resistance pathway for parvoviruses is yet to be demonstrated.

5. Transition to the late endosome

It is thought that four general biological alterations accompany the early-to-late endosomal transition: acidification of the endosomal lumen, formation of luminal vesicles, the switch of Rab GTPases, and microtubule-mediated transport between the organelles (Greber & Cosset, 2011). Some viruses escape to the cytoplasm under the effects of the acidic environment (pH 6.5 to 6.0) of the early endosome. Sensitivity to endosomal acidification raises a question regarding duration of the virus within the endosome. That is, whether the virus particles are directly released from the early endosomal compartment to the cytosol or are routed farther into the late endocytic compartment or even to the late endosome-lysosome complex. If virus does not escape from the early endosomal station, it would follow the transition pathway to the late endosome. In this transition, the early endosome becomes a transport intermediate recruiting ADP ribosylation factor-1 (Arf1)-dependent coatamer proteins (COPI, clathrin, and AP-1) and converts to the late endosome. Arf 1 or small GTPase's activities are catalyzed by Sec7-type GTP-exchange factors (GEFs). GEFs are primarily targets of BFA. Therefore, tubulation of maturation of early endosomes is delayed, and virus transition to the late endosome is blocked (Brandenburg et al., 2007; Nebenfuhr et al., 2002; Stuart & Brown, 2006). Studies reported that BFA also affects transport between Golgi and endoplasmic reticulum with the same mechanism through Arf1. BFA was chosen as an inhibitor of pH-dependent endocytosis to investigate early events of murine polyomavirus, SV40, AAV, DNV, MVM, PV, and FCV interaction with the host cell (Blanchard et al., 2006; Damm et al., 2005; Douar et al., 2001; Gilbert & Benjamin, 2004; Guinea & Carrasco, 1995; Mani et al., 2006; Ros et al., 2002; Stuart & Brown, 2006; Vendeville et al., 2009). That BFA blocks infection by these viruses is suggestive of late endosomal involvement in the entry process.

Rab proteins are small GTPases that regulate vesicular transport in endocytosis and exocytosis. They are considered master regulators of transport. Early endosomes are converted to late endosomes as a shift occurs in their linked Rab GTPases from the early endosome-associated Rab5 to the late endosome-associated Rab7 (Cabrera & Ungermann, 2010; Rink et al., 2005; Rodman & Wandinger-Ness, 2000). Rab conversion is the mechanism by which cargo moves from early to late endosomes. The Rab7 domain grows on the early endosome and converts the Rab5-positive endosome to a Rab7-positive endosome (Poteryaev et al., 2010). Participating in this process are the cofactors SAND-1 and Mon1

(Nordmann et al., 2010; Poteryaev et al., 2007). The Rab7 complex is activated by the Class C VPS/HOPS complex (vacuolar protein sorting/homotypic fusion and vacuole protein sorting) complex which is a GEF (guanine nucleotide exchange factor) for Rab7. It interacts with Rab5 and is required for the Rab5 to Rab7 conversion (Rink et al., 2005). Activated Rab5 is important for sequestering ligands into clathrin-coated pits and subsequent fusion of these vesicles with early endosomes. The actin cytoskeleton plays a prominent role in both the early stages of endocytosis and the late Rab5 function (Rodman & Wandinger-Ness, 2000) and actin facilitates fusion among late endosomes and between late endosomes and phagosomes (Kjeken et al., 2004). Materials destined for degradation are delivered to early endosomes then segregated for transport to late endosomes, then to lysosomes (Rodman &Wandinger-Ness, 2000). The transition of the Rab5 early endosome to the Rab 7 late endosome, mediated by the Class C VPS/HOPS complex, is facilitated by Syntaxin-7 which is localized to the late endosome and is required for late endosome and lysosome fusion (Kim et al., 2001). Moreover, the endosomal membrane protein Ema interacts with Class C VPS/HOPS to promote endosomal maturation (Kim et al., 2010).

