JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 122

Sanna Suikkanen

Cell Biology of Canine Parvovirus Entry

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Villa Ranan Blomstedt-salissa
lokakuun 10. päivänä 2003 kello 11.

Academic dissertation to be publicly discussed, by permission of
the the Faculty of Mathematics and Science of the University of Jyväskylä,
in the Building Villa Rana, Blomstedt Hall, on October 10, 2003 at 11 o'clock.

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2003

Cell Biology of Canine Parvovirus Entry

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 122

Sanna Suikkanen

Cell Biology of Canine Parvovirus Entry

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2003

Editors
Jukka Särkkä, Department of Biological and Environmental Science, University of Jyväskylä

Pekka Olsbo, Marja-Leena Tynkkynen
Publishing Unit, University Library of Jyväskylä

URN:ISBN 9513917851
ISBN 951-39-1785-1 (PDF)

ISBN 951-39-1508-5 (nid.)
ISSN 1456-9701

Jyväskylä University Printing House, Jyväskylä
and ER-Paino Ky, Lievestuore 2003

The Microbe
By Hilaire Belloc

The Microbe is so very small
You cannot make him out at all,
But many sanguine people hope
to see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of theeth;
His seven tufted tails with
lot of lovely pink and purple spots,
On each of which a patern strands,
composed of forty separate bands;
his eyebrows of a tender green.
All these have never yet been seen-
but Scientist, who ought to know,
assure us that they must be so...
OH! Let us never, never doubt
what nobody is sure about!

ABSTRACT

Suikkanen, Sanna
Cell biology of canine parvovirus entry
Jyväskylä: University of Jyväskylä, 2003, 88 p.
(Jyväskylä Studies in Biological and Environmental Science,
ISSN 1456-9701; 122)
ISBN 951-39-1785-1
Yhteenveto: Koiran parvovirusinfektion alkuvaiheiden solubiologia
Diss.

Canine parvovirus (CPV), a host range variant of feline panleukopenia virus (FPV), was first recognized in 1978 as a new virus infecting dogs. The CPV virion is formed from a single stranded DNA genome packed into a non-enveloped protein capsid. This capsid is composed of two structural proteins VP1 and VP2, formed by alternative splicing. The N-terminus of VP1 contains a potential nuclear localization signal (NLS) and phospholipase A_2 (PLA_2) like motif. In newly formed capsids the N-terminus of VP1 is enclosed within the capsid, but this sequence can be exposed by treatments with urea or heat without complete disintegration of the capsid. To initiate infection, CPV binds to transferrin receptors (TfRs) on the cell surface, and enters the cells by clathrin-mediated endocytosis. Productive infection is dependent on the acidic pH of endocytic vesicles as well as the integrity of microtubular the network.

Although many details about CPV entry have been solved so far, the endocytic entry pathway, the course of events leading to penetration to the cytoplasm and the steps preceding nuclear import are still unknown. This thesis was designed to clarify the missing links of the CPV entry process using cell biological methods.

The findings of this thesis suggest that after CPV enters the cells by clathrin-mediated endocytosis some receptor bound viruses are transported to recycling endosomes and some of those virus TfR complexes are redirected to the degradative pathway, ending up in lysosomes.

Parvoviral phospholipase A_2 (PLA_2) activity located in the N-terminal part of viral capsid protein-1 (VP1) is triggered by treatments of acidic buffers or by heating. This amino acid sequence is exposed during the entry at 3-8 h post infection (p.i.) in lysosomal vesicles, indicating that the acidic lysosomal environment may trigger the activation of viral PLA_2. This further suggests that viral PLA_2 activity might be utilized in the membrane penetration process. Endosomal vesicles were not found to be disrupted by CPV infection, but the CPV was found to modify permeability of the endosomal/lysosomal membranes causing the release of the small (M_r 3000) dextran particles to the cytoplasm after 8 h of infection. After penetration into the cytosol, CPV capsids were transported towards the nucleus along microtubules in a dynein-dependent manner.

Key words: Canine parvovirus; dynein; endocytosis; microtubules; phospholipaseA_2 activity; viral entry.

Sanna Suikkanen, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FIN-40014 University of Jyväskylä, Finland

Author's address Sanna Suikkanen
University of Jyväskylä
Department of Biological and Environmental Science
P.O. Box 35
FIN-40014 University of Jyväskylä, Finland
E-mail: sansuik@cc.jyu.fi

Supervisors Professor Matti Vuento
University of Jyväskylä
Department of Biological and Environmental Science
P.O. Box 35
FIN-40014 University of Jyväskylä, Finland
E-mail: vuento@gwia.jyu.fi

Dr. Maija Vihinen-Ranta
University of Jyväskylä
Department of Biological and Environmental Science
P.O. Box 35
FIN-40014 University of Jyväskylä, Finland
E-mail: mvihinen@jyu.fi

Reviewers Docent Eeva-Liisa Eskelinen
University of Kiel
Institute of Biochemistry
Olshausenstr. 40
D-24098 Kiel, Germany
E-mail: eleskelinen@biochem.uni-kiel.de

Docent Esa Kuismanen
University of Helsinki
Division of Biochemistry
Viikinkaari 5
FIN-00710 Helsinki, Finland
E-mail: ekuisman@mappi.helsinki.fi

Opponent Professor Klaus Hedman
University of Helsinki
Haartman institute
Division of Virology
P.O. Box 21
FIN-00290 Helsinki
E-mail: klaus.hedman@helsinki.fi

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals:

I Suikkanen, S., Sääjärvi, K., Hirsimäki, J., Välilehto, O., Reunanen, H., Vihinen-Ranta, M. & Vuento, M. 2002. Role of recycling endosomes and lysosomes in dynein-dependent entry of canine parvovirus. J. Virol. 76, 4401-4411.

II Suikkanen, S., Aaltonen, T., Nevalainen, M., Välilehto, O., Lindholm, L., Vuento, M. & Vihinen-Ranta M. 2003. Exploitation of microtubule cytoskeleton and dynein during parvoviral traffic towards the nucleus. Accepted for publication in J. Virol. 77: 10270-10279

III Suikkanen, S., Antila, M., Jaatinen, A., Vihinen-Ranta, M., & Vuento, M. 2003. Release of canine parvovirus from endocytic vesicles. Accepted for publication in Virology.

ABBREVIATIONS

AAV	adeno-associated virus
$AACOCF_3$	arachidonyl trifluoromethyl ketone
Ad	adenovirus
AMI	Amiloride(N-amidino-3,5-diamino-6-chloropyrazinecarboxamide)
AP-1	adaptor protein-1
AP-2	adaptor protein-2
ARF1	small GTPase ADP ribosylation factor
B19	human parvovirus B19
BFA	bfafeldin A
BFLA	bafilomycin A_1
BSA	bovine serum albumin
Ci-MPR	cation independent mannose-6-phosphate receptor
COP	coatomer protein
CPV	canine parvovirus
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
ECV	endosomal carrier vesicle
EEA1	early endosomal antigen-1
EM	electron microscopy
FPV	feline panleukopenia virus
GA	glutaraldehyde
GTP	guanosine triphosphate
HIV-1	human immunodeficiency virus type 1
IgG	immunoglobulin G
kDa	kilodalton
LAMP	lysosomal membrane protein
LDL	low density lipoprotein
LSM	laser scanning microscopy, confocal microscopy
MEV	mink enteritis virus
MON	monensin
mRNA	messenger RNA
MT	microtubule
MuLV	Moloney murine leukemia virus
MVM	minute virus of mice
NLFK	Norden laboratory feline kidney
NLS	nuclear localization signal
NPC	nuclear pore complex
NS	nonstructural protein
OG	Oregon green
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
p.i.	post infection
PI(3)P	phosphatidylinositol 3-phosphate
PI(4,5)P	phosphatidylinositol 4,5-bisphosphate
PLA_2	phospholipase A_2

$pvPLA_2$	phospholipase A_2 of parvovirus capsids (N-terminal end of VP1)
$sPLA_2$	secreted type phospholipase A_2
PNS	post nuclear supernatant
PPV	porcine parvovirus
RNA	ribonucleic acid
RPV	raccoon parvovirus
SFV	Semliki Forest virus
Tf	transferrin
TfR	transferrin receptor
TGN	trans Golgi network
VP	viral capsid protein

1 INTRODUCTION

Parvoviruses are classified into three genera, *Parvovirus* (autonomous parvoviruses), *Dependovirus* and *Densovirus* (Siegel et al. 1985, Berns 1996). The autonomous parvoviruses infect humans and many animals including dogs, cats, pigs and mice, causing a number of diseases. To replicate in their host cells these viruses require many cellular factors produced during active cell division (Berns, 1996). Canine parvovirus (CPV), a member of the genus *Parvovirus,* was found as a new pathogen in 1978 when it caused new enteric and myocardial diseases in dogs, and spread rapidly throughout the world (Parrish 1990). CPV capsid is assembled from 60 copies of the overlapping viral capsid proteins –1 and –2 (VP1 and VP2), VP1 containing the complete sequence of VP2 and the unique N-terminal sequence of 143 amino acid residues (Xie & Chapman 1996). The unique region of VP1 contains both potential nuclear localization signal (NLS) and a phospholipase A_2 (PLA_2) like motif and this sequence has been shown to be essential for productive infection (Vihinen-Ranta et al. 1997, Zádori et al. 2001). In newly produced capsids the N-terminal end of VP1 is enclosed within the capsid, but it can be exposed by heat or urea treatments without total disintegration of the capsid (Weichert et al. 1998).

To initiate infection, CPV binds to the transferrin receptor (TfR), and uses it as a receptor to enter and infect the cell (Parker et al. 2001). After binding to the receptor, CPV enters the cells by dynamin dependent, clathrin mediated endocytosis (Parker & Parrish 2000). To accomplish viral entry, intact microtubules and the low pH of endosomal vesicles are essential; since nocodazole treatment and compounds elevating endosomal pH are able to block the viral proliferation (Basak & Turner 1992, Vihinen-Ranta et al. 1998, Parker & Parrish 2000). The specific endocytic organelles used by CPV during its entry and the mechanism by which CPV is released from endocytic vesicles are still poorly understood. Cytoplasmic transport of the capsids to the perinuclear area is dependent on the microtubular network (Vihinen-Ranta et al. 2000), but the precise steps in cytoplasm before the nuclear entry are unclear. Instead, parvoviral DNA replication, gene expression and assembly are known to take place in the nuclei of host cells (Tattersall 1972). The CPV capsid is theoretically small enough to pass through the nuclear pore complex (NPC) without capsid disassembly or deformation (Xie & Chapman 1996, Feldherr et al. 2001), but the form in which virus crosses the nuclear membrane is not clear.

The literature review in this thesis will briefly describe the common features of cellular elements and their functions involved in viral journey into the nucleus of the cells, as well as the entry processes of the viruses,

concentrating on nonenveloped viruses and gives updated review of the parvoviruses and their life cycle. The aim of the experimental work was to find (at least some of the) missing pieces of the CPV entry puzzle. The results propose a theory for the CPV intracellular entry pathway, define the role of parvoviral PLA_2 in the viral escape process through the endosomal membranes and further clarify the role of microtubules and motor proteins before and after viral escape to the cytoplasm. Ingeneral, the growing knowledge of viral entry routes will allow the development of anti viral drugs based on modification of distinct organelle specific cellular functions and facilitate the understanding of the action mechanisms of gene therapy vectors.

2 REVIEW OF THE LITERATURE

2.1 Endocytosis

Endocytosis is a process used by cells to internalize extracellular ligands in plasma membrane derived vesicles. Endocytic processes can be divided in two classes: phagocytosis and pinocytosis. Phagocytosis is a property of specialized cells, such as macrophages, mediating the uptake of large particles with diameter of 200 nm or more. Pinocytosis is a property of virtually all cell types mediating internalization of macromolecules and particles smaller than 150 nm in diameter. Pinocytosis includes both fluid phase and receptor-mediated endocytosis (Steinman et al. 1983, Mellman 1996). The ability of receptors to select and concentrate specific ligands enables an efficient intake of physiologically important macromolecules present in low concentrations (Steinman et al. 1983). In addition to physiological ligands, opportunistic ligands such as viruses and toxins are internalized by receptor-mediated endocytosis (Marsh 1984).

Clathrin-mediated endocytosis is the major pathway for selective internalization of plasma membrane receptors and the ligands bound to them (Simonsen et al 2001). A cargo selection by clathrin coated vesicles is attributed to endocytic signals present on the cytoplasmic tails of the plasma membrane receptors. The binding of the extracellular ligands to receptors is followed by interactions of adaptor protein-2 (AP2s) and accessory proteins with lipids and receptors. These interactions mediate the recruitment of the receptors into the coated areas of the plasma membrane and cause the clathrin lattice to force vesicle invagination of the membrane. Invaginated pits undergo vesicle fission with help of a dynamin (a GTPase) and other effector proteins (Marsh 2001). In addition to the clathrin-dependent pathway cells have also other mechanisms to internalize (macro-) molecules bound to their receptors. For example macropinocytosis, which is charcacteristic for macrophages and dendritic cells. In addition, in some tumor cells macropinocytosis can be induced by growth factors. Many authors have reported that the clathrin-independent pathways may integrate with clathrin-dependent pathway (Montesano 1982, Tran et al. 1987, Vilhardt et al. 1999). Furthermore, in many cases, molecules enter cells both by the clathrin-dependent and –independent routes (Marsh 2001).

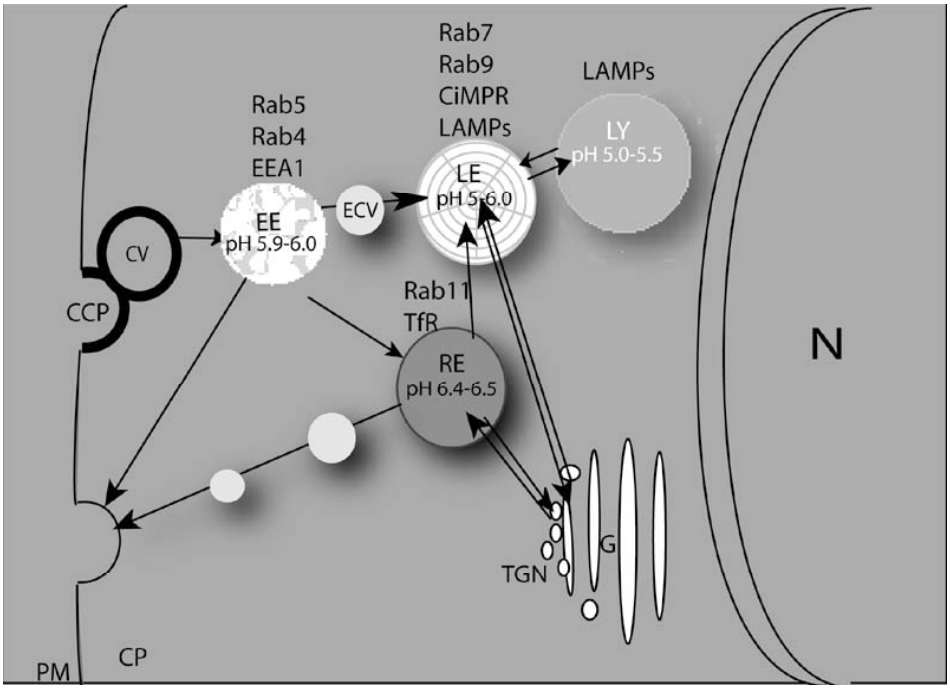

FIGURE 1 Simplified model of clathrin mediated endocytosis taking together the main points of the text. The major organelles are defined in terms of their kinetic relationships and in terms of generally used markers. Modified from Mukherjee et al. 1997.
PM, plasma membrane; CCP, clathrin coated pit; CV clathrin coated vesicle; EE, early endosome; RE, recycling endosome; LE, late endosome; LY, lysosome; G, Golgi apparatus; TGN, trans-Golgi network; N, nucleus.

Ligands taken into cells by clathrin-mediated endocytosis are transported to early endosomes (EEs) where the slightly acidic environment (pH 5.9-6.0) causes many ligands to dissociate from their receptors (Mukherjee et al. 1997). Specific signals on the cytoplasmic tail of receptors may cause them to be sorted and returned back to the plasma membrane for reuse, while others are transported together with dissociated ligands to the lysosomes for degradation (Mellman 1996, Riezman et al. 1997). Recycled molecules, targeted back to the plasma membrane, are rapidly shuttled back to the plasma membrane from EEs or from the tubulovesicular compartment near the nucleus, known as recycling endosomes or perinuclear recycling endosomes (RE) (Gruenberg & Maxfield 1995). The transit through RE is slow and requires an intact microtubular network (Hopkins & Trowbridge 1983, Ghosh et al. 1994). The boundary between EEs and REs is not clear and they are proposed to form a continuous tubular network. However, early endosomal membranes contain the small GTPases Rab4 (Daro et al. 1996), Rab5 (Bucci et al. 1992) and the early endosomal antigen-1 (EEA1) as specific marker proteins. These molecules are thought to confer directionality for endocytic transport (Simonsen et al. 2001). In addition, EEs contain proteins destined for recycling as well as degradation (Yamashiro et al. 1984), whereas REs have Rab11 (Ullrich et al. 1996) and proteins destined specifically for recycling (Yamashiro et al. 1984). REs have also more alkaline pH EEs (Yamashiro, et al. 1984, Sipe & Murphy 1987, Mukherjee et al. 1997). Late endosomes (LEs) are considered as an intermediate structure in the transport of endocytosed molecules, lysosomal hydrolases and membrane proteins to lysosomes. They differ from EEs by their lower luminal

pH (pH 5-6), different protein composition and association with different small GTPases of the Rab family (Fig. 1) (Somsel Rodman & Wandinger-Ness 2000). LEs and lysosomes (LYs) have lysosomal membrane proteins (LAMPs or lgps) associated in their membranes and both LEs and LYs have properties expected of functional degradative compartments including a low luminal pH and contentnts of acid hydrolases. Unlike lysosomes, LEs are enriched in mannose 6-phosphate receptors (MPRs) (Griffiths et al. 1990, Griffiths & Gruenberg 1991). In addition, LYs have a slightly lower pH (5-5.5) than LEs (Mukherjee et al. 1997). It has been shown that there is also retrograde traffic from terminal lysosomes to LEs suggesting dynamic equilibrium between LEs and lysosomes (Jahraus et al. 1994). In addition, receptor bound ligands (for example B subunit of Shiga toxin) can be transported from clathrin coated pits to ER (Gruenberg & Maxfield 1995, Mallard et al. 1998). After binding to its receptors on the cell surface toxin-receptor complex is internalized in clathrin coated pits (Sandvik et al. 1989) and transported *via* EEs and REs to the trans Golgi network (TGN) of the Golgi and finally to the ER (Mallard et al. 1998).

Although the receptor-ligand complex has a certain intracellular pathway multivalent ligands such as multivalent transferrin (composed of more than 10 transferrin molecules) may result redirecting of a ligand-receptor complex. The receptor-bound multivalent transferrin is rerouted to the degradative pathway, while transferrin is transported to the REs (Marsh et al. 1995). In addition, apo-B, E-receptor (Tabas et al. 1990, Tabas et al. 1991), M glycoprotein (as coronavirus receptor, (Weisz et al. 1993) and for Fc-receptors (Mellman & Plutner 1984) have been reported to be rerouted by the binding of multivalent ligands. All in all, the basic organization of the endocytic pathway has been elucidated, particularly in the case of the internalization of protein ligands bound to the cell surface receptors (Steinman, et al. 1983, Kornfeld & Mellman 1989, Trowbridge et al. 1993, Gruenberg & Maxfield 1995, Mellman 1996), there remains much to be studied to fully understand the complex processes of endocytosis.

During recent years, lipids have been found to contribute to the organization and functions of the vacuolar system (Gruenberg 2001). The membrane phospholipid phosphatidylinositol can be phosphorylated in different ways to produce distinct second messengers known as phosphoinositides. Localized changes of phosphoinositides are mediated by kinases and phosphatases providing temporal and spatial regulation of the membrane budding, motility and fusion (Simonsen et al. 2001). Phosphoinoitides seem to be rather well organized the organelles of the endocytic pathways (Gruenberg 2001). Clathrin coated vesicles (CCVs) are enriched in phosphatidylinositol 4,5-phosphates ($PI(4,5)P_2$), which seems to have the key role of regulating formation, scission and uncoating of CCVs (Jost et al. 1998). Phosphoinositides, in particular $PI(4,5)P_2$ and phosphatic acid, have been reported to participate in CCV formation as regulators of dynamin membrane activity (Lin et al. 1997, Burger et al. 2000). Phosphatidylinositol 3-phosphate, which interacts with proteins containing a FYVE or a PX domain, is concentrated in early endosomal membranes, where it is suggested to regulate the fusion events of EEs through Rab5-GTP (Simonsen et al. 1998, Gillooly et al. 2000, Rubino et al. 2000, Simonsen et al. 2001). Furthermore, lysophosphatidic acid and phosphatidylinositol 3, 5- bisphosphate ($PI(3,5)P_2$) are suggested to be typical lipids for endosomal carrier vesicles, multi vesicular bodies and late endosomes (Kobayashi et al. 1998, Shisheva et al. 2001, Kobayashi et al. 2002),

while sphingolipids and sulfatide are abundant in recycling endosomes (Gagescu et al. 2000 Höltta-Vuori et al. 2002). The roles of these lipids on membrane trafficking have not yet been clarified.