ADP-ribosylation factor (Arf) in association with Sec7 (Arf GDP/Sec7) is a GEF used in membrane traffic at the Golgi. Arf GDP/Sec7 is phosphorylated to the GTP level which may be used for activation of Class C VPS/HOPS-GDP to Class C VPS/HOPS-GTP that is used in the Rab5 to Rab7 conversion. BFA is a drug with specificity for the Arf-GDP/Sec7 complex and by binding at the interface between Arf-GDP and the Sec7 domains acts as an uncompetitive inhibitor of Arf activation and freezes (stabilizes) the complex that cannot proceed to nucleotide dissociation (Cherfils & Melancon, 2005; Zeghouf et al., 2005). BFA disrupts maturation of the early endosome to the late endosome (Douar et al., 2001; Vieira et al., 2002), but it also interferes with the secretory pathway (Greber & Way, 2006). However, inhibition of parvovirus infection with BFA is not due to interference with the secretory pathway as no parvoviruses are known to exit the infected cell in that way. So, viral susceptibility to BFA in the parvovirus replication cycle is most likely due to blockage of the early-to-late endosome transition. Thus, using BFA inhibition of Arf GTPase employs a strategy for disrupting early to late endosome traffic (Vieira et al., 2002).

A possible mechanism for BFA inhibition of late endosome formation is shown in Fig. 2. Illustrated in the figure is the molecular interaction between the ArfGDP and Sec7 subunits of the ArfGDP/Sec7 GEF complex. This interaction stabilizes the molecular complex preventing its activity as a GEF. Altogether, biochemical and structural data using isolated Sec7 domains provide a consistent explanation for the action of BFA, that its only target is the Arf-GDP/Sec7 interface (Cherfils & Melancon, 2005). This, in turn, inhibits the pathway responsible for the Rab5 to Rab7 conversion which is necessary for endosome maturation. The bovine parvovirus entry pathway goes through clathrin-associated endocytosis (Dudleenamjil et al., 2010) and it may go through extended compartments in this pathway. The results of inhibitor studies using BFA in BPV entry were consistent with virus persistence within the endosome until transition to late endosome is complete (Dudleenamjil & Johnson, unpublished data).

After internalization, generally both virus particles and vesicles that carry virus particles are able to interact with cell cytoskeletal structures and utilize their activities to reach specific sites in the cytoplasm or nucleus for replication. Virus-associated trafficking routes may involve actin filaments together with myosin motors or microtubules with their dynein and kinesin motors. Globular (G-) actin is polymerized to filamentous (F-) actin during synthesis of actin fibers. Synthesis begins with a loose association of three to four G-actin monomers to an unstable oligomer, a process called nucleation. Filaments are then elongated by addition of G-actin monomers. F-actin is involved in both cell movement and in the movement of cell organelles. Endocytic vesicles move at the tips of actin tails and appear to be pushed through the cytosol (Merrifield et al., 1999) and late endosomes can nucleate F-actin whereas early endosomes cannot (Kjeken et al., 2004). LAT A is a natural toxin secreted by red sea sponges, for example *Latrunculia magnifica* (Coue' et al., 1987). Lat A binds to G-actin and prevents it from adding to a filament end during synthesis of F-actin (Yarmola et al., 2000). Growing evidence has suggested a tight interaction between the actin network and acid-mediated endocytosis at the level of the late endosome (Kjeken et al., 2004; Rodman & Wandinger-Ness, 2000). Thus, inhibition of virus movement through the endocytic compartment by LAT A would be evidence that viral transit through the late endosome is essential in the process of getting the virus to the cell nucleus. Cells treated with increasing noncytotoxic concentrations of the inhibitor LAT A reduced bovine parvovirus infectivity (Dudleenamjil & Johnson, unpublished observations). The reduction of virus infection by this drug is evidence that acid-mediated endocytosis is a functional route of BPV internalization into the host cell and utilizes actin filaments in the trafficking of ligands contained in the late endosome.

6. Lysosomal interaction

In the viral entry process followed by some non-parvoviruses, exposure of stable capsids to low pH may allow proteolysis of capsid protein to occur creating a metastable configuration displaying sequences for membrane penetration (Cotmore & Tattersall, 2007). CPV enters the cell in association with its receptor, TfR, and the virus along with the TfRs is transported to late endosomes-lysosomes before escape into the cytoplasm (Suikkanen, 2003). In the pathway followed by MVM, a low pH environment fosters proteololysis of the VP2 N-termini which results in enhanced stability at low pH (Cotmore & Tatersall, 2007). Thus, these modified particles may be required to return to a pH-neutral environment before they undergo the structural transition that exposes the PLA2 activity required for membrane penetration. However, it would be expected that after late endosome-lysosome fusion this hazardous compartment would result in extensive viral polypeptide hydrolysis and DNA damage. Results indicate that CPV does not face this same requirement. CPV exposed to low pH *in vitro* develops PLA2 activity which persists when returned to neutral pH (Suikkanen et al., 2003). It seems clear that CPV requires PLA2 activity as PLA2 inhibitors block viral replication (Suikkanen, 2003).