TABLE 1 Properties of early endosomes (EEs), recycling endosomes (REs), late endosomes (LEs) and lysosomes (LYs). Modified from Mukherjee et al. (1997). Lipid data collected from Kobayashi et al. 1998, Gagescu et al. 2000, Shisheva et al. 2001, Simonsen et al. 2001, Hölttä-Vuori et al. 2002, Kobayashi et al. 2002.

	EEs	REs	LEs	LYs
Marker proteins:	Rab4 Rab5 EEA1	Rab11 TfR	Rab7 Rab4 Rab9 CiMPR LAMPs	LAMPs
Lipid markers:	PI(3)P	Sphingolipids Sulfatide	$PI(3,5)P_2$ Lysophosphatidic acid	
pH:	5.9-6.0	6.4-6.5	5.0-6.0	5.0-5.5
Distribution:	Peripheral	Variable	Mainly perinuclear	Mainly perinuclear
Morphology:	Tubular	Tubular/ Variable	Onion-like with internal membranes	Variable: tubular to LE-like Electron dense

2.1.1 Modification of endocytosis by disturbing pH gradients and coat protein binding

Receptor mediated endocytosis as well as receptor recycling require acidic luminal pH of endosomes in order to be functional (Pillay, et al. 2002). The formation of endosomal vesicles from early endosomes is a low pH dependent process (Tjelle et al. 1996). Moreover some membrane transporters (such as lysosomal cysteine transporter) and lysosomal hydrolases are functional only in acidic conditions (Gahl & Tietze 1985, Pisoni & Thoene 1991, Hasilik 1992). Acidic luminal enviroment is established by the vacuolar ATP-dependent proton pump (V-type H^+ ATPase) and a redox chain both of which pump protons to the lumen of the vesicles (Reviewed by Mellman, 1996, Pillay, et al., 2002). The pH of EEs has been reported to be regulated by the Na^+/H^+ exchangers that create a potential across the vesicle membrane and inhibit proton intake (Cain et al. 1989, Fuchs et al. 1989, Mukherjee et al. 1997, Pillay et al. 2002). Other ion transporters, such as chloride channels, may also affect and regulate the pH of the endosomes (Al-Awqati 1986, Pillay et al. 2002). Many compounds causing changes in the pH or in the ion balance of endocytic vesicles, have been shown to cause reduced rates of endocytosis, inhibition of receptor recycling, inhibition of diphteria toxin toxicity and reduction of infectivity of some viruses (Umata et al. 1990, Pérez & Carrasco 1994, Van Weert et al. 1995, Lichtman et al. 1996, Pless & Wellner 1996, Presley et al.

1997b, Chemello et al. 2002, Elliott & Dennison 2002, Meier et al. 2002, Pillay et al. 2002). However, in interpretation of these data one should take into consideration the possible side effects caused by these compounds on other cellular functions.

One of the best-known drugs modifying endocytosis is a macrolide antibiotic bafilomycin A_1, which is a specific inhibitor of V-H^+ATPase (Werner et al. 1984). The inhibition of V-H^+ATPases by bafilomycin A_1 blocks the acidification of endosomes, lysosomes and phagosomes and inhibits the degradation of endocytoced proteins (Umata et al. 1990, Yoshimori et al. 1991, Van Deurs et al. 1996). Bafilomycin A_1 has been reported to cause inhibition of receptor-ligand dissociation (Harada et al. 1997), to slow down receptor recycling (Johnson et al. 1993), to inhibit endosomal carrier vesicle formation (Clague et al. 1994) and late endosome-lysosome fusion (Van Weert et al. 1995) as well as to cause fragmentation of early endosomes (D'arrigo et al. 1997). Contrastingly, bafilomycin A_1 has been shown not to inhibit transferrin intake (Johnson et al. 1993, Stein & Sussman 1986). Another well known compound, monensin (Na^+/H^+ ionophore) raises the pH of all intracellular acidic compartments by exchanging the protons for monovalent cations (Pressman 1976). The intracellular effects of monensin on endocytic pathways are similar to those of bafilomycin A_1's. However, ionophores such as monensin dissipate pH gradients throughout the cell and may cause morphological changes in the vacuolar compartments due to osmotic swelling (Stein et al. 1984, Tartakoff 1983). In addition to bafilomycin A_1 and monensin, weak bases can be used to increase the pH of acidic compartment where the uncharged amines of weak bases are trapped by protons (Mellman 1996).

Na^+/H^+ exchangers, as mentioned above, regulate the pH of EEs but they also play an important role in the maintenance of intracellular pH and in the regulation of cell volume by translocating Na^+ in exchange for H^+ across the cellular membranes. Furthermore, Na^+/H^+ exchangers have been found in the plasma membrane as a part of the machinery that maintains the pH of the cytosol and in the endosomal and lysosomal membranes (Hilden et al. 1990, Sabolic & Brown 1990, Mukherjee et al. 1997, Pillay et al. 2002). The action of these exchangers can be blocked by amiloride, but the actual effects of amiloride on the endocytosis are not known.

Brefeldin A has been shown to interfere with trafficking between early and late endosomes and to have multiple effects on the morphology of endocytic organelles (Lippincott-Schwartz et al. 1991). It has been reported to inhibit the GDP-GTP exchange of the small GTPase ADP-ribosylation factor 1 (ARF1), needed for assembly of coatomer protein COPI, and β-adaptin (subunit of adaptor complex AP-1) (Donaldson et al. 1992, Randazzo et al. 1993, Kreis et al. 1995, Chardin & McCormick 1999). Coatomer proteins are involved in vesicular transport through the biosynthetic pathway between ER and Golgi (Lowe & Kreis 1998). AP-1 adaptor complexes are restricted to clathrin-coated areas of the TGN and they interact with proteins that exit the Golgi complex (Robinson & Pearse 1986, Mellman 1996). The COPs have been also proposed to be involved in endocytic pathways, since epsilon (ε)-COP-deficient cells exhibited deficient endocytosis as well as disturbed LDL -and transferrin receptors recycling (Guo et al. 1994, Hobbie et al. 1994, Daro et al. 1997). Moreover, ARF1 has been reported to be required for COP recruitment to endosomes and for the formation of endosomal carrier vesicles (Gu & Gruenberg 2000). Recently, coat proteins have been proposed to play a role in

endocytic sorting processes (Wang et al. 2001). The recruitment of COPs, the formation of ECVs and the recycling of transferrin receptor have been shown to be inhibited by brefeldin A (Whitney et al. 1995, Sonnichsen et al. 2000, van Dam & Stoorvogel 2002).

2.2 Cytoskeleton

Cytoplasmic transport and also cell morphology rely on cytosolic filaments: microtubules, intermediate and actin filaments (Sodeik 2000). These cytoskeletal structures are suggested to organize the endocytic machinery, provide the force to drive the membrane fission and to enable vesicle movement in the cytoplasm (Marsh 2001). The cytosolic filament network is a highly dynamic, reorganizing constantly as the cell responds to different stimuli, changes shape, migrates or divides (Sodeik 2000).

Microtubules are hollow cylinders with a diameter of 25 nm, having an intrinsic resistance to bending and compression. They are made of the GTPase tubulin and have a fast growing plus-end extending towards the cell periphery and a slow-growing minus end located at the microtubule organizing center, typically found in a perinuclear position (Mandelkow & Mandelkow 1995, Sodeik 2000). The directed transport of cellular components along microtubules is linked to large complexes called molecular motors.

The motor protein complexes dynein and kinesin are known to mediate organelle movement in opposite directions along the microtubules. Cytoplasmic dynein, a minus end directed motor is a multisubunit protein of 1 270 kDa consisting of two heavy chains (530 kDa), two or three intermediate chains (74 kDa), and a variable number of small subunits (Holzbaur & Vallee 1994, King & Schroer 2000). The ATPase and motor domains of dynein are located within the heavy chains whereas the specific cargo-binding activity involves the intermediate chains and several classes of light chains (Boylan et al. 2000, Vaughan et al. 2001). In many cases the dynein-dependent transport of the material is facilitated by the activator protein dynactin, which mediates dynein binding to microtubules (Allan 1996, Holleran et al. 1998). Dynein, in conjunction with dynactin, facilitates membrane transport from the early endosomes to the late endosomes and lysosomes (Gruenberg & Howell 1989, Aniento et al. 1993, Oda et al. 1995, Valetti et al. 1999) and also from the ER to the Golgi (Presley et al. 1997a). The plus end-directed motor protein, kinesin, is a microtubule-activated ATPase of 380 kDa (Brady et al. 1982, Vale et al. 1985) consisting of two 120 kDa heavy chains and two 64 kDa light chains (Vale et al. 1985). Three major types of kinesins are classified according to the position of the motor domain: N-terminal, middle and C-terminal, conventional kinesins having N-terminal motor domains. Heavy chains of kinesin have an ATP binding site, while light chains may play roles in interactions of kinesin with membranes and regulating the heavy chain ATPase activity (Hirokawa et al. 1998). Further, in various cell types kinesin associates with ER, Golgi, mitochondria, endosomes and lysosomes (Barton & Goldstein 1996, Vale & Fletterick 1997, Hirokawa et al. 1998).

Actin filaments are more flexible and much shorter than microtubules. Each filament has a diameter of 5-9 nm, consists of helical polymers of ATP-actin and has a fast-growing plus-end and a slow-growing minus-end, like

microtubules. Actin filaments are most abundant underneath the plasma membrane of the cells. Actin is suggested to be involved in the formation of clathrin coated pits. Actin filaments may need to be rearranged to make room for clathrin coat formation or they may provide organization of the plasma membrane allowing coat formation (Gruenberg & Howell 1989). Intracellular membrane transport along actin is mediated by ATP-driven motor proteins, myosins (types I, II, V and VI) (Mermall et al. 1998). Myosins and kinesins are suggested to generate movement according to similar principles (Vale 1996, Kikkawa et al. 2001). Cytosolic transport can also occur by binding to a subunit of treadmilling or depolymerizing filaments. For example, late endosomes and lysosomes have been shown to associate with treadmilling actin (Frischknecht et al. 1999, Merrifield et al. 1999, Rozelle et al. 2000, Taunton et al. 2000).

Intermediate filaments comprise a third family of fibrous proteins. They have a diameter of 8-10 nm and have similar intracellular distribution as the microtubules. Intermediate filaments provide stability and resistance to a mechanical stress. In contrast to microtubules and actin, intermediate filaments do not have a polarized structure and therefore they are not able to give directionality to intracellular transport (Sheetz 1999).

2.3 Viral entry into host cells

Viral entry is comprised of a series of steps that viruses must successfully complete to establish productive infection. Viruses bind to membrane receptors on the host cell surface. Next they have to penetrate cellular membranes to enter cytoplasm, after which the viral genome must be targeted to the place of replication and the viral capsid must be uncoated to allow replication and virus protein expression to start (Helenius et al. 1989, Whittaker & Helenius 1998, Whittaker et al. 2000).

2.3.1 Binding to membrane receptors

Viruses utilize a wide variety of cell surface molecules as their receptors to bind and enter the host cell. Proteins, carbohydrates as well as glycolipids can serve as viral receptors (Knipe 1996). The viral receptor binding site varies depending on the virus. Enveloped viruses bind to cell surface receptors by their envelope glycoproteins (Grewe et al. 1990) while nonenveloped viruses have been reported to use grooves, depressions, loops or spikes of the virion surface as their receptor binding sites (Marsh & Helenius 1989). In addition to the direct binding of virus to the plasma membrane, viruses might bind to the cell surface through soluble protein intermediates such as antibodies or $beta_2$-microglobulin (Marsh & Helenius 1989). Some viruses such as adenovirus utilize several receptors to enter the cells. Adenovirus exploits different receptors for attachment (coxsackie-adenovirus-receptor), for internalization (□V□5 and □V□3 integrins) and for penetration through endosomal membranes (□V□5 integrin) (Wickham et al. 1993, Bergelson et al. 1997, Tomko et al. 1997).

2.3.2 Entry

In general, two distinct mechanisms of virus entry occur. One where viruses enter cells by with fusion of the viral envelope with the plasma membrane, and the other where viruses enter cells by receptor-mediated endocytosis followed by penetration through endosomal membranes (White et al. 1983, McClure et al. 1988, Marsh 1993, Nussbaum et al. 1993). Many viruses require exposure to an acidic pH during infectious entry (Marsh 1993). Influenza virus and Semliki Forest virus are well known examples of enveloped viruses. They require the low pH (< 6) in the endosome to cause their glycoprotein spike complexes to undergo conformational changes needed for fusion of the viral envelope with endosomal membranes (Bullough et al. 1994, Kielian & Helenius 1985). In the presence of drugs disrupting endosomal acidification processes, the infection caused by these viruses is blocked (Matlin et al. 1981, Kielian 1995, Glomb-Reinmund & Kielian 1998). Also many nonenveloped viruses are affected by these drugs. For example adenovirus type 2 (Varga et al. 1991), rhinoviruses (Prchla et al. 1994) and reoviruses (Martinez et al. 1996) require a low pH step for their productive infection. Infection by some pH-dependent viruses may be inhibited by indirect effects of these drug treatments on endosomal protease activation or on endosomal trafficking of viral particles (Clague et al. 1994, Authier et al. 1996, Bayer et al. 1998). Generally, viruses that require exposure to low pH for penetration enter their host cells by clathrin-mediated endocytosis to ensure the delivery to acidic organelles (Marsh & Pelchen-Matthews 2000). Most viruses using endocytosis are adapted to escape from the EEs or the LEs to avoid delivery to lysosomes, which would cause inactivation and degradation (Marsh & Helenius 1989). However, some viruses such as reovirus need lysosomal proteolysis for productive infection (Sturzenbecker et al. 1987). For pH independent viruses endocytosis may also offer advantages, such as help with the bypass the cortical cytoskeleton and provide the services of the cellular delivery machinery, (Marsh & Pelchen-Matthews 2000). Recently, a caveolae-mediated entry pathway has also been reported for an increasing number of both enveloped and nonenveloped viruses (Werling et al. 1999, Richterov et al. 2001, Empig & Goldsmith 2002, Marjomäki et al. 2002).

Several viruses induce permeabilization of cellular membranes. Permeabilization has been observed for nonenveloped and enveloped viruses including rotavirus, poliovirus, reovirus, adenovirus, influenzavirus, vesicular stomatitis virus, Semliki Forest virus and Vaccinia virus (Fernandez-Puentes & Carrasco 1980, Almela et al. 1991, Perez & Carrasco 1994, Guinea & Carrasco 1995, Martinez et al. 1996). Viral permeabilization of cellular membranes has been investigated by cointernalization of viruses or viral proteins with fluid phase markers like dextran, calcein, carboxyfluorescein or HRP (Ruiz et al. 1994, Prchla et al. 1995, Suomalainen et al. 1999). Also BSA-conjugated SV40 nuclear localization signals have been used to detect endosomal disruption or pore formation (Meier et al. 2002). Evidence for the different escape mechanisms used by adenoviruses or human rhinovirus type 2 (HRV2) has been found by cointernalization of differently sized dextrans with the viruses or virus proteins. Adenovirus was found to be able to release endosomal dextran almost size-independently whereas release caused by HRV2 was shown to be highly size dependent (Prchla et al. 1995). It is known that enveloped viruses use fusion with the plasma membrane or with endosomal membranes to reach the cytoplasm (Marsh 1984). However, membrane interactions of nonenveloped

viruses are less well understood, but rhinovirus, poliovirus and coxsasckie virus are known to be able to interact with lipid bilayers (Pérez & Carrasco 1993, Van Kuppeveld et al. 1996, Tosteson & Chow 1997). Viral fusion domains display some similarities, such as a 15-30 amino acid region consisting of an alternating pattern of hydrophobic and hydrophilic amino acids. Most of these domains are able to form amphipathic □-helices (Plank et al. 1998).

2.3.3 Cytoplasmic trafficking and nuclear import

Penetration into the cytoplasm is insufficient to allow the replication of many virus species, because most DNA viruses (except poxviruses), as well as some RNA viruses (such as retroviruses and influenza viruses) replicate in the nucleus. The nucleus provides cellular factors needed for the amplification and transcription of their genomes as well as for the posttranscriptional processing of viral mRNA. Therefore, these viruses have to traverse through the cytoplasm and enter the nucleus after penetrating the cellular membranes (Cullen 2001). Cytoplasm restricts free diffusion of molecules larger than 500 kDa because of the presence of organelles, the cytoskeleton, and the high protein concentration (Luby-Phelps, 1994, Luby-Phelps 2000). Thus, passive movement of virions or subviral particles is virtually impossible, implying that their cytoplasmic trafficking must be an active process. Cytoplasmic trafficking depends on cytosolic filaments: microtubules, intermediate and actin filaments (Sodeik 2000). A well known form of cell-assisted pathogen movement is the actin polymerization-dependent motility of cytosolic *Vaccinia* virus as well as *Shigella* and *Listeria* bacteria (Cudmore et al. 1997, Ireton & Cossart 1997, Welch et al. 1997). The involvement of microtubules in cytoplasmic traffic has been reported for a number of viruses, and dynein-mediated transport has been described for adenovirus (Leopold et al. 2000, Suomalainen et al. 1999, Suomalainen et al. 2001), human foamy virus (Saib et al., 1997), herpes simplex virus type 1 (HSV-1) (Döhner et al. 2002, Sodeik et al. 1997), and African swine fever virus (ASFV) (Alonso et al. 2001). In the case of HSV-1, the viral nucleocapsid protein (U_L34) interacts with the cytoplasmic dynein intermediate chain (Ye et al. 2000), while for ASFV, viral protein p54 interacts with the cytoplasmic dynein light chain (Alonso et al. 2001).

To gain access to the nucleus viruses can wait until the cell undergoes mitosis, when the nuclear envelope is temporarily lost, allowing the viral genome to become part of a newly assembled nucleus. Viruses using this strategy (many retroviruses) are able to replicate only in dividing cells. More commonly, the genome of incoming viruses is transported to the nucleus, through the nuclear envelope of the interphase nucleus allowing replication also in non-dividing cells (Whittaker et al. 2000). Nuclear pore complex (NPC) has been shown to play a critical role in viral nuclear import processes (Whittaker & Helenius 1998, Cullen 2001). NPCs are large structures, which form gated aqueous channels through the nuclear membrane (Davis 1995). Generally all nucleocytoplasmic transport occurs *via* the aqueous channel of NPCs (Whittaker & Helenius 1998, Whittaker et al. 2000, Cullen 2001). Active nuclear import is mediated by nuclear transport receptors (termed importins or karyopherins) (Görlich & Mattaj 1996, Görlich 1997, Nigg 1997). Targeting and transport of viral genomes to the nucleus depends on a nuclear localization signal (NLS) exposed on the surface of the viral particle or virus protein (Whittaker et al. 2000). NLS-mediated translocation through the NPC of nuclear

envelope occurs by sequential steps involving a series of interactions between cargos, nuclear transport receptors and nuclear pore proteins of the central transport channel (Bayliss et al. 2000). Passive diffusion through the nuclear membrane is limited to substrates of approximately 25 Å or less, while NLS containing substrates up to 300 Å in diameter can enter the nucleus via the NPC (Feldherr et al. 2001). This suggests that the functional diameter of the NPC is substantially smaller than most viruses. Only few viruses are small enough to pass through the NPCs with their capsid shells. Viruses have different strategies to circumvent this size limitation. Most of the viruses undergo partial or full dissassembly before nuclear entry. For example the large nonenveloped adenovirus enters cells by endocytosis and the viral particles are delivered in partially dissassembled (fiber-free) form to the cytosol (Greber et al. 1993, Whittaker & Helenius 1998, Whittaker et al. 2000). In the cytosol, the virus undergoes a series of further disassembly events and a partially uncoated adenovirus capsid binds to the NPC. After docking to the NPC the viral DNA and viral DNA condensing proteins are imported to the nucleus (Greber et al. 1997).

2.4 Parvoviridae

2.4.1 Taxonomy

Viruses of *Parvoviridae* are divided to two subfamilies: the *Parvovirinae*, which infect vertebrates and *Densovirinae*, which infect insects. Both subfamilies are further divided to three genera (Siegel et al. 1985, Berns 1996). Only the *Parvovirinae* will be further discussed here. Parvovirinae includes three genera *Parvovirus, Erythrovirus,* and *Dependovirus* also known as adeno-associated virus (AAV) genus. Viruses of the genera *Parvovirus* as well as *Erythrovirus* are autonomous viruses replicating without helper virus infection. However, members of both *Parvovirus* and *Erythrovirus* needs functions of host cells supplied during cellular S phase for their DNA replication (Siegel et al. 1985, Cotmore & Tattersall 1987, Berns 1996). AAVs require helper a virus (adenovirus or herpesvirus) co-infection for their replication (Janik et al. 1981, Siegel et al. 1985, Berns & Bohenzky 1987). In the absence of helper virus AAV virion is able to infect the host cell efficiently but the genome undergoes an incomplete replication and integrates into the host genome to establish latency (Kotin et al. 1990, Samulski et al. 1991).

Parvovirinae can be divided to three evolutionary groups based on the analysis of virtually full length genomes of the subfamily members (Table 1). Parvovirus species appear to have evolved by both host dependent and independent evolution according to phylogenetic analysis (Lukashov & Goudsmit 2001). Canine parvovirus (CPV) is highly homologous to feline panleukopenia virus (FPV) and mink enteritis virus (MEV). These viruses are classified as host range variants of feline parvovirus (Parrish 1990). FPV and CPV have greater than 98% sequence identity but a distinct host range (Truyen 1992). However, CPV productively infects both canine and feline cell lines in cultures (Parrish & Carmichael 1986).

TABLE 2 The three evolutionary groups of Parvovirinae including virus species presented in this thesis with their abbreviations and host species. Modified from Chapman & Rossmann 1993, Berns 1995, Lukashov & Goudsmit 2001.

Virus species	Abreviated names	Host
Genus		
Parvovirinae		
1. PARVOVIRUSES OF CARNIVORES, RODENTS AND PIG		
Parvovirus		
Minute virus of mice	MVM	mice
Feline parvovirus	FPV	cat
Canine parvovirus	CPV	dog
Mink enteritis virus	MEV	mink
Porcine parvovirus	PPV	pig
Aleutian mink disease parvovirus	ADV	mink
2. ADENO-ASSOCIATED VIRUSES (AND AVIAN PARVOVIRUSES)		
Dependovirus		
Adeno-associated virus serotype 5	AAV-5	human
Adeno-associated virus serotype 4	AAV-4	human
Adeno-associated virus serotype 2	AAV-2	human
Adeno-associated virus serotype 1	AAV-1	human
Adeno-associated virus serotype 6	AAV-6	human
Adeno-associated virus serotype 3	AAV-3	human
3. AUTONOMOUS PARVOVIRUSES OF PRIMATE AND BOVINE PARVOVIRUS		
Parvovirus		
Bovine parvovirus	BPV	cattle
Human parvovirus B19	B19	human

2.4.2 Genome

Parvoviruses have a single copy of a linear single stranded DNA molecule about 5 000 nucleotides long. Most autonomous parvoviruses encapsidate primarily DNA strands of a negative polarity, while dependoviruses, AAVs, package strands of both polarities with equal frequency (Berns & Adler 1972, Cotmore & Tattersall 1987, Berns 1996). Terminal palindromic sequences are located at both ends of the parvoviral genome. These fold into stable hairpin structures. The protein coding regions appear to span almost the entire genome. The genome consists of two large non-overlapping open reading frames (ORFs) and a number of smaller ORFs, the exact size and location of which varies from virus to virus. The long left hand (5′) ORF encodes two nonstructural proteins and the right hand (3′) ORF encodes capsid proteins by alternate splicing (Cotmore & Tattersall 1987, Berns 1996).

2.4.3 Proteins

In general, the parvovirus capsid contains 60 protein subunits and consists of 90% of VP2 (67 kDa) and 10% VP1 (83 kDa). Proteins are encoded by alternative splicing of the messenger RNA (Tattersall et al. 1976, Cotmore & Tattersall 1987, Reed et al. 1988). In full capsids containing DNA, some N-termini of VP2 are exposed on the outside of the capsid. These are cleaved by host cell proteases from the N-terminus to form VP3 (65 kDa) (Paradiso et al. 1982, Tullis et al.

1992, Weichert et al. 1998). However, human parvovirus B19 and Aleutian disease viruses have only two capsid proteins, VP1 and VP2 (Cotmore & Tattersall 1987). VP1 contains the complete amino acid sequence of VP2 and an extra 150-230 amino acids at the N-terminal end (Jongeneel et al. 1986, Cotmore & Tattersall 1987).

In CPV and minute virus of mice (MVM) virions the unique N-teminal sequence of VP1 is enclosed within the capsid, but it can be exposed by heat or urea treatment without complete disintegration of the capsid (Weichert et al. 1998, Cotmore et al. 1999). The VP1 unique region of most parvovirus capsids contains a conserved motif that resembles the catalytic site of cellular secreted type phospholipase A_2 ($sPLA_2$) (Zádori et al. 2001). In porcine parvovirus (PPV), B19 and AAV capsids this parvoviral PLA_2 ($pvPLA_2$) motif has been reported to have catalytic activity (to catalyze the hydrolysis of phospholipid substrates at the 2-asyl ester position to relese lysophopholipids and free fatty acids) of $sPLA_2$ (Zádori et al. 2001, Dorsch et al. 2002, Girod et al. 2002). An amino acid sequence of PPV located on the C-terminal side of the $sPLA_2$-like site has been found to increase the catalytic activity of $pvPLA_2$. The $pvPLA_2$ of PPV or B19 does not exhibit substrate specificity hydrolyzing phosphatidylcholine, phosphatidylethanolamine and phophatidylinositol phospholipids in the mixed micelles assay (Zádori et al. 2001). The VP1 unique region also contains several basic sequences resembling the classical NLS, and one of these (PAKRARRGYK) containing N-terminal residues 4-13, has been shown to facilitate the nuclear import of bovine serum albumin (BSA) when coupled to this protein (Vihinen-Ranta et al. 1997). In CPV VP1, the PLA_2-domain and NLS containing regions are separated by 20 amino acids (Vihinen-Ranta et al. 1997, Zádori et al. 2001). A basic amino acid rich sequence (KGKLTMRAKLR) of MVM VP2 has been reported to be associated with nuclear import in a conformation-dependent manner (Lombardo et al. 2000). However, the equivalent sequence of CPV does not mediate nuclear import as shown by point mutation studies. Instead the assembly of the capsids was affected by these mutations (Vihinen-Ranta 1997, Yuan & Parrish 2000). The role of VP3 is not clear, but it has been suggested that VP3 might be needed for interactions with cellular membranes at some point of infection (Tattersall & Cotmore 1988).

In addition to capsid proteins, parvoviruses have two different nonstructural proteins named NS1 and NS2 (Cotmore & Tattersall 1987). NS1, or Rep 68/78 (AAV), is a large pleiotropic nuclear phosphoprotein (83 kDa) that exhibits site-specific DNA binding, ATPase, helicase and nickase activities (Nuesch et al. 1995, Ward & Berns 1995, Nuesch et al. 1998, Maxwell et al. 2002). NS1 is required for productive virus replication in all host cell types by serving as initiator for viral DNA replication and as a potent transcriptional activator of viral promoters (Naeger et al. 1990, Cotmore et al. 1995). NS1 initiates the DNA replication by binding to a specific sequence in the viral replication origin and introducing a single strand nick after which it appears to play an essential role in the establishment and maintenance of the replication fork (Nuesch et al. 1995). Furthermore, NS1 is described as the major factor of parvovirus-induced growth inhibition and cytotoxicity effects on the host cells (Caillet-Fauquet et al. 1990, Legrand et al. 1993, De Beeck et al. 2001, Morita et al. 2003).

A small nonstructural protein NS2 (25 kDa), formed from a spliced messager RNA, shares a common N-terminal region with NS1 (Cotmore & Tattersal 1986). NS2 may exist in at least two forms with a slightly different C-terminal amino acid sequence (Jongeneel et al. 1986). NS2 is expressed at high

levels early in infection and each subtype of NS2 exists in approximately equal amounts of phosphorylated and nonphosphorylated forms. The nonphosphorylated form of NS2 shows only cytosolic localization while the phosphorylated form shows a more uniform distribution in both cytosol and nucleus of infected cells (Clemens et al. 1990, Cotmore & Tattersall 1990). The role of NS2 is yet to be elucidated, but it has been shown to be required for productive MVM and H-1 virus infection in cells of natural host species (Naeger et al. 1990, Li & Rhode 1991, Brownstein et al. 1992). In contrast, NS2-null mutant CPV replicates efficiently, suggesting that CPV NS2 does not have a similar role (Wang et al. 1998).

2.4.4 Virion

The parvoviral genome is surrounded by a protein coat containing 60 copies of a mixture of three capsid proteins VP1, VP2 and VP3. One of the proteins (generally VP2) is the major structural protein composing about 90% of the capsid. The capsid is arranged with the icosahedral T=1 symmetry forming symmetrical structure in shape which has 20 faces (Fig. 1). Roughly spherical parvoviral capsids have a diameter of 18 to 26 nm (Chapman & Rossmann 1993). Interactions between DNA and capsid proteins are predominantly nonionic suggesting some sequence specificity (Xie & Chapman 1996). Parvoviral capsids lack lipids and carbohydrates (Berns 1990). The parvoviral particle molecular weight is 5.5 to 6.2 x 10^6 Da, 19-32% of which is viral DNA in infectious particles (Cotmore & Tattersall 1987, Siegel et al. 1985). Because of the high DNA to protein ratio, the buoyant density of the intact virion in CsCl is 1.39 to 1.47 g/cm^3 and the sedimentation coefficient in neutral sucrose gradients is 110-122. Parvoviruses are resistant to inactivation. Particles remain stable between pH 3-9 and at 56 °C for 1 h. The viruses can be inactivated by formalin, beta-propiolactone, hydroxylamine or by oxidizing agents (Cotmore & Tattersall 1987).

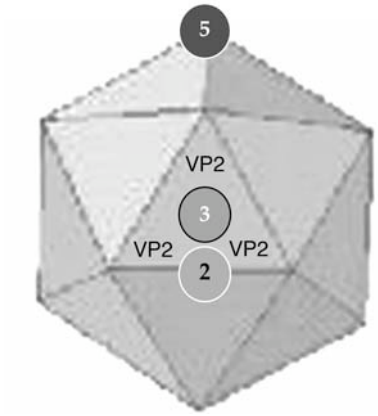

FIGURE 2 Scematic model of CPVcapsid. VP2, show the organization of them in one face of the capsid related to threefold symmetry axes. Numbers indicating (one example of each) axes of symmetry: 2, twofold; 3, threefold; 5, fivefold.

The atomic structures of CPV, FPV, MVM and AAV capsids have been solved at better than 3.5 Å resolution, while B19 capsids have been determined to 8 Å (CPV: (Tsao 1991 Wu & Rossmann 1993), FPV: (Agbandje et al. 1993), MVM: (Agbandje-Mckenna et al. 1998), B19:(Agbandje et al. 1994). In general, the

surface of icosahedral parvoviral capsids include protrusions (called spikes) at the threefold axes, canyon-like depressions and channels at fivefold axes and depressions (dimples) at the twofold axes (Tsao 1991 Agbandje et al. 1993, Chapman & Rossmann 1993, Wu & Rossmann 1993). The channels at the fivefold axis appear to be closed in ADV and B19 capsids (McKenna et al. 1999). The special loop structures of the protrusions distinguish the parvovirus capsid structure from earlier known RNA virus structures having a similar antiparallel β-barrel structure ("jelly-roll") of capsid proteins (Rossmann & Johnson 1989, Xie & Chapman 1996). These loops make up the most of the exposed surface of the capsid (Tsao 1991, Agbandje et al 1993, Chapman & Rossmann 1993, Wu & Rossmann 1993, Agbandje-Mckenna et al. 1998). Conformation of the loops varies significantly from in *Parvoviridae*. In CPV one of the loops has been suggested to bind two to three divalent ions, most probably calcium ions (Simpso et al. 2000). In full capsids the N-terminal 19-20 amino acid residues of about 50% of VP2 are exposed to the outside of the virion (Weichert et al. 1998), probably by passing through channels at the fivefold symmetry axes (Xie & Chapman 1996). Also 24 nucleotides of the 5´end of the DNA genome are exposed outside of the capsid. Large nonstructural protein attaches to these nucleotides during the assembly of the capsid (Cotmore & Tattersall 1989, Wang & Parrish 1999). A lysosomal protease, cathepsin B, has been reported to cleave VP2 to VP3 at pH < 5.5 (Weichert et al. 1998).

The sequences of the capsid proteins that control the host range of CPV, MEV, FPV and MVM are on, or close to, the capsid surface, located at the top and shoulder of the spikes and in or near the dimples (Gardiner & Tattersall 1988, Ball-Goodrich et al. 1991, Parrish et al. 1991, Ball-Goodrich & Tattersall 1992, Chang et al. 1992, Truyen et al. 1996, Horiuchi et al. 1994).

2.4.5 Clinical manifestations

In general, autonomous parvoviruses are teratogenic agents. They cause fetal and neonatal abnormalities by destroying specific cell populations, which proliferate rapidly during the normal course of development (Siegel 1984). For example human pathogen of the *Erythrovirus* genus, human parvovirus B19, is known to cause rash, arthropathy, fetal hydrops and death, and haematological complications (Naides et al. 1990). In contrast, AAVs have not been found to be associated with disease in either humans or animals (Srivastava 1994, Rabinowitz & Samulski 2000). Because AAVs are nonpathogenic, able to integrate in a site specific way and have a wide host ranges, theyhold promise for human gene therapy applications (Kotin et al. 1991, Samulski et al. 1991, Chatterjee et al. 1995).

CPV appears to cause disease only in canidae (Pollock & Carmichael 1990). The clinical signs are first observed 4 to 7 days after oro nasal exposure (Macartney et al. 1984, Pollock & Carmichael 1990). In natural infections, CPV starts its replication in the oropharyngeal lymphoid tissue and virus spreads hematogenously to other lymphoid organs and the intestine (Cotmore & Tattersall 1987). As parvovirus requires cycling cells for viral replication, infection of the fetus or newborn (dog) may be pantropic infecting a wide range of cells in various organs (Fenner et al. 1987). Furthermore, three distinct age related syndromes have been reported including rare generalized infections of neonatal dogs, myocarditis as an acute disease of pups and a panleukopenia enteritis of dogs of all ages (Fenner et al. 1987). There is no treatment for

parvoviral myocarditis and treatment of the enteric form of the infection is only supportive. Generally, domestic canines are vaccinated against the parvovirus enteritis. Vaccines have been developed from attenuated live CPV or FPV strains, or from inactivated CPV, FPV or MEV (Pollock & Carmichael 1982, Saliki et al. 1992). More recently vaccines have also been developed from synthetic peptides and recombinant proteins (Langeveld et al. 1995).

2.4.6 Life cycle of parvoviruses

2.4.6.1 Cell surface receptors

Several molecules, mostly carbohydrates or proteins have been reported to be the cell receptors for different parvoviruses. Adeno- associated virus serotype 2 (AAV-2) can use a heparin sulphate proteoglycan (HSPG) for attachment to the cell surface and to enter host cells (Summerford & Samulski 1998), although mutants that do not bind to the HSPG are still infectious (Shi et al. 2001). Some other receptors including the □V□5 integrin and the basic fibroblast growth factor receptor 1 have also been suggested to be involved in the interaction between AAV-2 and the host cell (Summerford & Samulski 1998, Sanlioglu et al. 2000). For AAV-4 and AAV-5 sialic acids have also been suggested to function as a receptors (Kaludov et al. 2001, Walters et al. 2001, Bantel-Schaal et al. 2002).

Human parvovirus B19 uses the glycolipid globoside as a receptor (Brown et al. 1993, Chipman et al. 1996). However, a number of cells expressing globosides are not susceptible to B19 infection (Liu et al. 1992, Brunstein et al. 2000). Moreover, bovine parvovirus (BPV) binds to sialylated erythrocyte membrane glycoproteins and to the major membrane glycoprotein, glycophorin A (Thacker & Johnson 1998).

CPV and FPV have also been reported to interact with sialic acids on some cell lines. However, sialic acid binding does not play a significant role in the infection of cultured cells since non-sialic acid binding mutants of CPV and FPV infect the same range of cells as wild type viruses (Barbis & Parrish 1994). Instead, CPV and FPV are able to use the transferrin receptor (TfR) to bind and infect the cells. Differences between the feline and canine TfR control the binding of viruses to cells. Both CPV and FPV are able to bind and enter feline cells, but only CPV was bound to the canine cells when capsids were incubated with feline or canine cells at 37°C. The feline TfR expressed in Chinese hamster ovary (CHO) cells allowed CPV and FPV to bind and infect the cells efficiently (Parker et al. 2001).

2.4.6.2 Endocytosis

Both autonomous parvoviruses including CPV and AAV have been reported to enter cells by clathrin-mediated endocytosis (Duan et al. 1999, Bartlett et al. 2000, Parker & Parrish 2000). Endocytosis of both CPV and AAV-2 has been shown to be inhibited by the overexpression of a dominant negative mutant of dynamin (Duan et al. 1999, Parker & Parrish 2000). However, AAV may also use noncoated vesicles to enter host cells (Douar et al. 2001, Bantel-Schaal et al. 2002). CPV, MVM, PPV and AAV infections can be inhibited by drugs increasing the pH of endosomal vesicles such as NH_4Cl and bafilomycin A_1

(Basak & Turner 1992, Bartlett et al. 2000, Parker & Parrish 2000, Douar et al. 2001, Hansen et al. 2001, Zádori et al. 2001, Ros et al. 2002) indicating that a low pH step during viral entry is required for productive infection. CPV and AAV capsids have been shown to colocalize with endocytoced transferrin, suggesting that the recycling pathway is part of their entry route (Duan et al. 1999, Parker & Parrish 2000) (Fig. 3). MVM and AAV capsids have been reported to traffic through both early and late endosomes prior to their nuclear import (Douar et al. 2001, Hansen et al. 2001, Bantel-Schaal et al. 2002, Ros et al. 2002) (Fig. 3). AAV-5 was detected in lysosome-like structures in immuno-EM studies and PPV was found to colocalize with lysosomal membrane protein 2 (LAMP-2) suggesting late endosomal or lysosomal location of these viruses during their entry process (Bantel-Schaal et al. 2002, Zádori et al. 2001) (Fig. 3). AAV-5 has also been detected in the TGN and in Golgi-associated vesicles at 3 to 22 h p.i. (Bantel-Schaal et al. 2002) (Fig. 3). Capsids of CPV and FPV remain associated with TfR-positive structures for several hours after uptake into cells as cytoplasmically microinjected antibodies against the TfR cytoplasmic tail reduced CPV and FPV infection when injected into cells 4 h p.i. or earlier (Parker et al. 2001) (Fig. 3). Moreover, antibodies against the CPV capsid injected into the cytoplasm prevented viral infection when injected as late as 6 hours p.i., indicating that release from endocytic vesicles begins several hours after the inoculation (Vihinen-Ranta et al. 2000, Vihinen-Ranta et al. 2002). Also other parvovirus capsids such as MVM, PPV and AAV have been reported to stay in perinuclear vesicles for several hours (Duan et al. 1999, Zádori et al. 2001, Ros et al. 2002).

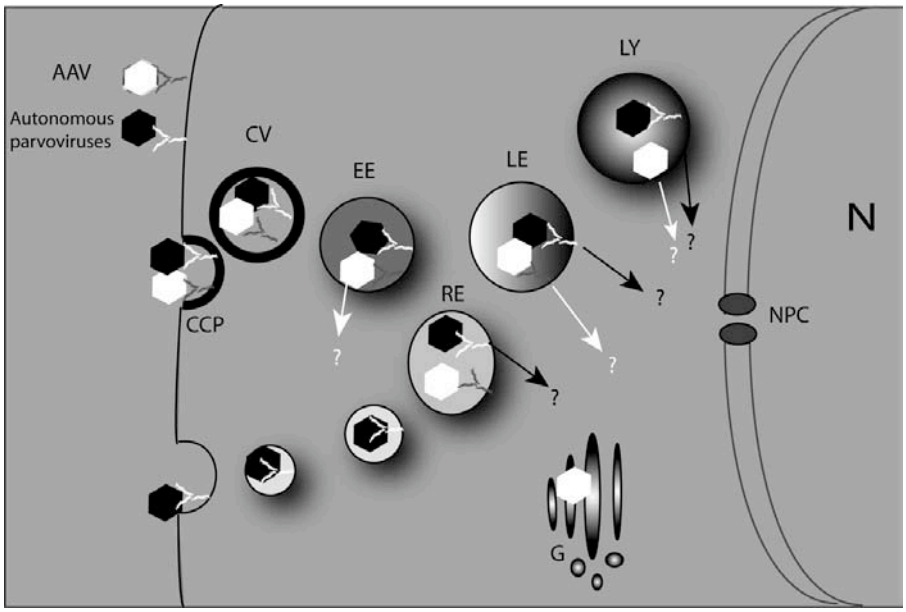

FIGURE 3 Potential entry routes of parvoviruses indicating the organelles of the endocytic system where parvoviruses have been observed, and the organelles from which viruses have been suggested to be released to the cytoplasm.
AAV, white; Autonomous parvoviruses, black. CCP, clathrin coated pit; CV, clathrin coated vesicle; EE, early endosome; RE, recycling endosome; LE, late endosome; LY, lysosome; G, Golgi apparatus; NPC, nuclear pore complex; N, nucleus, arrows indicate suggested organelles of endosomal escape.