Cells infected with viable parvovirus, empty capsids, or entry-defective mutants accumulate virus in large, crescent-shaped, peri-nuclear vesicular clusters that are probably microtubule organizing centers. The vesicles appear to be late endosomes-lysosomes as the processing of early endosomes to these late structures utilizes microtubule transport. Nocodazole, a microtubule depolymerizing drug, inhibits CPV infection and leaves vesicles containing CPV near the cell surface (Vihinen-Ranta et al., 1998) and they fail to accumulate in the perinuclear crescents. Likewise, in the MVM model, microtubule polymerization moves virus to the perinuclear late endosome-lysosome complex and depolymerization of microtubules scatters virus toward the cell periphery. Upon repolymerization of the microtubules the virus returns to crescent complexes (Cotmore &Tattersall, 2007). Similarly, BPV infection is sensitive to nocodazole treatment.

Some viruses that infect the gastrointestinal tract, notably rotavirus, require proteolytic activation in order to promote virus entry and infection. The proteolytic enzymes in the gastrointestinal tract provide this service to the virus. Influenza A viruses require proteolytic cleavage of the hemagglutinin (H) molecule to separate HA1 and HA2 exposing the fusion peptide located on the N-terminal end of HA2 which is required for bridging the endosomal membrane. Also, the acidic pH of the endosome promotes conformational shift in the HA structure resulting in functional availability of the hydrophobic fusion domain for penetration into the endosomal membrane. Many parvoviruses target the enteric tract and/or the respiratory tract of their natural hosts. Although parvoviruses, many of which may be exposed to the proteolytic enzymes in the gastrointestinal tract or to respiratory proteolysis such as that mediated by tryptase clara, may not require proteolysis for cell entry, but one considers a possible role for proteolysis as well as low pH conformational shifts in capsid protein structure for enhancement of infection relating to the display and activity of the PLA_2 and NLS motifs. On the other hand, exposure to low pH may circumvent the infectious pathway. Acid pHs promote virus aggregation and crystal formation (see Fig. 3). The pH of the respiratory tract is acidic resulting from the CO_2 reaction with H_2O to form carbonic acid. Parvoviruses that infect the respiratory tract such as Parvovirus B19, HuBoV, BPV, AAV and others may aggregate in the respiratory tract. Viruses infecting the gastrointestinal tract such as PPV, MVM, and others may aggregate in the gastric environment. In infected cells, intranuclear newly assembled virions clearly form large crystalline arrays, then, upon escape from the infected cell these newly produced aggregates may not be easily dispersed even at physiological pH. Moreover, regarding entry, it is possible multiple virions may be engulfed in single CCVs and transport together within the endosomal system and upon acidification form aggregates. Because aggregates become too large to penetrate the nucleus as is the case for individual particles, the fate of the virions within the aggregate may not include nuclear entry but they may be digested in the lysosome complex. As demonstrated for PPV, viral aggregates enter by macropinocytosis. The outcome of this pathway may be virus destruction rather than nuclear entry. In this regard, most viral progeny in parvoviral infections may not end up as infectious units but may be cleared and destroyed in the lysosome. More work will be required to decipher the probability of multiple routes of cell entry, and multiple intra-cellular outcomes.
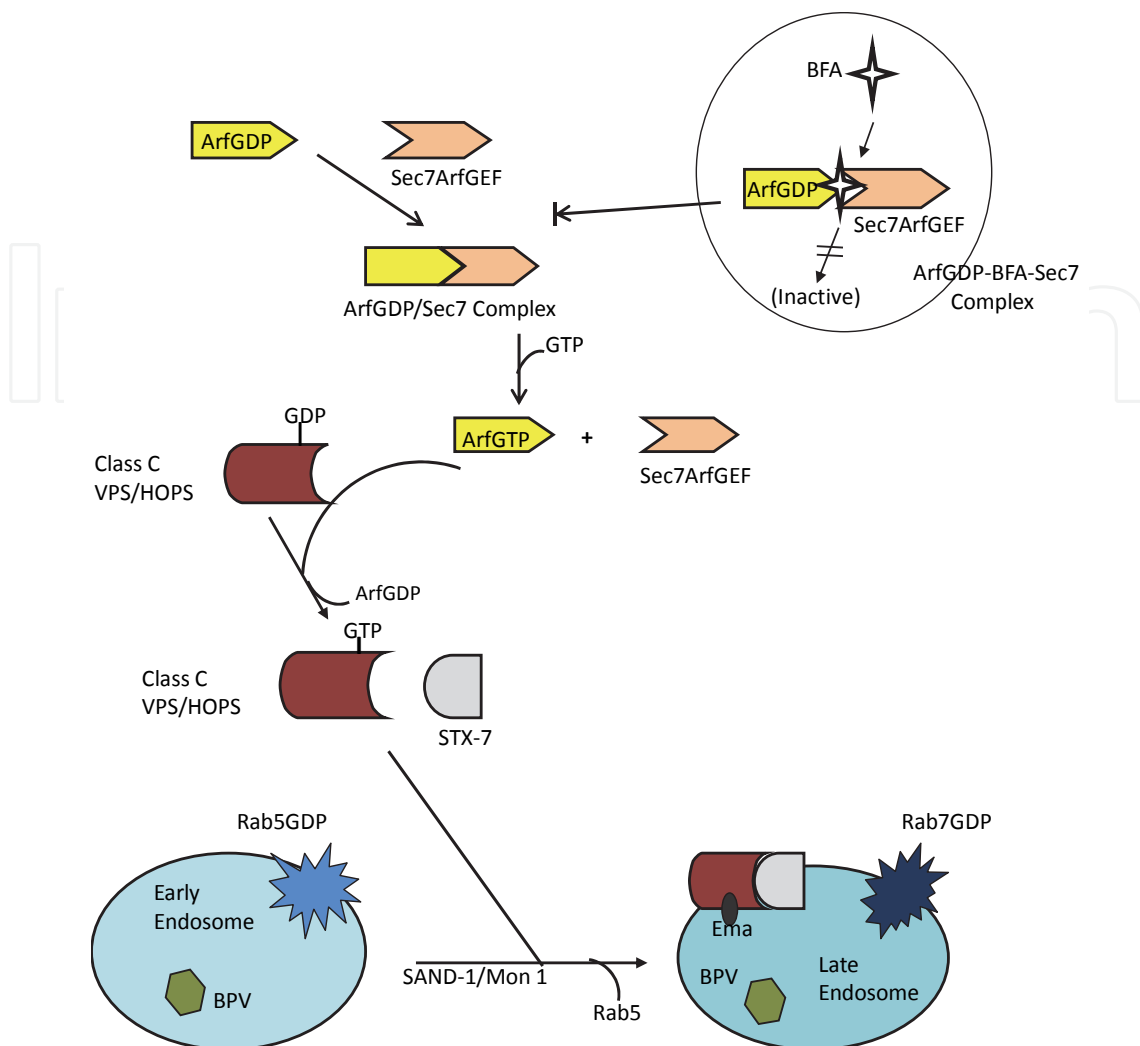
Figure 2. A possible mechanism of inhibition of the early to late endosome transition by brefeldin A (BFA). Rab5GDP is a marker on the early endosome. It is phosphorylated and gradually replaced by Rab7GDP which is a marker of the late endosome. The Mon1-Ccz 1 complex with SAND-1 control this process (Cabrera & Ungermann, 2010; Poteryaev et al., 2010). During this event, Class C VPS/HOPS is activated by phosphorylation, complexes with STX-7, and binds to the late endosome with Ema. The guanine exchange factor ArfGTP may activate the Class C VPS/HOPS complex but it can perform this function only through the activation of the ArfGDP/Sec7 complex. It is at the ArfGDP/Sec7 complex where BFA works. The drug binds to the ArfGDP-Sec7 interface and stabilizes the complex making it inactive as an ArfGDP/Sec complex preventing Arf activation (Cherfils & Melancon, 2005; Zeghouf et al., 2005), thus inhibiting the early-to-late endosome conversion. **Abbreviations:** Arf (ADP-ribosylation factor, a GTPase). ArfGDP/Sec 7 (a guanine nucleotide exchange factor, the target of BFA). BFA (brefeldin A). GEF (guanine nucleotide exchange factor, a GTPase). Class C VPS/HOPS (vacuolar protein sorting/homotypic fusion and vacuolar protein sorting, a GEF for Rab7). STX-7 (Syntaxin-7, a vsnare). Ema (an endosomal membrane protein).