The mechanism behind parvoviral escape from endocytic vesicles into the cytosol is still unknown. However, it has been reported that penetration of CPV through endosomal membranes does not cause the disruption of the vesicles, as coendocytoced -sarcin is not released to the cytoplasm with CPV (Parker & Parrish 2000). The role of the PLA_2 activity of the VP1 unique region of most parvoviruses has been studied by using PLA_2 inhibitors, and by mutants of the predicted active site suggesting that the viral PLA_2 activity is needed in the virus lifecycle between perinuclear accumulation and early gene expression (Li et al. 2001, Zádori et al. 2001, Dorsch et al. 2002, Girod et al. 2002).

2.4.6.3 Cytoplasmic trafficking and nuclear import

CPV seems to be intact upon release from endosomal vesicles, as antibodies against intact capsids microinjected to the cytoplasm were able to inhibit infection (Vihinen-Ranta et al. 2000). The cytoplasm is known to efficiently restrict the free diffusion of complexes larger than 500 kDa (Luby-Phelps 1994, Seksek et al. 1997, Luby-Phelps 2000). Thus, although endosomal trafficking carries the parvoviruses close to the nucleus, it is most probable that parvovirus particles (5 500 kDa) require an active transport mechanism to move to the site of nuclear entry. Furthermore, the transport of microinjected capsids to the perinuclear region was prevented by treatment of cells with the microtubule depolymerizing drug, nocodazole, suggesting that the cytoplasmic transport is a microtubule-mediated process (Vihinen-Ranta et al. 2000). After endosomal escape MVM capsids have been reported to be modified by the chymotrypsin activity of cellular proteosomes suggesting that proteasomes are needed for virus dissassembly or nuclear transport (Ros et al. 2002). Since the VP2 N-terminus contains a chymotryptic cleavage site (Maroto et al. 2000), it is possible that proteasomes are needed for the formation of VP3 (Ros et al. 2002) (Fig. 3). Also cytoplasmic AAV capsids have also been shown to be susceptible to proteosomes. In these reports proteosomal inhibitors considerable enhanced the AAV transduction, suggesting that the ubiquitin-proteasome pathway would be a barrier for AAV life cycle (Douar et al. 2001, Yan et al. 2002).

Small macromolecules can diffuse freely through the nuclear pore complex, but usually the transport of larger molecules requires GTP and soluble cytosolic factors, and is mediated by NLSs (Feldherr & Akin 1990, Nigg 1997, Gorlich & Kutay 1999, Feldherr et al. 2001). A VP1 sequence between residues 4 and 13 functions as NLS when conjugated to BSA (Vihinen-Ranta 1997). Also MVM capsids have NLS sequences in both VP1 and VP2 proteins (Tullis et al. 1992). Two NLS sequences are found in the VP1 N-terminus, while the VP2 appears to have a conformation-dependent basic NLS (Lombardo et al. 2000, Lombardo et al. 2002). The importance of the VP2 N-terminus in the nuclear translocation of AAV-2 virus like particles (VLP) has also been verified by N-terminal deletion analysis (Hoque et al. 1999).

Although parvovirus capsids are stable, many considerably mild treatments are able to change the capsid structure. The exposure of internal sequences and structures including the VP1 N-terminal region and the 3' end of the viral DNA of MVM and CPV takes place when capsids are heated or treated with urea (Christensen et al. 1997, Cotmore et al. 1999, Vihinen-Ranta et al. 2002). Having exposed VP1 N-termini makes it possible for the virus to use of the $pvPLA_2$ activity and the potential NLSs during entry (Vihinen-Ranta 1997, Zádori et al. 2001, Vihinen-Ranta et al. 2002), while the exposed 3' end of viral

genome has been suggested to act as a template for the DNA synthesis (Christensen et al. 1997) (Fig. 4). Cytoplasmically microinjected antibodies against the N-terminal unique region of VP1 block infection and specific point mutations or deletions in the VP1 N-terminal sequence reduce the infectivity of the capsids, suggesting that exposure of VP1 N-termini most probably occurs in endocytosed virions and that the VP1 N-terminus plays an essential role in the productive infection (Vihinen-Ranta 1997, Vihinen-Ranta et al. 2002) (Fig. 4). CPV capsids microinjected to the cytoplasm enter the nucleus slowly and they are recognized in the nucleus by antibodies against the nondegraded capsids, indicating that the whole parvovirus capsid may pass through the NPC (Vihinen-Ranta et al. 2000) (Fig. 4). AAV has also been suggested to be transported into the nucleus through the NPC (Sanlioglu et al. 2000).

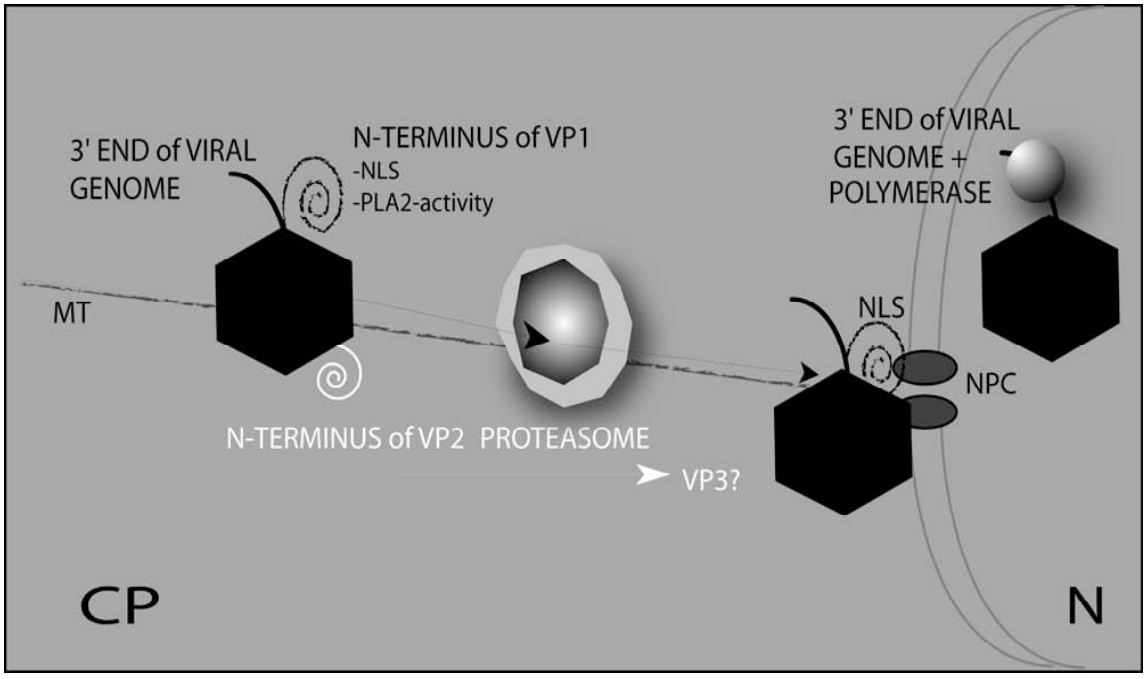

FIGURE 4 Schematic model of cytoplasmic trafficking and nuclear import of the autonomous parvoviruses.
CP, cytoplasm; MT, microtubule; N, nucleus; NLS, nuclear localization signal; NPC, nuclear pore complex; PLA_2, phospholipaseA_2.

2.4.6.4 DNA replication, gene expression and assembly

Parvoviral DNA replication occurs in host cell nucleus *via* double stranded replicative form, and is initiated by a self priming mechanism from 3' OH group (Rhode 1974, Cotmore & Tattersall 1987, Cotmore & Tattersall 1994, Berns 1996). The amplification of DNA proceeds with help of cellular DNA polymerases, through the formation of multimeric intermediates by the undirectional leading strand synthesis mechanism, named the modified rolling hairpin model (Tattersall et al. 1976, Astell et al. 1985, Cotmore & Tattersall 1992). Complementary strand synthesis of MVM has been shown to be activated by cyclin A and has been proposed to be dependent on DNA polymerase-□ (Cossons et al. 1996, Bashir et al. 2000). To be successfully replicated parvoviruses require viral nonstructural protein NS1 (Christensen et

al. 1997). Thus, nonstructural proteins are expressed first (Berns 1990). NS1 protein has been shown to serve both as an initiator protein for viral DNA replication, and as a transcriptional activator of viral promoters (Nuesch et al. 1995). In addition, accumulation of MVM NS1 protein has been shown to interfere with host cell DNA replication and cause cytostatic effect arresting cell cycle in S-phase and thus providing an ideal environmen for the virus (Op De Beeck & Caillet-Fauquet 1997). Newly expressed capsid proteins are transported to the nucleus where DNA packaging is suggested to take place (Wistuba et al. 1995, Tattersall & Cotmore 1988). The viral DNA is thought to be encapsidated into at least partly preformed empty capsids (Hoque et al. 1999, Tattersall & Cotmore 1988, Lombardo et al. 2000).

Parvovirus life cycle has been considered to be lytic leading to the degeneration of both of the nucleus and the plasma membrane (Richards et al. 1977). Recently, NS2 of MVM was found to contain a nuclear export signal and the importance of NS2 protein in nuclear egression of progeny virions was shown by point mutations (Eichwald et al. 2002). NS1 proteins of many parvoviruses (such as MVM, H-1, B19) have been found to be cytotoxic for their host cells (Ozawa et al. 1988, Li & Rhode 1990, Legendre et al. 1992). B19 infection, as well as, expression of B19 NS1 protein in erythroid cells has been shown to induce apoptosis suggesting that progeny virions may be released from the host cell by mechanism as programmed cell death (Sol et al. 1999).

3 AIM OF THE STUDY

The specific aims of this study were:

1. To define the mechanisms of receptor mediated endocytic entry of CPV.
2. To study the role of parvoviral PLA_2 in endosomal escape of CPV.
3. To define the role of microtubules and motor proteins in CPV entry process.
4. To clarify the macromolecular composition (DNA, DNA and some viral proteins, or whole virion) of the viral material released into the cytoplasm from endosomes.

4 SUMMARY OF MATERIALS AND METHODS

4.1 Cells and viruses

Norden laboratory feline kidney (NLFK) cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, Paisley, United Kingdom). The canine parvovirus type 2, CPV-d (Parrish et al. 1982) was derived from infectious clone of CPV (p265) by transfection of NLFK as previously described (Parrish 1991, Parker et al. 2001). CPV was grown in NLFK cells in 175 cm^2 cell culture flasks (Nunc, Roskilde, Denmark) for 5 to 7 days and 300 ml of culture medium from infected cells was clarified by centrifugation and concentrated by ultrafiltration (500 kDa filter, Millipore Corporation, Bedford, MA). The CPV was pelleted by ultracentrifugation at 245 000 x g for 1 h and resuspended in 0.9 ml of phosphate buffered saline, pH 7.4 (PBS). Suspension was sonicated using low power and extracted with chloroform. Full (H-CPV, with DNA) and empty (L-CPV, without DNA) capsids were separated from the aqueous phase of chloroform extraction by isopycnic centrifugation in a 45% cesium chloride gradient, opalescent bands were collected with a syringe and capsids were pelleted by ultracentrifugation at 245 000 x g for 2 h and the pellets were resuspended in 100 μl of PBS. Plaque forming unit (PFU) titer of the full capsids stocks was approximately $1x10^9$ and empty capsids formed no plaques (I, II, III).

4.2 Antibodies

TABLE 3 Antibodies used in this study.

Antigen	Antibody made in	Antibody source
PRIMARY ANTIBODIES		
CPV capsid	Rabbit	Gift from Colin Parrish (Cornell University, Ithaca, New York, N.Y.)
CPV capsid (Mab 8)	Mouse	Gift from Colin Parrish
NS1	Mouse	Obtained from Caroline Astell (Yeung et al. 1991)
CPV unique sequence of VP1 N-terminus	Mouse	Gift from Colin Parrish
A part of the unique region of VP1	Rabbit	Gift from Colin Parrish (Originated from Prof. P. Tattersals laboratory (Yale University, New heaven, Conn.))
TGN-38	Mouse	Sigma, St. Luis, Mo.
LAMP-2	Mouse	Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA.
ß-COP	Rabbit	Gift from Albrecht Gruhler (R. W. Johnson Pharmaceutical Research Institute, San Diego, CA)
CiMPR	Rabbit	Gift from Varpu Marjomäki (University of Jyväskylä, Jyväskylä,Finland)(Juuti-Uusitalo, et al., 2000)
Caveolin	Rabbit	Gift from Varpu Marjomäki

Continues

Table 3 continues

Antigen	Antibody made in	Antibody source
Disulphide Isomerase (PDI, clone 1D3)	Mouse marker for ER	Gift from Varpu Marjomäki Originated from Stephen Fuller (Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK).
Dynein (Intermediate chain)	Mouse	Chemicon, Temecula, CA.
Kinesin	Mouse	Chemicon, Temecula, CA.
Tubulin	Mouse	Amersham, Buckinghamshire, UK.
TfR (CD71)	Mouse	Dako, Carpinteria, CA.
TfR	Mouse	Molecular Brobes, Eugene, Oreg.
Trout IgG	Mouse	Gift from Ilmari Jokinen (University of Jyväskylä, Jyväskylä, Finland)

SECONDARY ANTIBODIES

Antigen	Antibody made in	Antibody conjugated to	Antibody source
Streptavidin	Goat	Biotin	Molecular Probes, Eugene, OR.
Mouse IgG	Goat	FITC	Cappel, ICN Pharmaceuticals, Inc., OH.
Rabbit IgG	Goat	TRITC	Cappel, ICN Pharmaceuticals, Inc., OH.
Mouse IgG	Goat	Alexa fluor-546	Molecular Probes
Rabbit IgG	Goat	Alexa Fluor-488	Molecular Probes
Mouse IgG	Goat	Alexa Fluor-488	Molecular Probes

Continues

Table 3 continues

SECONDARY ANTIBODIES

Antigen	Antibody made in	Antibody conjugated to	Antibody source
Goat IgG	Donkey	Alexa Fluor-488	Molecular Probes
Mouse IgG	Rabbit	Nanogold	Nanoprobes, Yaphank, N.Y.
Rabbit IgG	Goat	Nanogold	Nanoprobes
Rabbit IgG	Swine	Alkaline phosphatase	Dako, Glostrup, Denmark
Rabbit IgG	Swine	horse raddish peroxidase	Dako, Glostrup, Denmark

4.3 Immunofluorescence microscopy

For immunofluorescense studies, 80% confluent cells grown on coverslips (diameter 13 mm) were infected with CPV particles containing DNA. After different times post infection (p.i.), coverslips were rinsed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. After incubation in permeabilization buffer (1% BSA, 0.1% Triton X-100 and 0.01% sodium azide in PBS) for 15 min, the coverslips were incubated with primary antibodies diluted in permeabilization buffer for 45 min at room temperature, and then rinsed with permeabilization buffer before incubation with secondary antibody (45 min at room temperature). Finally, the coverslips were rinsed again with permeabilization buffer and embedded with Mowiol containing the antifading agent DABCO (30 mg/ml). Confocal microscopy was conducted with a Zeiss LSM 510 (equipped with Nomarski DIC) (I, II, III).

4.4 Fluorecence *in situ* hybridization (FISH)

The DNA probe for FISH to label viral DNA was made from plasmid clone of CPV p265, containing the total genomic sequence of the virus (gift from Colin Parrish, Cornell University, Ithaca, New York, NY.) by Biotin Nick-Translation Mix (Boehringer Mannheim, Mannheim, Germany). The size of the labeled probe was analyzed with electrophoresis to be the optimal 200-500 nucleotides. The probe was separated from the labeling mixture by precipitation with ethanol. NLFK cells were grown on coverslips (diameter 13 mm) to 80% confluency and inoculated with purified CPV for 15 min at 37 °C and then incubated in DMEM for 0-24 h. After which cells were fixed with methanol and after washes with PBS, the capsid proteins were labeled with polyclonal antibody and TRITC-conjugated anti-rabbit antibody. The samples were then post-fixed with 1% PFA, washed with PBS, and then dehydrated and

rehydrated with alcohol series (70, 90, 100, 90, 70%) for 30 s each. Hybridization was carried out in buffer containing 60% deionized formamide, 1.5 M NaCl, and 0.15 M sodium citrate, 10 mM EDTA, 25 mM NaH_2PO_4, 5% dextran sulphate, 250 ng/μl herring sperm DNA and 30 ng/μl probe. The probe was denatured for 5 min at 80 °C. The chamber was humidized with 50% deionized formamide, 0.3 M NaCl and 0.03 M sodium citrate and hybridization was carried out overnight at 37 °C. Coverslips were washed two times with 1.5 M NaCl and 0.15 M sodium citrate in 50% deionized formamide and two times with PBS. Samples were blocked with 3% BSA-PBS for 10 min and all antibodies were diluted in 3% BSA-PBS. The incubations were carried out in a humid chamber for 1 h at room temperature. The biotinylated DNA probe was first labeled with streptavidin conjugated Alexa Fluor-488, which was then recognized with biotinylated goat anti-steptavidin antibody. To get maximal labeling anti-goat Alexa Fluor-488 and Alexa Fluor-488 conjugated streptavidin were added to label both biotin and Fc-part of the biotinylated goat anti-streptavidin antibody (Fig. 5). Samples were mounted in Mowiol-DABCO (30 mg/ml) and examined using a LSM.

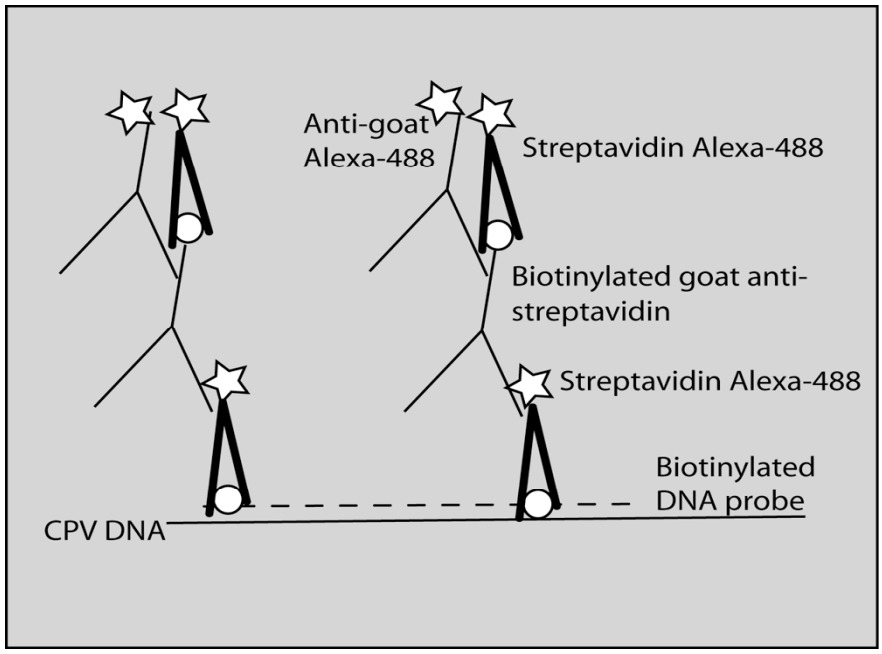

FIGURE 5 Schematic representation of FISH labeling.

4.5 Immunoelectron microscopy

4.5.1 Pre-embedding labeling with gold-conjugated protein A

Cells were grown on 35 mm plastic culture dishes to 80% confluency. After rinsing with ice cold 0.1 M phosphate buffer (pH 7.4), the cells were treated with CPV (0.05 μg/ml) in DMEM for 1 h on ice. After washes with ice-cold 0.1 M phosphate buffer, polyclonal rabbit antiserum to CPV diluted in 0.1 M phosphate buffer was applied for 1 h at 0 °C, followed by protein-A gold conjugate for 45 min at 0 °C. After washes, the cells were either fixed

immediately with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 4 °C, or first warmed up in DMEM to 37 °C for from 0.5 to 3 h periods of time and then fixed. After three rinses in 0.1 M phosphate buffer (pH 7.4) the cell cultures were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4 °C, dehydrated in ethanol, stained with uranyl acetate, and embedded in Epon LX-112. Thin sections were cut and poststained with lead citrate and uranyl acetate (I).

4.5.2 Pre-embedding labeling with antibody-conjugated nanogold

Cells were washed twice with PBS and then fixed for 2 h at RT in PLP (2% PFA, 0.01 M periodate, 0.075 M lysine, and 0.075 M phosphate buffer) fixative (Brown & Farquhar 1989). Fixed cells were prepared for pre-embedding labeling as described previously (Sawada & Esaki 1994, Sawada & Esaki 2000). Shortly, cells were treated with 0.01% saponin and 0.1% BSA in 0.1 M phosphate buffer (pH 7.4) for 8 min at RT before adding the antibody diluted in buffer A for 1 h. Some samples were treated with 0.05% Triton X-100 in phosphate buffer before adding the antibody to ensure that the nuclear membrane became permeabilized. After washes with buffer A, nanogold-conjugated secondary antibody was applied for 1 h, followed by washes with buffer A and 0.1 M phosphate buffer (pH 7.4). Cells were post-fixed with 1% glutaraldehyde in phosphate buffer for 10 min at RT. Then cells were quenched with 50 mM NH_4Cl in phosphate buffer, washed with phosphate buffer and water. Silver enhancement was carried out in the dark with HQ-silver for 2 to 4 min. Then cells were washed with distilled water and silver was protected with gold toning (2% sodium acetate 3 x 5 min, 0.05% gold chloride 10 min on ice, 0.3% sodium thiosulphate 2 x 10 min on ice). After washes with water, the cells were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4 °C and dehydrated with a descending concentration series of ethanol, and stained with 2% uranyl acetate. Plastic capsules filled with Epon LX-112 (Ladd, Research industries, Williston, VT) were placed upside-down on top of the cells. After polymerization, the capsules were warmed up to 100 °C, removed carefully and horizontal sections were cut with an ultramicrotome (Ultracut 8008, Reichert-Jung), picked up on a copper or nickel grid, and stained with 2% uranyl acetate and lead citrate (II, III).