7. Virus escape from endosomes

Paradoxically, parvoviruses must have a capsid shell rugged enough and stable enough to protect their single stranded DNA genomes from damage and degradation during transit

from host to host and from portal of entry to ultimate target tissue, yet sensitive enough to navigate the cell-entry process leading to final uncoating within the proper viral replication compartment. Intracellular navigation, in the case of parvoviruses, requires subtle reorientations of capsid structural proteins exposing functional domains on the VP1u motif (Cotmore & Tattersall, 2007). Conceptually, a T=1 virus capsid would be constructed of 60 copies of a structural polypeptide. MVM empty capsids are constructed, on average, of 50 molecules of VP2 and 10 copies of VP1. In parvoviruses, the major (most abundant) structural polypeptide is the smallest structural protein (VP2 or VP3). Altogether, as noted above, the capsids contain either two or three related structural polypeptides that are coterminal at their carboxyterminals and have small unique sequences on their amino terminals. Unique to the larger versions of the polypeptides are elements on the VP1u region that are required for trafficking through the host's entry pathways (Cotmore & Tattersall, 2007). VP1 is dispensable for capsid assembly and genome packaging, but is absolutely required for infectivity. The VP1u sequence, which is within the N-terminal unique region, contains both the PLA_2 phospholipase domain and nuclear localization signals. The proteins are translated from a large mRNA and initiate either by leaky scanning or after differential splicing so that the upstream initiation codon is removed and leaves a downstream codon available for starting translation of the smaller capsid protein. One of these elements, PLA_2 which is a lipolytic enzyme is employed to breach the endosomal membrane allowing virus escape from the endocytic organelles, releasing it into the cytosol. An MVMp PLA_2 mutant deficient in this enzyme is unable to escape from its vacuolar confine and these particles accumulate in the endosomes. Once the virus is released, the virus targets the nucleus presumably by cellular factors that facilitate transport to the nuclear membrane, penetration across the nuclear membrane, capsid disassembly, exposure of the genome, and movement to an appropriate intranuclear replication compartment. Preliminary capsid conformational change, which may allow eventual endosomal escape, may occur in the early or late endosome but remains to be clarified for the various parvoviruses. It's likely that parvoviruses as a group utilize their phospholipase activity to escape the endosomal pathway. The parvovirus capsid is structurally dynamic undergoing multiple conformational changes during its replication cycle including the externalization of the VP1 N terminus during entry. In such a condition, VP1 remains tethered to the viral shell and appears to be active in particle escape from the endosome. Interestingly, an active particle may also operate in *trans* allowing for escape of VP1u-deficient particles if contained in the same endosome (Farr et al., 2005). It has also been shown that parvovirus B19, the human virus that causes erythema infectiosum (fifth disease) has a VP1-unique region that contains PLA_2 activity (Dorsch et al., 2002) and presumably operates in a manner similar to that described for MVM.

When studies use high multiplicities of virus and track the particles in infected cells, many of the particles enter dead-end pathways and never enter the nucleus. Moreover, in cases where ratios of numbers of physical particles to infectious particles have been studied, the ratio is quite high, 300:1 for MVMp and 1000:1 for CPV (Cotmore & Tattersall, 2007). Since only a small number of particles actually arrive at their nuclear destination, it makes the

interpretation of vacuolar transport studies that use high viral multiplicities somewhat difficult. Many of these particles may be flagged for destruction and not infection. In studies on BPV entry, virus was labeled with fluorescein, cells exposed to high multiplicities of virus, then observed microscopically over time to assess virus transport through the cell. Labeled virus appeared in vacuolar structures but whether virus was visible in the nucleus was not conclusive (Dudleenamjil & Johnson, unpublished results).
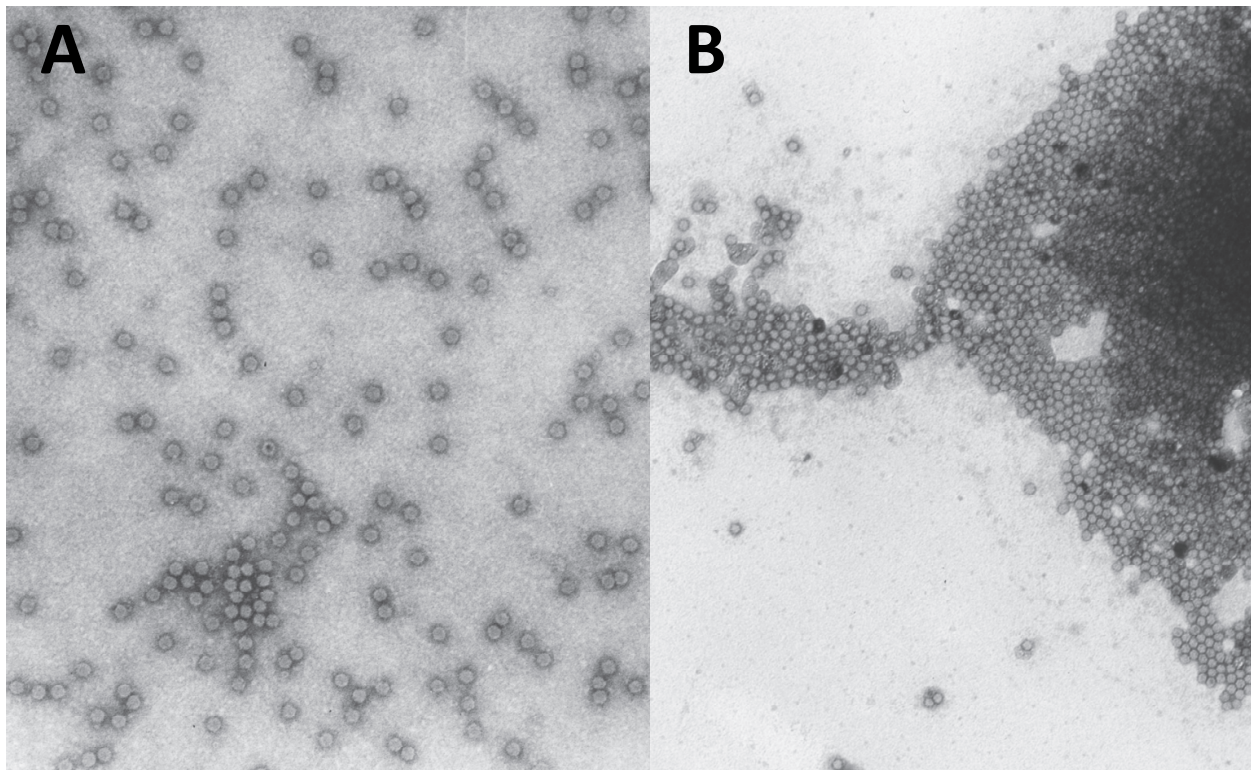
Figure 3. Aggregation of parvovirus particles at acid pH. A) Adeno-associated Virus Type-3 at pH 7.4 negatively stained with uranyl acetate. B) AAV-3 particles shifted to pH 6.1 also negatively stained with uranyl acetate, showing extensive particle aggregation and formation of crystalline array. Original micrograph magnification: 141,000x.

8. Transport to the nucleus and nuclear invasion

In the parvovirus life cycle, structural proteins must be transported at least twice from cytoplasm to the nucleus: following structural protein synthesis so that nuclear capsid assembly can be accommodated and also after virus entry during infection for invasion of the host cell nucleus. Both MVM and CPV have nuclear localization signals (NLS) on their capsid proteins (Cotmore & Tattersall, 2007; Lombardo et al., 2002). Because capsid-associated VP1 undergoes structural rearrangement during entry exposing functional signals, it is possible that the VP1 NLS motif participates in penetration of the invading capsid across the nuclear membrane. Thus, a life-cycle advantage that parvoviruses have over other nuclear-dependent viruses is their small size, small enough at 26 nm to be able to import into the nucleus as an intact (although modified) virion controlled by a bifunctional

set of NLS motifs. In support of capsid transport across the nuclear membrane, CPV virions microinjected into cells were found to translocate into the nucleus intact and initiate gene expression (Suikkanen et al., 2003; Vihinen-Ranta et al., 2000). Entry of the microinjected virions circumvented the natural endocytic pathway and placed them in position to penetrate the nucleus, but it is unclear if the NLS motifs were active in the process. As an alternative mechanism of nuclear entry, MVM nuclear penetration in mouse fibroblast cells was tracked by fluorescence microscopy and electron microscopy (Cohen et al., 2006). It was found that this virus caused marked changes in nuclear shape, alterations of nuclear lamin immunostaining and breaks in the nuclear membrane. These changes may allow direct physical access to the nuclear interior for the virus.