4.5.3 Post-embedding labeling of cryosections

Cells were inoculated with CPV at 37 °C for 15 min and incubated in DMEM for 1 h or 3 h. Dishes were then washed with PBS and the cells were fixed with 4% PFA, 0.1% GA, 0.01 sodium azide and 7.5% sucrose in PBS for 1 h at room temperature. After fixation, the cells were washed with PBS and scraped off with a rubber policeman, sedimented, resuspended in 10% warm gelatin in PBS and sedimented again and placed on ice for 1 h. Then cells were cut out of the tubes, fixed with 4% PFA in PBS for 1 h and infused a mixture containing 15% polyvinylpyrrolidone and 1.7 M sucrose. Blocks were placed on a cutting pin, trimmed and immedeately frozen under liquid nitrogen, where they were stored until sectioning (Tokuyasu 1973, Tokuyasu 1978). The sections were labeled with mouse monoclonal antibody to CPV capsids followed by rabbit anti-mouse antibody and by gold-conjugated protein A. The sections were incubated with antibodies for 45 min at room temperature and washed with

PBS after each labeling step and finally grids were embedded with methyl cellulose (1.5%)/uranyl acetate (0.4%) (I).

A JEOL JEM-1200EX transmission electron microscope operated at ~60kV was used in the pre-embedding or cryosectioning electron microscopy (I, II, III).

4.6 Microinjection

Cytoplasmic microinjection into NLFK cells was performed using a Transjector 5246 and Micromanipulator 5171 (Eppendorf, Hamburg, Germany) connected to an inverted IMT-2 microscope (Olympus Optical Co., Tokyo, Japan). Needles were pulled from glass capillaries (diameter 1.2 mm, Clark Electromedical Instruments, Reading, UK) using a P-97 needle puller (Sutter Instruments, Novato, CA) or, in some cases commercial microinjection needles (Eppendorf) were used (I, II, III). Cells were grown to 80% confluency on microgrid coverslips (175 nm grid size; Eppendorf) (II, III) or on 35 mm plastic culture dishes for EM studies (II). Cells were injected with antibodies (2.5-5 mg/ml) in PBS or in 10 mM Tris HCl, 120 mM KCl (pH 7.4) or with full capsids of CPV (2.5-5 mg/ml) in PBS (I, II, III). To study the role of motor proteins on cytoplasmic trafficking, full capsids were injected together with anti-dynein or anti-kinesin antibodies, or with control mouse IgG (II). Cells were fixed with 4% PFA (20 min at room temperature) (I, III) for immunofluorescence studies and with PLP for immuno-EM (II).

4.7 Drugs

For EM experiments cells were grown to 80% confluency on 35 mm plastic culture dishes, while cells for fluorescence microscopy were grown on coverslips or for microinjection studies on microgrid coverslips. Cells were pretreated with 0.4 mM amiloride, 500 nM bafilomycin A_1, 20 nM brefelfin A or 500 nM menensin for 30 min in 37 °C before inoculation with the virus, except for the experiments where the drugs were added at different time points post infection. Viral infections were carried out in the presence of the drugs at 37 °C for 16 h after which cells were fixed with 4% PFA. EM samples were fixed with PLP. Control infections were carried out in the absence of the drugs (III).

4.8 Microtubule binding assays

Commercial Texas red labeled tubulin was polymerized in Eppendorf tubes and stabilized with taxol as instructed by the manufacturer. Full capsids were used for preparation of Oregon green labeled capsids according the instructions for amine reactive probes (Molecular Probes, Eugene, OR.) and the infectivity of labeled capsids was tested by an immunofluorescence assay. For LSM microscopy, Oregon green labeled capsids were mixed with polymerized MTs and post nuclear supernatant (PNS) prepared as described in (Sojakka, et al.,

1999) in a 1:1:1 ratio (final concentration of 1.5 μg/ml of each) in a buffer containing 35 mM piperazine-N,N-9-bis(2-ethanesulfonic acid) (PIPES), 1 mM EGTA, 0.5 mM EDTA, 1-4 mM GTP and 10 μM taxol (King & Schroer 2000), and incubated for 30 min at room temperature. The mixture was transferred to glass chambers coated with DEAE-dextran (2mg/ml) (Murray et al., 2000) and viewed without fixation (II).

For electron microscopy, capsids were mixed with microtubules and PNS in a 1:1:1 ratio. The mixture was incubated on the grids for 1 h. The grids were then stained with uranyl oxalate and embedded in a methyl cellulose/uranyl acetate (Griffiths, et al., 1984). For immunolabeling of EM-specimens, cells were fixed with 4% PFA. CPV was detected with a polyclonal capsid antibody followed by gold-conjugated protein A (5 nm), after which free protein A binding sites were blocked with unconjugated protein A. Dynein was then detected with anti-dynein monoclonal antibody followed by gold-conjugated protein A (10 nm). The samples were then negative stained with uranyl oxalate and embedded in methyl cellulose/uranyl acetate (II).

4.9 Coimmunoprecipitation and Western blotting

For coprecipitation experiments, concentrated viral capsids purified from infected cells were immunoprecipitated with anti-dynein antibodies. Lysates prepared from noninfected cells were used as a control. Before precipitation, viruses and cell lysates were mixed with Protein A beads (Prosep-A High capasity, Bioprocessing Ltd., Durham, England) for 1 hour at 4 °C, followed by centrifugation at 12 000 x g for 20 s. Supernatants were used for immunoprecipitation. Viruses were immunoprecipitated with a mixture of two monoclonal anti-dynein antibodies or with a control monoclonal antibodies against trout IgG (2-5 μg antibody/1 mg protein). Immuno complexes were collected with Protein-A beads (1.5 h, 4°C). For Western blotting, precipitated proteins were separated by polyacrylamide gel electrophoresis as described (Towbin et al. 1979) and the proteins were transferred to nitrocellulose membranes. The membrane was incubated with an antibody against denatured CPV capsids followed by horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark). The signal was detected using aminoethylcarbazole as a substrate (II).

4.10 Assays for viral phospholipase A_2 activity

4.10.1 Treatment of capsids with elevated temperature and with PLA_2 inhibitors

Purified full capsids (0.3-0.7 mg/ml in PBS) were incubated for 2 min in a heat block thermostated at 50, 55, 60, 65, 70 or 75 °C (± 2 °C), after which the samples were cooled gradually. To introduce PLA_2 inhibitors to the heated capsids manifesting PLA_2 activity, capsids were incubated with manoalide (5–10 μM), quinacrine (0.02-2 mM), or $AACOCF_3$ (0.02-10 mM) for 1 h at room temperature with agitation. Heat treated capsids and heat- and PLA_2 inhibitor treated capsids were used for assays of $sPLA_2$ activity and viral proliferation assays (III).

4.10.2 Treatment of capsids with acidic buffers

Purified full CPV capsids dissolved in 0.9% NaCl were diluted in 0.1 M citrate buffer pH 4.0, 5.0, 6.0, and in PBS pH 7.0, and incubated for 5 min at room temperature, and brought to neutrality by adding predetermined aliquots of 1 M Tris base before running the $sPLA_2$ assay or used for the assay without neutralization (III).

4.10.3 Assay for PLA_2 activity

A commercial kit obtained from Cayman Chemical (MI, USA) was used to assay the phospholipase A_2 activity of CPV capsids *in vitro* according to the instructions provided by the manufacturer. The assay uses the 1,2-dithio analog of diheptanoyl phosphatidylcholine, which serves as a substrate for most PLA_2 s (Hendrickson, et al. 1983). Approximately 4.5 μg of CPV capsids were used for these measurements and the same amount of nontreated full capsids was used as a control (III).

4.11 Release of dextran particles from endosomal vesicles

Cells were grown on coverslips to 80% confluency. The cells were pulsed with rhodamine conjugated lysine fixable dextran (Mr 3000 or 10 000, 3 mg/ml) together with H-CPV, and chased for 2, 8 or 16 h in the presence or absence of amiloride, bafilomycin A_1, brefeldin A or monensin. Controls were done without the virus. Cells were fixed with 4 % PFA and imaging was made with Leitz DM RBE fluorescence microscope (Leica, Wetzlar, Germany) equipped with Spot CCD camera and Spot RT software (Diagnostic instruments inc., Sterling Heights, MI). Percentage of cells showing diffuse cytoplasmic and/or nuclear dextran was calculated (approx. 1500 cells/sample). The presence of the CPV was tested by indirect immunofluorescence labeling as described above. In addition, anti-LAMP-2 antibody was used to show late endosomal/lysosomal localization of the dextrans and viruses (III).

4.12 Binding of CPV to lipids

To study the binding of CPV capsids to lipids we used lipid containing membranes (PIP-strips and sphingo-strips) purchased from Molecular Probes. Membranes were first blocked over night at 4°C with 3% fatty acid free bovine serum albumin in Tris buffered saline (TBS, pH 7.4). Then membranes were incubated two times O/N at 4°C in TBS-Tween (0.1%) containing 3% fatty acid free BSA and 1 μg/ml purified CPV capsids. Bound viruses were detected using monoclonal anti capsid antibody and alkaline phosphatase-conjugated secondary antibody. Signal was visualized using 5-bromo-4-chloro-3-indoyl phosphate/Nitro blue tetrazolium substrate (5 minutes) (III).

5 REVIEW OF RESULTS

5.1 General course of CPV infection

To get a time frame for CPV entry studies, a laser scanning microscopy (LSM, confocal microscopy) was used to define the general course of CPV infection (I, Fig. 1). CPV was found to bind to plasma membrane and to enter cells in small vesicles. During the next 3 hours, CPV concentrated at the perinuclear area. Capsid proteins were detected in perinuclear vesicles for several hours. At 10 h post infection some capsid proteins were still detectable in the perinuclear area. Viral DNA visualized by FISH was found to colocalize with viral capsid proteins in perinuclear vesicular structures as late as 8 to 10 h after inoculation with the virus (I, Fig. 11). At 9-12 h p.i., but not before 9 h p.i., viral DNA and capsid proteins were detected in the nucleus indicating accumulation of virus in the nucleus, virus encoded protein/DNA synthesis, or both. When an antibody against NS1 protein, which is expressed in early stages of infection, was used instead of antibody to capsid protein, some fluorescent nuclei were found at 8 h p.i. indicating onset of viral protein synthesis at that time. According to these results intracellular trafficking of CPV from the cell surface to the nucleus took approximately 6 to 8 hours (II, Fig. 2)

5.2 Participation of endocytic compartments and TfR in CPV entry

Three different immunoelectron microscopy techniques, pre-embedding labeling with protein A-gold (I) or with nanogold (III) as well as post-embedding with cryosectioning (I) were used to identify intracellular organelles used by CPV during its entry process. Furthermore, LSM double labeling experiments were carried out to confirm the EM results (I, III). After binding of CPV to the cell surface, gold particles indicating CPV antigens were found in pits having an electron dense coating, which resembles clathrin in pre-embedding samples. Several virus particles were detected in each pit (I, Fig. 2A). After 5 minutes of incubation, some of the gold particles were found in coated vesicles, while others were already detected in tubular noncoated vesicles resembling early endosomes (I, Fig. 2B & C). During the first 30 min of

infection no CPV was detected in noncoated vesicles resembling caveolae in immunoelectron microscopy, and no colocalization was found between caveolae and CPV antigens by LSM double labeling experiments (I, Fig. 3A & B). These results were in agreement with the idea that CPV enters the cells *via* clathrin-coated vesicles.

Pre-embedding immuno-EM analysis of cells fixed 1 h p.i. revealed gold particles in endosomes having an onion like structure of internal membranes, which resembles internal membranes in late endosomes (Mukherjee et al. 1997). Gold particles were also found in simple vesicles with homogeneous interior, located next to microtubules (I, Fig. 4). At 3 h p.i., gold particles showed presence of CPV in vesicles with complex internal membranes and heterogeneous interiors (I, Fig. 7B; III, Fig. 4E (nanogolgd labeling), resembling lysosomes (Mukherjee et al. 1997). To control whether gold labeling on the cell surface had changed the route of the virus particles in pre-embedding studies, cryosections prepared from infected cells were immunolabeled. These postembedded samples confirmed that CPV was found at 1 h and at 3 h p.i. in late endosome like and lysosome like structures, respectively (I, Fig.7C & D).

LSM-double labeling studies were carried out to further identify the intracellular organelles used by CPV. In these studies CPV infection was found not to change the subcellular distribution of the organelles as detected with the antibodies used in this study (data not shown). To find out whether intracellular trafficking of empty capsids differs from that of full capsids, empty (L-CPV: I, Fig. 10) and full (H-CPV: I, Fig 8) capsids were prepared and their entry routes were followed separately. No differences were found between intracellular trafficking of empty and full capsids in LSM studies. CPV was found to colocalize with endocytosed transferrin, a marker of recycling endosomes. This colocalization was most intense from 0.5 to 3 h p.i. when both transferrin and CPV appeared to move to the perinuclear area. An antibody against CiMPR was used as a marker of late endosomes and it was found to colocalize with CPV at 0.5 h p.i. to 1 h p.i. In addition, some colocalization was detected at 3 h p.i. The lysosomal membrane protein LAMP-2, a marker of both late endosomes and lysosomes, showed strong colocalization with CPV at 1 h to 8 h p.i. In contrast, CPV was not found to colocalize with the trans-Golgi marker, TGN-38 or with the ER marker PDI (I, Fig. 9).

To study the interaction of CPV with TfR, infected cells were fixed at different time points post infection and labeled with a mixture of a two different monoclonal antibodies against different epitopes of TfR and with a polyclonal antibody to CPV capsids. CPV was found to colocalize with TfR for at least 10 h (III, Fig. 6A) indicating CPV was associated with its receptor when it is trafficking inside endosomal vesicles. Furthermore, TfR was found to colocalize in infected cells with LAMP-2 at 8 h p.i., while at 1 h p.i. as well as in noninfected cells only barely detectable colocalization was found between TfRs and LAMP-2 positive vesicles (III, Fig 6B).

5.3 The role of microtubules and motor proteins in CPV entry

As explained in sections 5.1 and 5.2 CPV was transported to a perinuclear area during the first 3 hours of infection and by immuno-EM CPV was detected in vesicles located along microtubules at 1 h p.i. (I, Fig. 4). The vesicles seemed to be connected to microtubules by electron dense material (I, Fig. 4).

When microtubules were disrupted with nocodazole the viral antigens remained in the cellular periphery, while in control cells viral capsid proteins were detected in the nucleus at 12 h p.i. indicating the importance of microtubules for CPV entry (I, Fig. 5). To demonstrate productive infection, the NS1 production of CPV infected cells was analyzed by immunofluorescence 10 h after inoculation. Only 1.5% of nocodazole and 0% vinblastine-treated cells expressed NS1, whereas 43% of untreated and 16% taxol-treated cells showed the presence of NS1 (II, 2B).

Taking in to account of the known roles of dynein and kinesin in transport of vesicular cargo along microtubules, it was of interest to study whether these motor proteins were involved in transporting vesicles containing CPV. Antibodies against motor proteins were microinjected to the cytosol of cells before inoculation of CPV, and cells were fixed and prepared for immunoluorescence detection at 2 h after inoculation to avoid viral escape from endosomes into the cytosol before fixing. In cells microinjected with anti-dynein antibody, vesicles containing CPV were found in the cellular periphery, while in noninjected control cells CPV was detected in the perinuclear area (I, Fig. 6A & B). In contrast, cytoplasmic microinjection of anti-kinesin antibody did not cause notable effects (I, 6C & D).

5.4 PLA_2 activity of CPV capsids and exposure of VP1 N-terminus during entry

The amino acid sequence of the CPV VP1 capsid protein has been found to contain a PLA_2 like domain at the N-terminal end (Zádori et al. 2001). When CPV capsids were tested for phospholipase A_2 activity, no activity was found in non treated full capsids or in capsids incubated at temperatures of 50 °C or below. However, when capsids were heated up to 55 °C they started to show PLA_2 activity (III, Fig 1A). Activity increased as capsids were heated to higher temperatures. The highest temperature used was 70 °C as it has been shown that capsids are degraded by heating to 75°C (Vihinen-Ranta et al. 2002). PLA_2 activity was also tested after pretreatment at pH 4.0 to 7.0 with or without a neutralization step. CPV capsids showed PLA_2 activity when treated with buffers of pH 4-6 (III, Fig. 1C). Activity was detected in both neutralized and non neutralized capsids. The activity of heated CPV capsids was abolished in the presence of 5 mM EDTA and in the presence inhibitors of secreted type PLA_2; quinacrine (QN) (2 mM, note that free QN had to be removed before conducting the assay due to its strong yellow color) or manoalide (MAN, 5 μM) (III, Fig. 1B). PLA_2 activity was also decreased by binding of polyclonal anti VP1 antibody (2.5 mg/ml) to heated capsids while anti-capsid antibody used at the same concentration had no significant effect (III, Fig. 1B). Alterntively, an inhibitor of cryptic pospholipase A_2, $AACOCF_3$, had no effect on the PLA_2

activity of CPV capsids (III, Fig 1B). The effect of PLA_2 -inhibitors on CPV proliferation was tested by infecting cells with CPV capsids that were heated (2 min, 65°C) and incubated with phospholipase A_2 inhibitors. QN (0.2 mM) reduced CPV infectivity from 74% to 13% and MAN (5 μM or 10 μM) totally abolished infectivity (III, Fig. 1C). However, no decrease of infectivity was found when full capsids were treated with of 0.2 mM $AACOCF_3$ (III, Fig. 1C). Higher concentrations could not be tested because of the toxicity.

To determine whether the N-terminus of VP1 is exposed during the CPV entry process, cells were inoculated with full capsids, fixed at 1 to 8 h p.i. and double labeled with polyclonal anti-capsid antibody as a primary antibody to stain capsids and with monoclonal anti-VP1 antibody to specifically detect VP1 proteins with exposed N-termini, then analyzed with confocal immunofluorescence microscopy. Monoclonal anti-VP1 antibody showed no staining at 1 h p.i. (III, Fig 2A) indicating that VP1 N-terminal ends were still buried in capsids. At 2-8 h p.i. monoclonal anti-VP1 antibody showed perinuclear staining colocalizing with polyclonal anti-capsid antibody (III, Fig. 2A), suggesting that N-termini of VP1 were exposed. At 6 to 8 h p.i. the two perinuclear staining patterns colocalized more extensively compared to earlier time points (III, Fig. 2A), indicating that more of endocytoced virus capsids showed exposed N-terminal ends of VP1.

5.5 The effects of drugs modifying endocytosis on CPVentry

Inoculated cells chased for 20 h in the presence of 0.4 mM amiloride (inhibitor of Na^+/H^+ exchangers), 500 nM bafilomycin A_1 (inhibitor of vacuolar type-H^+ -ATPase) 20 nM brefeldin A (inhibitor of GDP-GTP exchange of the small GTPase ADP-ribosylation factor 1) or 500 nm monensin (Na^+/H^+ ionophore) showed significantly reduced proliferation of CPV when these compounds were added 2 h p.i. or earlier (III, Fig. 3A). When added 0 h p.i. the drugs caused a complete inhibition of proliferation (III, Fig. 3A). Amiloride and brefeldin A also inhibited CPV proliferation when they were added at 8 h p.i., at which time the two other drugs had only minimal effect on viral proliferation (III, Fig. 3A). Amiloride was able to inhibit the proliferation also when it was added as late as 16 h p.i. (III, Fig 3A).

When inoculated cells were chased for 20 h p.i. in the presence of 0.4 mM amiloride, 500 nM bafilomycin A_1, or 500 nm monensin, a double labeling immunofluorescence analysis showed that CPV labeled with polyclonal anti-capsid antibody was present in perinuclear vesicles that extensively costained with antibodies against LAMP-2 (III, Fig. 3C), but not with antibody against early endosomal marker EEA1 (III, Fig. 3B). Instead, in the presence of 20 nM brefeldin A, CPV was found to colocalize to a certain extent both with antibodies to EEA1 and to LAMP-2 (III, Fig. 3B & C). Some confocal images suggested that CPV forms aggregates at the vesicular membrane (data not shown). The aggregation seemed to take place even in the presence of the above drugs. Clusters of label were found also by immunoelectron microscopy (III, Fig. 4). In the presence of bafilomycin A_1, monensin and amiloride, nanogold label indicating the presence of capsids was found in clusters at the membranes of late endosomal and lysosomal vesicles (III, Fig. 4 A, B, C & D). In controls without drugs added, a proportion of the label was found to be in the

cytoplasm and some label was detected at the nuclear membrane, while some labeling remained in contact with the lysosomal membranes (III, Fig. 4E). These results support the idea that amiloride, bafilomycin A_1, monensin and brefeldin A inhibit the escape of CPV from lysosomal vesicles. However, these compounds did not affect binding of CPV to the lysosomal membrane.

To determine whether the above drugs can block some post endocytic steps of CPV infection, full CPV capsids were microinjected to the cytoplasm of drug treated NLFK cells. After 16 h of incubation capsid localization or presence of early expressed viral NS1-protein was studied with LSM. Nuclear NS1 proteins and viruses were observed in the presence of 500 nM bafilomycin A_1, 500 nM monensin and 20 nM brefeldin A, as well as in controls (no drugs added). In contrast, in the presence of 400 nM amiloride no nuclear capsid proteins or NS1 expression was detected. The inhibitory action of bafilomycin A_1, monensin and brefeldin A was therefore due to effects they exerted on the endocytic route. Further, amiloride appeared to also block some steps in the entry route or proliferation of CPV after release of CPV from the endosomal/lysosomal vesicles.

5.6 The effects of drugs modifying endocytosis on exposure of N-terminal ends of VP1

As bafilomycin A_1 and related drugs are known to modify the intravesicular conditions by elevating the luminal pH, it was of interest to test whether an acidic environment is needed for exposure of the VP1 N-terminus. Intrestingly, the exposure of the VP1 N-terminal end was also detected in the presence of 0.4 mM amiloride, 500 nM bafilomycin A_1, or 500 nm monensin (III, Fig. 2B) indicating that the inhibition of the exposure of VP1 N-terminus is not the target of any of these drugs.

To further elucidate the importance of VP1 N-terminus in release of the virus from vesicles, cells were infected with heated (65 ^{o}C, 2 min) capsids showing PLA_2 activity. Infectivity was determined by immunofluorescence labeling of NS1 or CPV capsid proteins, after which, the percentage of cells showing nuclear fluorescence was determined from 300 cells/sample. Heated capsids fully retained their ability to infect the cells, and the infection was still strongly inhibited by added drugs (III, Fig. 5). Thus the drugs were able to inhibit proliferation of heat-treated CPV having exposed VP1 N-termini, supporting the view that exposure of VP1 N-terminus was not the target of the inhibitory effect of these drugs.

5.7 Interactions of CPV capsids with lipids

Because membrane lipids have been found to be rather well organized along organelles of the endocytic machinery, it was of interest to study the lipid-binding properties of CPV (Simonsen et al. 2001). Interactions of CPV capsids with membrane lipids were studied with arrays of phosphatidylinositides and sphingolipids. CPV was found to bind to many phosphatidylinositol phosphates, such as phosphatidylinositol 4-phosphate (PI(4)P), phosphatidylinositol 5- phosphate (PI(5)P) and phosphatidylinositol 3, 5-bisphosphate (PI(3,5)P_2). Some binding was detected also with PI(3)P, PI(3,4)P, PI(4,5)P, PI(3,4,5)P and with phosphatidyl serine. EDTA (5 mM) abolished CPV's ability to bind to these lipids. EDTA independent binding of CPV to sulfatide was also found. Non treated capsids, heated capsids and VP1 antibody treated capsids had an equal ability to bind to these lipids (data not shown) (III).

5.8 CPV induced membrane permeabilization

To study the parvoviral penetration through cellular membranes, CPV was coendocytosed with rhodamine conjugated dextrans (M_r 3000 or M_r 10 000). The cells were incubated for 2-20 h, fixed with PFA and analyzed by LSM. In non infected control cells, the rhodamine label of the M_r 10 000 as well as M_r 3 000 dextrans were found in vesicular structures after 20 h of incubation (III, Fig. 7A). In infected cells both virus and dextran colocalized with LAMP-2 after 2 h of infection (data not shown). Both entry and proliferation of the virus seemed to be unchanged despite of the presence of the dextran. In cells infected with CPV, dextran with molecular size of 3000 was found in the cytoplasm and nucleus after 8-20 hours of infection (III, Fig.7B). Release of the small dextran was detected before CPV was observed in the nucleus. The larger dextran M_r of 10 000 was not released into the cytoplasm even after 20 h of infection. Instead, it was still detected in the perinuclear vesicles (III. Fig 7B).

To quantify the dextran release and study effects of amiloride, bafilomycin A_1, brefeldin A and monensin, the percentage of cells showing released (cytoplasm/nuclear) dextran was counted both in the presence and in the absence of the drugs. In noninfected controls, only few cells released the smaller dextran (III, Fig. 7C). Practically no release of M_r 10 000 dextran was detected in control cells or in infected cells even after 20 h of infection (III, Fig. 7C). In infected cells, M_r 3000 dextran was released into the cytoplasm in some infected cells at 8 h p.i. and more cells showing cytoplasmic and nuclear dextran were detected at 20 h p.i. (III, Fig. 7C). In infected cells treated with 0.4 mM amiloride, 500 nM bafilomycin A_1, 20 nM brefeldin A or 500 nM monensin, the M_r 3000 dextran was released into the cytoplasm (III, Fig. 7C), suggesting that these drugs were not able to inhibit CPV induced endosomal membrane permeabilization. In noninfected cells amiloride, bafilomycin A_1, brefeldin A or monensin caused no increase in the amount of cells showingdextran release. The results suggest that CPV appears to modify endosomal/lysosomal membranes after 8 h of infection so that small sized dextrans (M_r 3000) could

leak out, while larger dextrans (M_r 10 000) were mainly retained in the lysosomes.

5.9 The effect of microtubule disrupting compounds on the traffic of cytosolic CPV

To test for changes in the organization of the microtubule network for cytoplasmic trafficking of capsids, cells were incubated in medium containing 60 μM nocodazole, 20 μM vinblastine, or 2 μM taxol for 30 min prior to capsid injection. To detect nuclear transport of cytoplasmic capsids, high concentrations (2.5-5mg/ml) of DNA containing CPV capsids (H-CPV) were microinjected into cells in the presence or absence of microtubule-affecting drugs, cells were fixed 10 h after injection and capsids and microtubules were immunolabeled. Cycloheximide (0.2 mM) was used to prevent synthesis of viral proteins. In control cells, having normal microtubule networks, approximately 42% of injected cells showed a strong nuclear labeling 10 h after microinjection (II, Fig. 1A, nontreated &B, control) indicating nuclear accumulation of the capsids. Disruption of the microtubule network by nocodazole or vinblastine treatments reduced the nuclear import of CPV significantly. In nocodazole treated cells 8% and in vinblastine treated cells only 1% of cells showed nuclear accumulation of viral capsids (II, Fig. 1A & B). Stabilization of microtubules with taxol also reduced nuclear import of microinjected capsids. 22% of injected cells showed fluorescent nuclei (II, Fig. 1A& B)).

The same experiment was carried out without cycloheximide to demonstrate whether microinjected H-CPV was able to infect drug-treated or non treated cells and cells were immunolabeled for newly produced viral NS1 protein instead of capsid protein and microtubules.

NS1 label was detected in 25% of cells treated with nocodazole and 15% of cells treated with vinblastine, showing that at 12 h post injection the treatments did not totally abolish nuclear import of capsids. Some capsids were able to reach the nucleus despite the disruption of microtubules and cause expression of viral proteins. In the presence of taxol 35% and in absence of drugs 53% of injected cells showed detectable NS1 expression (II, Fig. 2A).

The consistency of the above results (carried out by microinjecting the capsids) with productive infection was studied by infecting the cells. When the kinetics of NS1 expression was compared between cells that were cytoplasmically injected with the CPV and cells that were inoculated with the virus, no difference was found. An increase in the percentage of NS1 expressing cells was observed after 8 h in both injected cells and in inoculated cells and the maximum percentage of infection was reached after 12 h (II, Fig. 2C).

5.10 Motor protein dependence of CPV cytosolic transport

Effects of antibodies against motor proteins on nuclear transport of capsids were tested. Cells were coinjected with capsids and monoclonal antibody to the 74 kDa intermediate chain of the dynein motor complex. In the presence of anti-dynein antibody only 7 to 13% of cells showed nuclear accumulation of the capsids, whereas 40 to 50% of cells injected with capsids and either monoclonal anti-kinesin antibody or control mouse IgG showed nuclear localization of the capsids (II, Fig. 3).

5.11 CPV binding to microtubules *in vivo* and *in vitro*

The association of CPV capsids with microtubules within the cytosol was studied in living cells by microinjecting fluorescently labeled capsids into cells containing fluorescent microtubules. The fluorescent capsids were localized in close proximity to microtubules throughout the cytoplasm (II, Fig. 7).

To analyze virus microtubule interaction in more detail, the binding of the capsids to microtubules was studied *in vitro*. In LSM studies, Oregon green-labeled capsids and taxol stabilized TxR labeled microtubules with or without PNS in FDB buffer were carefully transferred to DEAE-dextran coverslips chambers to adhere the microtubules (Murray et al. 2000). The capsids could be seen being clearly aligned on microtubules in the presence of PNS (II, Fig. 5). Movement of the capsids was also detected in the presence of PNS (data not shown), but live imaging was hard to carry out because the low amount of the label per virus caused quick fading. More intensive labeling of capsids with succinimidyl esters caused dramatic decrease of infectivity. When the same experiment was carried out in the absence of PNS, capsids showed only little interaction with microtubules.

In *in vitro* EM analysis of stabilized microtubules and viral capsids, negative stained capsid-like structures were in contact with microtubules (II, Fig. 6A). Immunoelectron microscopy with the anti-capsid antibody followed by protein A-5 nm gold showed gold-labeled CPV capsids bound to microtubules. A double labeling of the samples with both capsid antibody (5nm gold) and anti-dynein antibody (10nm gold) showed the dynein associated with the capsids (II, Fig. 6B). In contrast, capsids purified by isopycnic centrifugation in a CsCl gradient showed no labeling for dynein and only little binding to microtubules (data not shown).

To further establish the interaction of capsids with dynein, antibodies against cytoplasmic dynein intermediate chain were used to immunoprecipitate CPV-dynein complexes from virus concentrate. Precipitates were studied by immunoblotting with an antibody against denatured viral capsids and a band with the size of VP2 (67 kDa) was observed (II, Fig. 8). When capsids were purified using CsCl centrifugation they did not coprecipitate with the anti-dynein antibody, indicating that dynein-capsid complexs disintegrate during capsid purification and confirming that dynein is needed for the precipitation of the capsids.

5.12 Cytoplasmic transport of CPV towards the nucleus

To follow cytoplasmic transport of the virus, both inoculated and microinjected viruses were used. Capsids were labeled with a monoclonal antibody recognizing intact viral capsids (MAb8, (Strassheim et al. 1994, Wikoff et al. 1994)) followed by nanogold immunolabeling with silver enhancement to detect viruses by EM. Experiments were performed in the presence of 0.2 mM cycloheximide to block the synthesis of new viral proteins. Purified DNA-containing capsids were microinjected into cytoplams of the cells, and capsids recognized by MAb8 which binds intact capsids. Microinjected capsids were detected inside the nucleus at 2 h after injection. Some capsids seemed to be attached to the nuclear membrane, while some of the injected viruses were detected in the cytoplasm (II, Fig. 4A & B).

In cells inoculated with CPV, as late as 10 h p.i. cytoplasmic capsids appeared to be associated with the nuclear membrane and only very few capsids were observed in the nucleus of infected cells (II, Fig. 4C). Triton-X-100 treatment (0.05%) used to permeabilize the nuclear membrane to allow detection of antigen inside the nucleus caused a slight degeneration of the fine structures of the cellular membranes (II, Fig. 4D).

6 DISCUSSION

Although CPV is one of the most intensively studied parvoviruses, still today its intracellular entry process is far from understood. In this thesis a variety of approaches were used to investigate the CPV journey from the cell surface to the nucleus. The time course of entry of CPV in to NLFK cells was characterized by following the intracellular pathways of CPV capsid proteins and CPV DNA during entry, and by following the expression level of newly synthetized viral NS1 protein. NS1 plays a role in the initiation of viral DNA replication as well as in activation of the viral promoters (Cotmore & Tattersall 1994, Nuesch et al. 1995). It is found in infected cells before structural proteins (Molitor et al. 1985) and can be used as an efficient marker for productive infection indicating that also very small amounts of capsids imported to the nucleus lead to productive infection. After binding to the cell surface, CPV was internalized in clathrin coated vesicles within minutes. During next few hours CPV capsids entered perinuclear vesicles, where CPV was detected still at 10 h p.i. Viral proliferation, detected as the presence of CPV NS1 protein, was active at 8 h p.i. indicating that some viruses had entered the nucleus and virus proteins were already synthetized. Thus the time frame of the entry phase extends 7-8 hours from the viral inoculation. During that time CPV must find its way to the correct endosomal compartment(s), from where it may penetrate the cytoplasm to take its genome to the nucleus for replication (I).

An important aim of our study was to define the vesicular compartments that are utilized by CPV during its entry. CPV was initially detected as clusters at the plasma membrane using immuno-EM (protein-A, pre-embedding). Clusters containing a number of gold particles were observed in pits with an electron dense coating resembling clathrin (I). In immuno EM studies and double-labeling immunofluorescence analysis, no colocalization of CPV with caveolae was found (I). Thus, the clathrin-dependent route seemed to be the exclusive route used by CPV. The results agree with earlier findings showing that the cellular uptake of AAV (Duan et al. 1999) and CPV (Parker & Parrish 2000) was blocked by overexpression of dominant negative mutant dynamin (I).

In LSM studies CPV was found to colocalize with transferrin in NLFK cells at 0 to 3 h p.i. and to a lesser degree at 8 h p.i. CPV has been reported to colocalize with transferrin up to 4 h p.i. in Mv1Lu cells (Parker & Parrish 2000). In our study both transferrin and CPV moved from the plasma membrane to the perinuclear area during first 3 h of infection. As transferrin is known to be transported through the tubulovesicular compartment near the nucleus known

as recycling endosomes (RE) (Mayor et al. 1993, Prekeris et al. 1998), this data indicates that CPV reached this compartment during its entry (I). In immunoelectron microscopy, CPV antigens were detected in both onion-like late-endosomes and lysosomal vesicles with heterogenous interiors (I). LSM studies supported the EM data by the observed colocalization of CPV with the Ci-MPR, a marker of late endosomes, and with LAMP-2, a marker of late endosomes and lysosomes (I). Cross-linking of transferrin receptors by a multivalent ligand can cause redirection of ligand-receptor complex to the late endosomes and lysosomes (Marsh et al. 1995). Therefore, the results suggest that CPV may act as multivalent ligand and cause CPV-TfR complex to be redirected from REs to the lysosomes *via* late-endosomes (Fig. 6). The lysosomal location of CPV during its entry was evident for the following reasons. Even though the location of the vesicles occupied by CPV resembled that of the Golgi apparatus, they did not colocalize with the trans Golgi marker, TGN-38. No colocalization was found between the ER marker (PDI) and CPV containing vesicles at 0.5-8 h p.i (I). In immuno-EM studies at 3-8 h p.i. the vesicles showing the majority of CPV-labeling had internal membranes and a polymorphous interior different from the onion-like vesicles in which CPV was observed at earlier time points (I). The characteristics of the observed vesicles agree with the description given for lysosomes (Mukherjee et al. 1997). Both cryosectioning and nanogold immuno EM labelings showed that labeling CPV with antibody and colloidal gold before endocytic uptake did not change its entry route (I). Finally, LSM imaging showed that at 6-8 h after inoculation CPV colocalized strongly with the lysosomal membrane protein LAMP-2, but no longer with the CiMPR, found only in late-endosomes (I). Moreover, as viral DNA colocalizes with capsid proteins in perinuclear vesicles at 8 h p.i. suggesting that CPV had not yet released its genome to the cytoplasm. These results indicate that CPV seems to enter the lysosomes before it penetrates into the cytoplasm (I). The behavior of CPV resembles that of the autonomous parvovirus PPV, which has been shown to colocalize with LAMP-2 before it escapes from vesicular structures. The CPV entry is considerable different from the entry pathway of AAV (Bartlett, et al., 2000), as AAV has been shown to penetrate the endosomal membrane quickly following internalization. Reovirus, on the other hand, is also known to need lysosomal proteolysis to cause productive infection (Sturzenbecker et al. 1987). The routing of CPV to the degradative route might be necessary for parvovirus in order to go through the conformational changes needed for membrane penetration. Alternatively, acidic pH might be needed for the release CPV from transferrin receptors. Empty CPV capsids (without DNA, L-CPV) seemed to use the same endocytic pathway as full capsids (I). This finding indicates that, at least up to the point of release from the endosomal compartment, the DNA of CPV or VP3 missing from empty capsids do not have a role in the vesicular transport of the virion.

It has been shown earlier that CPV-TfR interaction persists at least 4 h from the inoculation of the virus by cytoplasmic microinjections of antibodies against the TfR cytoplasmic tail (Parker et al. 2001). In the present study we defined the role of transferrin receptors in the later steps of the entry process. CPV capsids were found to colocalize with TfR from the beginning of the entry to 10 h p.i. (III), the point when viral NS1 and capsid proteins are already expressed in host cells (Cotmore & Tattersall 1994, Cotmore et al. 1995, Nuesch et al. 1995). As CPV was shown to be transported to the lysosomes, it was of interest to check if CPV changed the routing of TfRs in NLFK cells. In double

labeling LSM experiments more TfRs were detected in lysosomal vesicles at 8 h after inoculation with CPV than at 1 h after inoculation (III). The results support the view that CPV redirects TfRs towards the lysosomes by acting as a multivalent ligand (Marsh et al. 1995). Normally TfR is mostly recycled back to the plasma membrane through the recycling endosomes (Mayor et al. 1993).

Since intact microtubules and functional motor proteins are essential for endosomal trafficking, endosomal transport and sorting, and for maintenance of the intracellular location of LEs and REs (Brown et al. 1980, Matteoni & Kreis 1987, Gruenberg & Howell 1989, McGraw et al. 1993, Oda et al. 1995, Bananis et al. 2000), the disintegration of microtubules might cause disturbance in the endocytic entry of viral capsids. In earlier studies, CPV infection could be blocked by microtubule disrupting drugs or by low temperatures, suggesting the involvement of microtubule dependent vesicle traffic (Vihinen- Ranta 1998). In the present study, the role of microtubules in intracellular trafficking was further supported by many results. First, disruption of the microtubule network with nocodazole inhibited infection and caused vesicles containing CPV to be dispersed to the cell periphery (I). Second, CPV containing vesicles were found in close proximity to microtubules and in immunoelectron micrographs electron dense material was seen connecting the vesicles to microtubules (I). In contrast, the disruption of actin cytoskeleton by cytochalasin D treatment did not significantly effect on the entry or proliferation of CPV, suggesting that actin is not essential for CPV entry (I). Because the plus-end directed motor protein kinesin and minus-end directed motor dynein are needeed for trafficking along microtubules, anti-dynein and anti-kinesin antibodies were microinjected into the cytoplasm before inoculation of CPV. Microinjected anti-dynein antibody caused endocytosed CPV to stay in vesicles located in the cellular periphery, while in noninjected cells CPV was found in perinuclear vesicles (I, II). The effect caused by microinjected anti-dynein antibody appeared quite similar to that caused by nocodazole (I, II). In contrast, cytoplasmically injected anti-kinesin antibody or control antibody had no effect on the intracellular transport of CPV (I, II). These findings, and the results of previous studies (Vihinen- Ranta 1998, I), support the idea that vesicular transport of CPV to the perinuclear area takes place along microtubules and is dependent on dynein. However, the results do not imply the use of a particular route of the intracellular transport machinery, because microtubules as well as dynein motors are known to participate in many functions related to endocytic processes, such as trafficking from early endosomes to the late or the recycling endosome (Gruenberg et al. 1989, Sakai et al. 1991, Ren et al, 1998).

Altogether, the results suggest that CPV entry involves at least the following steps (Fig. 6), 1) binding and clustering of the virus to the coated pits on the cell surface followed by uptake into coated vesicles (I), 2) entry to early endosomes (I), 3) dynein-dependent transport from the peripheral cytoplasm to the perinuclear region in endosomes along microtubules (I), 4) rerouting TfR-virus complexes to the degradative pathway presumably due to CPV-mediated clustering of the TfRs (I, III).