Microtubule-associated activity in parvoviral trafficking is involved with dynein-dependent endosomal trafficking of CPV capsids before escape. Further, dynein dependency is seen in movement of CPV capsids after escape through the cytoplasm to the nucleus (Suikkanen, 2003). It was reported that AAV-2 trafficking to the nucleus utilizes the PI13 kinase activation cascade directing virus along microtubules and microfilaments (Sanlioglu et al., 2000). Surprisingly, one study found AAV's trafficking to the nucleus appears to be independent from the microtubule network (Hirosue et al., 2007). These authors found that overexpression of dynamitin which results in a functional inhibition of the minus-end-directed micotubule motor protein dynein did not inhibit transduction of rAAV2. Treatment of HeLa cells with nocadazole or vinblastine disrupted microtubules but did not significantly affect virus transduction. In contrast, high concentrations of taxol resulted in microtubule stabilization and high vinblastine concentrations caused formation of tubulin paracrystals both reducing rAAV2 transduction. These authors concluded that these results demonstrate that AAV2 can infect HeLa cells independent of dynein function or an intact microtubule network. In another report (Johnson & Samulski, 2009), a population of rAAV2 virions entered the nucleus and accumulated in the nucleolus after infection but empty capsids were excluded from nuclear entry. Interestingly, virions trafficked to the nucleolus were found to retain infectivity in secondary infections. Thus, in the case of AAV, mobilization from the nucleolar site to nucleoplasmic locations likely permits uncoating and gene expression. Also in this study proteosome inhibitors were found to potentiate nucleolar accumulation.

Proteosome activity is essential for PPV infection (Boisvert et al., 2010). In the presence of lactacystin and MG-132, two commonly used proteosome inhibitors, the virus remains in a more diffused state in the perinuclear area of the cell and low-level replication occurs. Supportive data showed that PPV capsid proteins were ubiquitinated early in infection. These observations suggest proteosomal interaction during the last stages of transport prior to nuclear entry. Interaction with the proteosome has also been demonstrated for MVM (Ros et al., 2002; Ros & Kempf, 2004). The exact role for ubiquitination and proteosomal interaction for these viruses remains to be elucidated.

Because the autonomously replicating parvoviruses require the S-phase nuclear environment for replication, after they penetrate the nucleus they remain in a

genomic nonsynthetic state until S-phase occurs. The Dependoviruses do not have this constraint as the helper virus provides the synthetic environment, yet these viruses have a constraint of their own, the necessary co-infection with the helper virus. Once in the nucleus, AAV appears to undergo subnuclear mobilization accumulating in the nucleolus (Johnson et al., 2010), uncoating and genome replication. Although the purpose of this review is to only take the entering virus to the intranuclear environment, it is interesting to note that in CPV infection the nuclear replication compartment expands and is accompanied by chromatin marginalization to the vicinity of the nuclear membrane, virus capsids move by passive diffusion, intranuclear structure and dynamics are extensively affected enlarging the interchromosomal domain which contains viral proteins, genomes, and capsids (Ihalainen et al., 2009). After parvovirus assembly and maturation within the nucleus of infected cells, they egress by processes that include apoptosis (Poole et al., 2004; Poole et al., 2006) and cell necrosis (Abdel-Latif et al., 2006).

9. Conclusion

Not all parvoviruses have been examined in detail for their entry and trafficking pathways, but the model viruses that have been studied reveal some commonalities shared by the viruses in this family. Among these are:

1. Parvoviruses penetrate their host cell through receptor-mediated endocytosis, interacting with a host cell receptor, and entering in clathrin-coated vesicles.
2. The viruses are processed in the early to late endosome and possibly lysosome prior to nuclear entry.
3. In preparation for vacuolar escape, the low pH environment induces capsid protein conformational shifts which display the viral VP1 PLA_2 domain and nuclear location signals.
4. Viral escape from the endosome is mediated by PLA_2.
5. Microtubules and actin generally play a role in virus particle transport to the nuclear membrane where capsid penetration to the intranuclear environment occurs.
6. Intranuclear mobility occurs by random diffusion for some and for others (AAV) by tracking to the nucleolus.

Alternative pathways may exist for some of these viruses, as seen by macropinocytosis for PPV. There is yet much to be learned about the virus-cell interactions utilized by parvoviruses, mechanisms of bridging the nuclear membrane, intranuclear localization and the uncoating process.

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