Acidification is known to be an important factor for successful parvovirus entry as the drugs raising endosomal pH block productive infection (Basak & Turner 1992, Vihinen-Ranta 1998, Bartlett et al. 2000 Douar et al. 2001, Parker et al. 2001). However, the more detailed mechanisms behind the inhibitory effect of these drugs have not been clarified. In the present study the conditions that are needed for a successful entry process were explored in more detail using

four different drugs (amiloride, bafilomycin A_1, brefeldin A, and monensin) all of them capable of modifying endocytosis (III). Amiloride is an inhibitor of Na^+/H^+ exchangers known to regulate the pH of EEs. These exchangers also play a role in the maintenance of the intracellular the pH and in the regulation of cell volume. Na^+/H^+ exchangers have been found in the plasma membrane as a part of the machinery that maintains the pH of cytosol (Mukherjee et al. 1997, Pillay et al. 2002). In addition, Na^+/H^+ exchangers are also present in endosomal and lysosomal membranes (Hilden et al. 1990, Sabolic & Brown 1990). Both bafilomycin A_1, a specific inhibitor of vacuolar type H^+ ATPase, and monenisn, Na^+/H^+ ionophore, are known to raise the pH of intracellular acidic compartments (Pressman 1976, Mellman 1996, Pillay et al. 2002) and by doing so they interfere with many phases of endocytosis. Bafilomycin A_1 has been shown to specifically inhibit acidification of organelles having vacuolar H^+ATPases in their membranes. These organelles include all endosomes except early endosomes, lysosomes and phagosomes (Umata et al. 1990, Yoshimori et al. 1991, Johnson et al. 1993). However, monensin and bafilomycin A_1 have been shown not to inhibit Tf intake (Stein & Sussman 1986, Johnson et al. 1993). Brefeldin A, an inhibitor of the small GTPase ARF1 (Donaldson et al. 1992, Randazzo et al. 1993, Kreis et al. 1995, Chardin & Mccormick 1999), has been reported to inhibit recruitment of the COPs, formation of the ECVs and recycling of transferrin receptor (Whitney et al. 1995). Moreover, brefeldin A has multiple effects on the morphology of endocytic organelles and the Golgi apparatus (Lippincott-Schwartz et al. 1991). In the present study as well as in earlier studies bafilomycin A_1 and monensin were shown to block CPV infection (III). Brefeldin A and amiloride were also found to be potent inhibitors of CPV proliferation. In the presence of amiloride, bafilomycin A_1 or monensin, viral capsids were detected in the lysosomal membrane protein-2 (LAMP-2) positive, late endosomal/lysosomal vesicles even after 20 h of infection, while no colocalization was detected with the early endosomal marker (EEA1) even though the drugs were added 30 min before inoculation of the virus. The results suggested that these drugs blocked CPV infection before escape into the cytosol from the lysosomal compartment, but did not block the trafficking of the capsids from EEs to lysosomes. (III) Since the neutralization of endosomal compartments is known to block trafficking between EEs and LEs (Clague et al. 1994), and inhibit late endosome-lysosome fusion (Van Weert et al. 1995), but not to block the transferrin receptor recycling (Johnson et al. 1993), this result supports the view that CPV may enter the cells *via* recycling route from which CPV is delivered to the lysosomal pathway. In the presence of brefeldin A, capsids colocalized with early endosomal and with lysosomal markers, suggesting that brefeldin A partially inhibited or slowed down the trafficking from EEs (III).

To study if these drugs have effects on later steps of infection, taking place after viruses are released into the cytoplasm, we microinjected CPV into the cytoplasm of the cells in the presence of the above drugs to bypass endocytosis (III). This was a relevant method because CPV has been shown to be released to the cytosol in a rather intact form (Vihinen-Ranta et al. 2002). Only amiloride was able to inhibit viral proliferation in these conditions, suggesting that amiloride inhibited also some step(s) taking place after the endocytic phase of entry. This conclusion is supported by our findings that amiloride was able to inhibit CPV infection even when added after 16 h of the infection, when CPV had already started its replication (III). Similar results were obtained using the

amiloride analogue EIPA (5-(N-ethyl-N-isopropyl)amiloride), that was shown to inhibit the escape of adenovirus type 2 from endosomes as well as to inhibit adenoviral gene expression (Meier et al. 2002). Instead, we found that bafilomycin A_1, monensin and brefeldin A were only able to inhibit the endocytic phase of CPV entry (III).

The N-terminal unique region of the capsid protein VP1 of CPV and MVM has been shown to be essential for infectivity. Deletion of the N-terminal end from VP1 leads to the formation of noninfectious viruses (Tullis et al. 1993, Vihinen-Ranta et al. 2002). In intact capsids of CPV and MVM, the N-terminus of VP1 is not accessible but it is externalized in specific conditions, such as heating or urea treatment, without complete disintegration of the capsids (Cotmore et al. 1999, Weichert et al. 1998, Vihinen-Ranta et al. 2000). Moreover, sequence comparisons showed the presence of motifs found at active sites of $sPLA_2$ at the N-terminus of CPV VP1 (Zádori et al. 2001). In the present study we showed that PLA_2 activity of CPV capsids could be triggered by heating the capsids (III). No PLA_2 activity was found in purified full CPV capsids or capsids treated at temperatures of 50 °C or below. PLA_2 activity of the capsids was triggered by heating to 55 °C. PLA_2 activity of CPV capsids seemed to increase with increasing temperature, so that capsids treated at 70 °C, the highest temperature used in this study, showed the highest activity (III). It was shown earlier that the N-terminus of VP1 is exposed by heating CPV capsids to 55 °C and capsids were shown to be disrupted at 75 °C (Vihinen-Ranta et al. 2002). Thus it appears that the PLA_2 activity could be used as a measure of the exposure of the N-terminal unique sequence. Inhibitors of $sPLA_2$, quinacrine and manoalide, as well as 5 mM EDTA, were able to abolish the PLA_2 activity of the capsids. In addition, an antibody against the N-terminal end of VP1 reduced the PLA_2 activity of heat treated capsids. These results indicated that the PLA_2-like domain of the VP1 N-terminus gives the $sPLA_2$ function in CPV capsids. In addition to heating, low pH treatments were able to trigger parvoviral PLA_2 activity as capsids incubated at acidic pH (4-6) buffer for 5 minutes showed PLA_2 activity. Therefore, it seems probable that capsid PLA_2 activity could be triggered by the low pH in early endosomes, recycling endosomes or lysosomes. To study the role of the PLA_2 activity in CPV entry we treated capsids showing exposed VP1 N-termini with PLA_2 inhibitors then used the capsids to inoculate cells. MAN and QN, which were shown to inhibit viral PLA_2 activity, reduced the percentage of cells showing productive infection by immunofluorescence analysis at 20 h after inoculation. These results suggest that viral PLA_2 activity was essential for productive infection. (III)

In the present study it was also shown that the VP1 N-terminal sequence became exposed during the endocytic entry of CPV (III). Detectable amounts of VP1 N-termini were exposed at 2 h p.i. and the amount of exposed VP1 N-termini increased at least up to 8 h p.i. We showed earlier that at 2 h p.i. CPV capsids had reached the lysosomal compartment and proposed that lysosomes might be the site of the CPV penetration into the cytosol. Thus, the result suggests that parvoviral PLA_2 activity can be used for the membrane penetration process of the virus. The same experiment was carried out in the presence of endocytosis modifying drugs (amiloride, bafilomycin A_1, brefeldin A, monensin) to test whether these drugs blocked CPV infection by inhibiting the exposure of VP1 N-termini of the capsids. However, the exposure of N-termini was still detected in the presence of all of these drugs, suggesting that the ability of these drugs to block the infection was due to some factors other

than preventing the PLA_2 activity of the capsids. Infection by heat treated CPV was inhibited in the presence of the drugs even though heat treated CPV (N-termini of VP1 exposed) caused infection like nontreated CPV in the absence of the drugs. This suggests that exposure of the VP1 N-terminus is not sufficient for membrane penetration (III).

Taken together, these results suggest that the PLA_2 -activity located in the N-terminal part of VP1 was triggered by incubation of CPV capsids at an acidic pH. The N-terminus was exposed during the entry at 3-8 h p.i. when CPV has reached lysosomes, probably due to the acidic pH of the lysosomal interior. PLA_2 inhibitors inhibited CPV entry, indicating that PLA_2 activity was essential for successful infection. However the exposure of VP1 N-termini was not sufficient to allow CPV to proliferate in the presence of lysosomotropic drugs, indicating that PLA_2 activity alone is not adequate to cause CPV penetration through the lysosomal membranes. (III)

Mechanisms behind the penetration of nonenveloped viruses through the cellular membranes are not well understood. However, some nonenveloped viruses such as rhinovirus, poliovirus and coxsasckie virus are known to be able to interact with lipid bilayers (Pérez & Carrasco 1993, Van Kuppeveld et al. 1996, Tosteson & Chow 1997). Because membrane lipids are not randomly distributed along endocytic pathways and their organelles, it was of interest to study the lipid binding properties of CPV (Simonsen et al. 2001). In the present study we assayed the CPV binding to sphingolipids and to phosphoinositides (III). Results showed that CPV was able to interact with several lipid components of the intracellular membrane compartments such as $PI(4,5)P_2$ found from CCVs, PI(3)P concentrated to early endosomal membranes, sulfatide enriched in recycling endosomal membrane components and $PI(3,5)P_2$ and lysophosphatic acid found in late endosomal membranes, suggesting that CPV might be able to interact with at least the membranes of these organelles also *in vivo* (Simonsen et al. 2001, III). Binding to lipids, excluding sulfatide, was reduced by EDTA, suggesting that the binding is dependent on divalent cations. Heated capsids and VP1 antibody treated capsids had an equal ability to bind to lipids than non-treated capsids, suggesting that VP1 N-terminus is not responsible for membrane binding. This further suggests that lipid binding and PLA_2 activity of CPV capsids are independent activities associated with the capsids (III). The possible role of these lipids as coreceptors required for membrane penetration warrants further investigation.

Two different mechanisms of escape from endosomal vesicles were found, when adenovirus and human rhinovirus type 2 (HRV2) were studied by cointernalization of differently sized dextrans with these viruses or virus proteins. Adenovirus was found to release endosomal dextran size independently, whereas the release by HRV2 was shown to be highly size dependent process (Prchla et al. 1995). The escape mechanism of CPV has been studied earlier in MDCK cells and in tTA-Mv1Lu cells by cointernalization of α-sarcin (M_r about 16 000), but entering CPV did not permeabilize endosomes to α-sarcin (Parker & Parrish 2000). In the present study CPV was cointernalized with dextrans smaller than α-sarcin to detect changes in membrane permeability (III). In LSM analysis, cointernalized dextran and viruses colocalized strongly with LAMP-2, suggesting that both dextran and CPV entered the lysosomal vesicles. When rhodamine labeled dextrans (M_r 3000 or M_r 10 000) were coendocytosed with CPV, the M_r 3000 dextran was able to penetrate into the cytoplasm and migrate further into the nucleus. The M_r 10

000 dextran was not able to escape from endosomal vesicles even after 20 h p.i. In noninfected control cells both dextrans remained in endocytic vesicles and were not detected in the cytoplasm or in the nucleus. This suggests that CPV modified the permeability of the late endosomal or the lysosomal membrane, allowing the smaller dextran to leak out (III).

To quantify the CPV induced membrane permeabilization the percentage of cells showing released dextran in the cytosol and in the nucleus was determined (III). This assay was used to define the time when the membrane permeabilization by CPV takes place and to investigate the effect of the drugs (amiloride, bafilomycin A_1, brefeldin A or monensin) on the membrane permeabilization process. None of the drugs were able to inhibit dextran release despite the fact that they arrested CPV in the LAMP-2 positive vesicles (III). Earlier rotavirus, reovirus and HRV14 were reported to also cause membrane permeabilization in the presence of befilomycin A_1, indicating pH independency of endosomal disintegration (Martinez et al. 1996, Liprandi et al. 1997, Bayer et al. 1998). This result suggests that membrane permeabilization, probably caused by the viral PLA_2 activity, is not the only function needed for release of CPV from vesicles. This is in line with our earlier results indicating that exposure of VP1 N-terminus leading to activation of viral PLA_2 activity was not enough to cause membrane penetration by CPV. Furthermore, it seems unlikely that CPV, which escaped to the cytoplasm in rather intact form (Vihinen-Ranta et al. 2002), would be able to penetrate through a pore of a size that would allow M_r 3000 dextran but not M_r 10 000 dextran to pass through the membrane. Thus, the leakage of dextran may not necessarily indicate pore formation, and the mechanism of parvoviral penetration through the endosomal/lysosomal membranes remains unresolved. VP3 N-terminal end has a conserved polyglysine region capable for membrane insertion (Cotmore & Tattersall 1987, Bloom et al. 1988, Cortes et al. 1993). The role of the VP3 protein on the viral membrane penetration process needs to be investigated. Also the involvement of receptor(s) in parvoviral membrane penetration should be clarified as the results of this thesis indicated that CPV and TfR are associated when CPV escapes to cytosol.

Transport of viral particles in the cytosol generally requires interactions with the host transport system as the passive cytosolic trafficking of virus sized particles is restricted by the properties of the cytoplasm (Luby-Phelps 1994, Seksek et al. 1997, Luby-Phelps 2000). Although nocodazole blocked the CPV infection (Vihinen- Ranta 1998), it is difficult to determine whether the inhibition was due to effects on endocytosis or to disturbed interactions between viral components and microtubules. As CPV has been shown to be released into the cytoplasm in an intact form, the microinjection technique offers a possibility to study cytosolic transport of the virus. The nuclear transport of cytoplasmically microinjected CPV capsids has been shown to be relatively slow and dependent on microtubules (Vihinen-Ranta et al. 2000). To define the role of microtubules in nuclear localization in detail we tested three microtubule affecting drugs for their effect on nuclear transport of cytoplasmically injected capsids (II). Both nocodazole, and vinblastine caused injected viral capsids to be dispersed in the cytoplasm, while in the absence of the drugs, injected capsids accumulated in the nucleus and perinuclear area, suggesting that the viruses were able to use microtubules for their transport towards the nucleus. When microtubules were stabilized with taxol, the nuclear import of the capsids was slightly decreased, but not abolished. This implies

that microtubules can serve as tracks to mediate virus transport towards the nucleus, and that the polymerization and depolymerization events at microtubule ends might also affect nucleus oriented viral movements (II).

To demonstrate successful infection after microinjection and after inoculation of the capsids in the presence and in the absence of microtubule-affecting drugs, cells were immunostained for viral NS1 protein (II). The production of NS1 started 8 h after either the cytoplasmic microinjection or the inoculation of the cells. It has been shown that inoculated CPV stays for several hours in endosomal vesicles before escaping to the cytoplasm. Thus it is possible that endosomal entry not only provides CPV a useful entry route, but also prepares the virus for gene expression. Even though cytoplasmically microinjected viruses were detected within the nucleus 2 h post microinjection in immuno-EM studies, they appear to require several hours to reach levels of NS1 expression detectable by fluorescence microscope. In the presence of microtubule affecting drugs, in cells injected with capsids the expression of NS1 protein was significantly decreased although not completely inhibited. In cells inoculated with the virus NS1 expression was inhibited almost completely by these drugs. These results suggest that when cells are inoculated with the virus, microtubule affecting drugs are able to inhibit both microtubule-dependent endocytotic entry phase as well as microtubule-dependent cytoplasmic transport towards the nucleus. Thus, causing double level entry block (Fig. 6). In contrast, in injected cells only cytoplasmic transport is affected. Furthermore, injected capsids may reach the nucleus in a microtubule-independent fashion by the pressure of the microinjection, or due to the large numbers of capsids injected. The present results suggest that capsids entering microtubule-deficient cells *via* endocytosis were not able to traffic through the endosomal pathway to the site of release and thus remained inside endosomal structures without being able to cause a productive infection. These results also showed that in the absence of microtubules, fever viral genomes were transported into the nucleus even though they were microinjected to the cytoplasm, suggesting that cytoplasmic transport after viral release from endocytic vesicles was also microtubule-dependent (II).

To accomplish microtubule-dependent movement towards the nucleus, viral capsid are likely to benefit from the inherent polarity of microtubules by using microtubule associated motor proteins. Since microtubule minus-ends are anchored at the perinuclear microtubule organizing center, it seems likely that dynein is used for transport of incoming viral capsids towards the nucleus. However, adenovirus has been shown to use both kinesin- and dynein motors for its trafficking in the cytosol (Suomalainen et al. 1999). Involvement of cellular motor proteins in the cytoplasmic trafficking of CPV was studied by the coinjection of an antibody against the dynein intermediate chain along with capsids (II). The transport of capsids into the nucleus was decreased considerably by the presence of the antibody, suggesting that the intermediate chain of dynein is involved in capsid transport towards the nucleus. Contrastingly, antibody against the plus end-directed motor kinesin had no effect on CPV trafficking to the nucleus. (II)

In vitro binding assays confirmed the microtubule binding activity of capsids (II). As the presence of PNS was essential for the virus to be able to bind to microtubules, cytoplasmic factors, most probably dynein, are required for the binding. EM double labeling assays showed that capsids were simultaneously bound to both dynein and microtubules. The association of capsids with dynein

was further verified by coimmunoprecipitation of capsids isolated from infected cells with antibodies to dynein. The interaction of capsids with microtubules *in vitro* is consistent with the *in vivo* observation that cytoplasmic viruses were found to be associated with microtubules in living cells. The data imply that capsids were bound to microtubules both *in vitro* and *in vivo* in addition to dynein being involved in this interaction. (II) Whether the binding of dynein to CPV capsids involved other cellular components remains unclear.

NLS-mediated translocation through the NPC of the nuclear envelope occurs by sequential steps that involve a series of interactions between cargos, soluble transport factors and nuclear pore proteins of the central channel (Whittaker & Helenius 1998, Whittaker et al. 2000). NPC restricts the passive diffusion of molecules larger than approximately 25 Å in diameter, but allows NLS containing substrates as large as 300 Å or more to rapidly enter the nucleus (Feldherr et al. 2001). Parvoviruses are potentially small enough to pass through the nuclear pore without capsid disassembly or deformation. It has not yet been established whether parvoviruses are able to pass through the NPC in an intact form without capsid dissassembly during early steps of infection. The VP1 N-terminal end, containing several potential NLS, appears to play a role in the nuclear import process because the VP1 N-terminal sequence was exposed after cytoplamic injection in a manner correlating with the nuclear transport of the capsids (Vihinen-Ranta 1997). The EM studies presented here demonstrate that viral capsids injected into the cytoplasm enter the nucleus 2 hours post microinjection in a form recognized by the antibody recognizing only intact capsids (Strassheim et al. 1994, Wikoff et al. 1994), suggesting that nuclear entry can occur without extensive uncoating of the capsids (II). However, in cells inoculated with the virus only a few cells showed trace amounts of the intranuclear labeling suggesting that only a few virions are imported to the nucleus, whereas most capsids remain in the endosomal vesicles or in the cytoplasm (II).

To summarise (Fig. 6), the data reported in this thesis suggest that CPV entry is comprised binding into the cell surface and clustering to coated pits, followed by uptake into coated vesicles and entry to early endosomes (I). The virus is next transported along microtubules to the perinuclear region in a dynein-dependent manner (I). TfR-CPV complexes are routed to the degradative pathway ending up in lysosomes, presumably due to CPV-mediated clustering of the TfRs (III). Parvoviral PLA_2 activity located in the N-terminal part of VP1 is exposed during entry at 3-8 h p.i. in lysosomal vesicles probably due to the acidic pH of the lysosomal interior (III). PLA_2 inhibitors were able to inhibit proliferation of CPV, indicating that PLA_2 activity is essential for successful infection (III). CPV is capable of binding lipids found in many organelles along its endocytic route, although in light of the results of this thesis the most probable site of membrane penetration is lysosomes. Heated capsids, VP1 antibody treated capsids and non treated capsids showed equal binding to the lipids, indicating that VP1 or its PLA_2 domain is not responsible for membrane binding, which further suggests that the lipid binding and the PLA_2 activity of CPV capsids are independent activities (III). CPV infection modified the permeability of the late endosomal or lysosomal membrane at 8-20 h p.i., allowing the small dextran (M_r 3000) to leak out. It remains unclear whether this indicates pore formation during the vesicular escape of the virus (III). Parvoviral capsids released into the cytoplasm are transported in a dynein-dependent manner along microtubules towards the nucleus since the

cytoplasmic trafficking of capsids, requiring intact microtubules, was blocked by anti-dynein antibody and the capsids showed binding to dynein motor proteins *in vitro* (II).

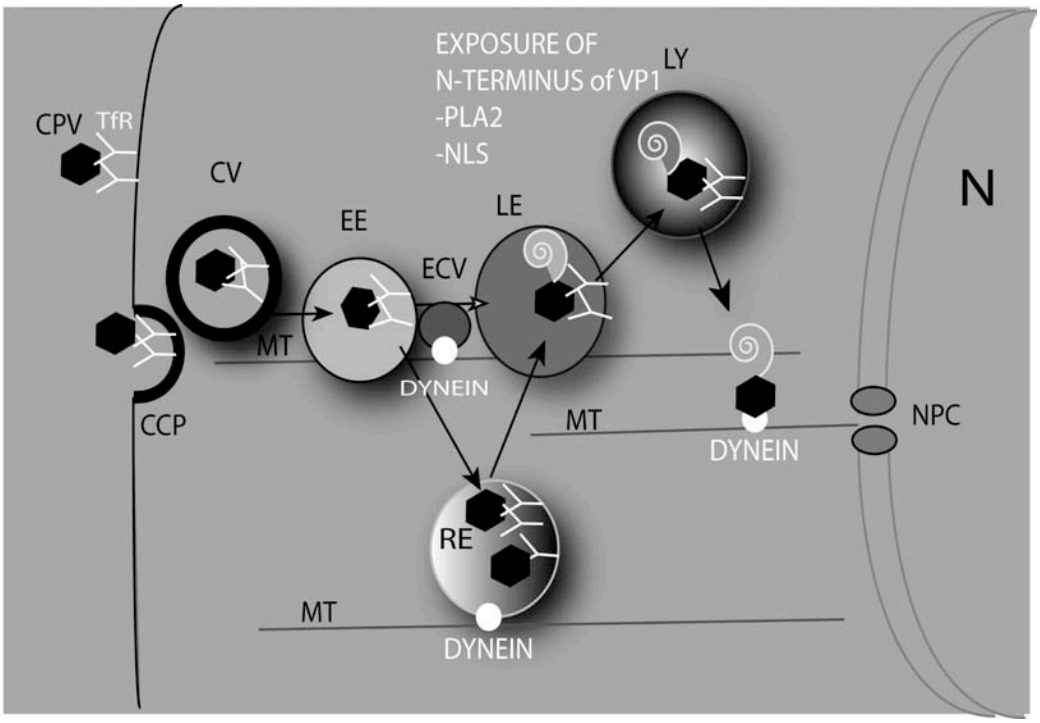

FIGURE 6 Proposed model of the CPV entry and cytoplasmic trafficking. Please see conclusions (below) as figure legend.
CPV, canine parvovirus; TfR, transferrin receptor; CCP, clathrin coated pit; CV, clathrin coated vesicle; MT, microtubule; EE, early endosome; ECV, endosomal carrier vesicle; LE, late endosome; RE, recycling endosome; LY lysosome; PLA2, phospolipase A_2; NLS, nuclear localization signal, NPC, nuclear pore complex, N, nucleus. Arrows, indicating suggested pathways of CPV.

7 CONCLUSIONS

The main conclusions of this thesis are:

1. CPV entered cells in clathrin-coated vesicles associated with its receptor, TfR, but before CPV penetration to the cytoplasm, the virus and TfRs were transported to late endosomes/lysosomes, possibly due to virus-mediated clustering of TfRs (I, III).

2. The PLA_2 activity of CPV capsids located in the N-terminal part of VP1 was required for successful infection because $sPLA_2$ inhibitors inhibited viral proliferation (III).

3. CPV showed the ability to bind several membrane lipids, suggesting that CPV has affinity for almost all membranous organelles along its entry pathway. The lipid binding and PLA_2 activity of CPV capsids seemed to be independent activities (III).

4. CPV infection did not completely disrupt endosomal vesicles but modified the permeability of the endosomal/lysosomal membranes (III).

5. Dynein dependent trafficking along microtubules was needed in two different phases during CPV entry.

    i) During endosomal trafficking of CPV capsids (I, II).

    ii) After viral penetration into the cytosol, when CPV capsids were trafficked towards the nucleus in cytoplasm (II).

Acknowledgements

The work described in this thesis has been carried out at the Division of Biochemistry, Department of Environmental and Biological Science, University of Jyväskylä, Finland.

First, I whish to thank professors Jyrki Heino and Matti Vuento for providing exellent research facilities.

I also want to thank my supervisors Dr. Maija Vihinen-Ranta and Prof. Matti Vuento for being excellent role models, and having enthusiastic and creative attitudes towards the science. They gave me support when I was tired or experiments failed and gave credit when it was earned or needed. I also appreciate the freedom that they gave me during the course of this work, in which I became involved because of Maija's inspiring teaching during my years already as a undergraduate.

I want to thank my external reviewers, Docent Eeva-Liisa Eskelinen and Prof. Esa Kuismanen for their valuable comments and advise for improvement of this thesis. I would also like to thank Dr. Daniel J. White and Leona Gilbert for reviewing my English.

Then I wish to thank my dearest colleagues, Katja Sääjärvi, Jonna Nykky, Mika Laitinen, Outi Välilehto and Jouni Toivola for support and all the discussions, some scientific and some not so scientific ones. I want to express my deepest gratitude to Katja, whose support and sympathy were irreplaceable. My warm thanks go to Mika, who patiently took care of computer problems. In addition, I would like to thank our warm hearted laboratory technician Pirjo Kauppinen for the excellent technical assistance and my progradu students Mia Antila, Anne Jaatinen, Marjukka Nevalainen and Salme Mujunen, and all the trainees for the help and for having personalities, giving new energy for the whole group.

I want to thank my dearest coworkers Mirka Salminen, Heli Lehtinen, Varpu Marjomäki, Paula Upla and Mira Tulla and all the staff of the Molecular Recognition team for creating a great atmosphere. My special thanks go to Leona Gilbert, who has brightened many of my days during all these years by sharing a common interest towards so many things including science. I would also like to warmly thank my dear friend and colleague Kirsi Ojala. She has been my support in many diverse ways. In addition, I would like to thank Laura Pitkänen and Raija Vassinen for the support and help they gave me during these years.

My warmest thanks go to my parents Tuula and Jorma Suikkanen, and also my grandparents, for their endless support and love during my life.

Finally, my very best and warmest thanks go to my love Harri Kirjavainen. His love and great attitude to life were indispensable during this work. I also want to express my thanks to my therapy dog, Nina. She provided me relaxing moments with many good laughs and took me out to get some fresh air.

This work was supported by grants from the Academy of Finland, The Finnish Foundation of Research on Viral Diseases, the Ellen and Artturi Nyyssönen Foundation, Galilaeus Oy and the National Technology Agency (TEKES).

YHTEENVETO (Résumé in Finnish)

Koiran parvovirus (CPV) on ainoa tunnettu koiraeläimille infektioita aiheuttava parvovirus. CPV eriytyi kissan panleukopeniaviruksesta 1970-luvun lopulla ja levisi nopeasti ympäri maailmaa aiheuttaen aikuisille koirille ankaraa ripulia ja pennuille tappavia sydänlihastulehduksia (Siegel et al. 1985, Parrish, 1990, Berns 1996).

CPV-partikkeli rakentuu yksijuosteisesta DNA-genomista ja sitä suojaavasta proteiinikuoresta (kapsidista). Proteiinikuoresta 90% on viruksen rakenneproteiini-2:ta (VP2) ja noin 10% rakenneproteiini-1:tä (VP1) (Cotmore & Tattersall, 1987). VP1:n aminopäästä on löydetty sekä potentiaalinen tumakuljetussignaali että eritettävän tyypin fosfolipaasi A_2:n ($sPLA_2$) aktiivista kohtaa muistuttava aminohapposekvenssi (Zádori et al. 2001, Vihinen-Ranta et al. 2002). Normaalisti VP1:n aminopää on kapsidin sisällä, mutta se saadaan paljastumaan urea-käsittelyllä tai kuumentamalla (≥55 °C) kapsideja ilman kapsidin hajoamista (Tullis et al. 1993, Cotmore et al. 1999, Vihinen-Ranta et al. 2002).

CPV tunnistaa isäntäsolunsa ja aloittaa matkansa solun sisään sitoutumalla solun pinnalla transferriinireseptoreihin. Reseptoreihin sitoutuneet virukset tunkeutuvat isäntäsoluihin klatriinipeitteisissä rakkuloissa ja seuraavat transferriinin solunsisäistä reittiä (kierrättävä endosytoosi) (Parker et al. 2001). CPV tarvitsee endosomaalisen reitin happamia olosuhteita ja ehjää mikrotubulustukirankaa pystyäkseen tuottoisaan infektioon (Vihinen-Ranta 1998, Parker & Parrish 2000). Vaikka CPV on yksi tutkituimmista parvoviruksista, sen solunsisäinen infektioreitti on vielä suurelta osin tuntematon. Myös viruksen vapautuminen endosomaaliselta reitiltä sytoplasmaan ja eteneminen kohti tumaa endosomeista vapautumisen jälkeen ovat huonosti tunnettuja, mutta infektion kannalta kriittisiä vaiheita.

CPV:n havaittiin samoissa rakkuloissa lysosomaalisen merkkiproteiinin (LAMP-2:n) kanssa. Myös virus-DNA näytti etenevän lysosomeihin asti, sillä virus DNA:n fluoresenssi-*in situ*-leima ja kapsidileima havaittiin päällekkäisinä. Näiden tulosten perusteella CPV tunkeutuu soluun klatriinipeitteisten vesikkelien kautta kierrättäviin endosomeihin, joista virukset ilmeisiesti siirtyvät myöhäisten endosomien kautta lysosomeihin. CPV näyttäisi olevan sitoutuneena transferriinireseptoriin koko endosomaalisen kuljetuksen ajan, sillä konfokaalimikroskoopilla tarkasteltuna CPV:n ja transferriinireseptorin vasta-aineleimat pysyivät päällekkäisinä koko seuratun 10 tunnin ajan infektion aloituksesta lähtien. Lisäksi infektion edetessä transferrinireseptoreja havaittiin kertyvän lysosomeihin.

CPV:n sekvenssin on osoitettu sisältävän $sPLA_2$:n kaltaisen alueen VP1:n aminopäässä. Koska $sPLA_2$-entsyymiaktiivisuutta voidaan hyödyntää solun kalvojen läpäisyssä, tutkittiin onko viruskapsideilla PLA_2-aktiivisuutta ja hyödyntääkö CPV tätä vesikkeleistä vapautumiseen. Viruspartikkelien PLA_2-aktiivisuutta mitattiin kolorimetrisella testillä. Puhdistetut viruspartikkelit eivät osoittaneet aktiivisuutta, mutta kapsidien lämmitys yli 55 °C:een tai käsittely happamalla puskurilla (pH 4-6) saivat aikaan kapsidien PLA_2:n aktivoitumisen. VP1:n aminopään havaittiin paljastuvan infektoiduissa soluissa viruksen edettyä lysosomeihin. PLA_2-inhibiittorit estivät CPV-infektion, mikä kertoo aktiivisuuden välttämättömyydestä infektioprosessissa. PLA_2-aktiivisuus ei kuitenkaan yksin näyttäisi riittävän vapauttamaan virusta sytoplasmaan, sillä

amiloridi, bafilomysiini A_1, brefeldiini A tai monensiini eivät pystyneet estämään VP1:n aminopään paljastumista kapsidissa, vaikka ne estivät tuottavan infektion. Tätä tukee myös havainto kokeesta, jossa solut infektoitiin viruksilla, joiden VP1:n aminopää oli paljastettu lämpökäsittelyllä. Nämä virukset eivät pystyneet aiheuttamaan tuottoisaa infektiota amiloridi-, bafilomysiini A1-, brefeldiini A- tai monensiinikäsitellyissä soluissa. Näiden tulosten perusteella on todennäköistä, että viruksessa tai sen ympäristössä tapahtuu myös muita happamuudesta riippuvaisia muutoksia kuin VP1:n aminopään vapautuminen.

Viime vuosina on julkaistu useita tutkimuksia, joissa osoitetaan endosomaalisten vesikkelien kalvojen eroavan toisistaan lipidikoostumukseltaan. Halusimme selvittää onko CPV-partikkeleilla taipumusta sitoutua lipideihin, joita löytyy tarkasti rajatulta vesikkelipopulaatiolta saadaksemme lisää tietoa viruksen tunkeutumisesta ulos endosomaalisista rakkuloista. Tulos kuitenkin osoitti, että virus pystyy sitoutumaan kalvolipideihin, joita löytyy niin klatriinivuoratuista vesikkeleistä, varhaisista endosomeista, kierrättävistä endosomeista kuin myöhäisistä endosomeista. Tässä kokeessa käytettiin käsittelemättömiä puhdistettuja viruspartikkeleita, lämmitettyjä viruksia, joilla VP1:n aminopää on paljastunut ja viruspartikkeleita, jotka olivat sekä lämmitettyjä että VP1 aminopään tunnistavalla vasta-aineella käsiteltyjä. Eri tavoin käsiteltyjen virusten välillä ei havaittu eroja sitoutumisessa lipideihin, mistä voidaan päätellä, että CPV:n sitoutuminen kalvopideihin ei ole sidoksissa VP1:een tai sen PLA_2-aktiivisuuteen.

Vaipattomien virusten vapautuminen endosomaaliselta reitiltä solulimaan tunnetaan huonosti. Aikaisemmissa tutkimuksissa on osoitettu, että CPV-infektio ei aiheuta endosomaalisten rakkuloiden täydellistä hajoamista, sillä □-sarkiini ei vapautunut solulimaan CPV-infektion vaikutuksesta. Tässä väitöstyössä CPV:n vapautumista vesikkeleistä tutkittiin syöttämällä soluille kooltaan joko Mr 3 000 tai Mr 10 000 dekstraania, yhdessä viruksen kanssa. Infektion edetettyä 8 tuntia pienempi dekstraani alkoi vapautua sytoplasmaan, josta se eteni tumaan. Suuremman dekstraanin ei havaittu vapautuvan sytoplasmaan edes infektion edettyä 20 tuntia. Tulosten perusteella CPV näyttää muuttavan endosomaalisia kalvoja läpäiseviksi suhteellisen pienikokoisille molekyyleille, mutta infektio ei aiheuta varsinaisten reikien muodostumista endosomaalisiin rakkuloihin eikä myöskään vesikkelien hajoamista.

Soluliman suuri proteiinipitoisuus, soluelimet ja solun tukiranka rajoittavat vapaata diffuusiota tehokkaasti. Tästä johtuen on todennäköistä, että tehokkaaseen infektioon pystyvät virukset etenevät solulimassa hyödyntäen solun kuljetuskoneistoa. Tukirangan ja moottoriproteiinien osallistumista CPV:n solunsisäiseen kuljetukseen tutkittiin *in vivo* ja *in vitro* kokein sekä elektronimikroskooppisesti että konfokaalimikroskooppisesti. Sytoplasmista liikennettä tutkittaessa virukset saatettiin suoraan sytoplasmaan mikroinjektiolla. Mikroskooppisten tutkimusten tueksi tehtiin myös koimmunopresipitaatiokokeita. Tulokset osoittivat, että CPV-infektiossa on todennäköisesti kaksi erillistä kohtaa, joissa virus tarvitsee mikrotubulusten ja dyneiini-moottoriproteiinin apua kulkeakseen kohti tumaa. Ensimmäisessä vaiheessa virus on kierrättävissä endosomeissa, joiden liikkuminen kohti solun keskustaa on riippuvaista sekä mikrotubuluksista että dyneiinistä. Tämän lisäksi osoitimme CPV:n tarvitsevan mikrotubulusten ja dyneiinin apua kulkiessaan kohti tumaa viruksen vapauduttua sytoplasmaan. Tulosten perusteella virus näyttäisi kiinnittyvän dyneiiniin ja kulkevan viruspartikkelina kohti tumaa. On kuitenkin

epäselvää liittyykö dyneiini suoraan virukseen vai tarvitaanko onnistuneeseen matkaan myös muita komponentteja.

Tämän väitöskirjatyön tulokset antavat kehykset CPV:n ja sen läheisten sukulaisvirusten solunsisäisen reitin tarkemmalle tutkimukselle. Karttuva tieto virusten solunsisäisestä kulusta puolestaan tarjoaa mahdollisuuden virus-lääkkeiden ja rokotteiden suunnitteluun. Mielenkiintoa parvoviruksien tutkimukseen lisää myös kiinnostus parvopartikkeleiden geeniterapiavektori-sovellutuksiin.

REFERENCES

Agbandje, M., Mckenna, R., Rossmann, M. G., Strassheim, M. L. & Parrish, C. R. 1993. Structure determination of feline panleukopenia virus empty particles. Proteins 16, 155-171.

Agbandje, M., Kajigaya, S., Mckenna, R., Young, N. S. & Rossmann, M. G. 1994. The structure of human parvovirus B19 at 8 A resolution. Virology 203, 106-115.

Agbandje-Mckenna, M., Llamas-Saiz, A. L., Wang, F., Tattersall, P. & Rossmann, M. G. 1998. Functional implications of the structure of the murine parvovirus, minute virus of mice. Structure 6, 1369-1381.

Al-Awqati, Q. 1986. Proton-translocating ATPases. Annu. Rev. Cell Biol. 2, 179-99.

Allan, V. 1996. Motor proteins: a dynamic duo. Curr. Biol. 6, 630-633.

Almela, M. J., Gonzalez, M. E. & Carrasco, L. 1991. Inhibitors of poliovirus uncoating efficiently block the early membrane permeabilization induced by virus particles. J. Virol. 65, 2572-2577.

Alonso, C., Miskin, J., Hernaez, B., Fernandez, Z. P., Soto, L., Canto, C., Rodriguez, C. I., Dixon, L. & Escribano, J. M. 2001. African swine fever virus protein p54 interacts with the microtubular motor complex through direct binding to light-chain dynein. J. Virol. 75, 9819-9827.

Aniento, F., Emans, N., Griffiths, G. & Gruenberg, J. 1993. Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. J. Cell Biol. 123, 1373-1387.

Astell, C. R., Chow, M. B. & Ward, D. C. 1985. Sequence analysis of the termini of virion and replicative forms of minute virus of mice DNA suggests a modified rolling hairpin model for autonomous parvovirus DNA replication. J. Virol. 54, 171-177.

Authier, F., Posner, B. I. & Bergeron, J. J. 1996. Endosomal proteolysis of internalized proteins. FEBS Lett. 389, 55-60.

Ball-Goodrich, L. J. & Tattersal, P. 1992. Two amino acid substitutions within the capsid are coordinately required for acquisition of fibrotropism by the lymphotropic strain of minute virus of mice. J. Virol. 66, 3415-3423.

Ball-Goodrich, L. J., Moir, R. D. & Tattersall, P. 1991. Parvoviral target cell specificity: acquisition of fibrotropism by a mutant of the lymphotropic strain of minute virus of mice involves multiple amino acid substitutions within the capsid. Virology 184, 175-186.

Bananis, E., Murray, J. W., Stockert, R. J., Satir, P. & Wolkoff, A. W. 2000. Microtubule and motor-dependent endocytic vesicle sorting in vitro. J. Cell Biol. 151, 179-186.

Bantel-Schaal, U., Hub, B. & Kartenbeck, J. 2002. Endocytosis of adeno-associated virus type 5 leads to accumulation of virus particles in the Golgi compartment. J. Virol. 76, 2340-2349.

Barbis, D. P. & Parrish, C. R. 1994. Characterization of canine parvovirus (CPV) interactions with 3201 T cells: involvement of GPI-anchored protein(s) in binding and infection. Braz. J. Med. Biol. Res. 27, 401-407.

Bartlett, J. S., Wilcher, R. & Samulski, R. J. 2000. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. J. Virol. 74, 2777-2785.

Barton, N. R. & Goldstein, L. S. 1996. Going mobile: microtubule motors & chromosome segregation. Proc. Natl. Acad. Sci. U S A 93, 1735-1742.

Basak, S. & Turner, H. 1992. Infectious entry pathway for canine parvovirus. Virology 186, 368-376.

Bashir, T., Horlein, R., Rommelaere, J. & Willwand, K. 2000. Cyclin A activates the DNA polymerase delta -dependent elongation machinery in vitro: A parvovirus DNA replication model. Proc. Natl. Acad. Sci. U S A 97, 5522-5557.

Bayer, N., Schober, D., Prachla, E., Murphy, R. F., Blaas, D. & Fuchs, R. 1998. Effect of bafilomycin A1 and nocodazole on endocytic transport in He La Cells: implications for viral uncoating and infection. J. Virol. 72, 9645-9655.

Bayliss, R., Corbett, A. H. & Stewart, M. 2000. The molecular mechanism of transport of macromolecules through nuclear pore complexes. Traffic 1, 448-456.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. & Finberg, R. W. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275, 1320-1323.

Berns, K. I. 1990. Parvovirus replication. Microbiol. Rev. 54, 316-329.

Berns, K. I. 1996. Parvoviridae: The Viruses and Their replication. In: Fields, B. N. Knipe, D. M., Howley, P. M. et al. (eds), Virology 2: 2173-2220 Lippincott-Raven Publishers, Philadelphia.

Berns, K. I. & Adler, S. 1972. Separation of two types of adeno-associated virus particles containing complementary polynucleotide chain. J. Virol. 9, 394-396.

Berns, K. I. & Bohenzky, R. A. 1987. Adeno-associated viruses: an update. Adv. Virus. Res. 32, 243-306.

Bloom, M. E., Alexandersen, S., Perryman, S., Lechner, D. & Wolfinbarger, J. B. 1988. Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a nonpathogenic and a pathogenic strain of ADV. J. Virol. 62, 2903-2915.

Boylan, K., Serr, M., & Hays, T. 2000. A molecular genetic analysis of the interaction between the cytoplasmic dynein intermediate chain and the glued (dynactin) complex. Mol. Biol. Cell 11, 3791-3803.

Brady, S. T., Lasek, R. J., & Allen, R. D. 1982. Fast axonal transport in extruded axoplasm from squid giant axon. Science 218, 1129-1131.

Brown, K. D., Friedkin, M. & Rozengurt, E. 1980. Colchicine inhibits epidermal growth factor degradation in 3T3 cells. Proc. Natl. Acad. Sci. U S A 77, 480-4.

Brown, K. E., Anderrsson, S. M. & Young, N. S. 1993. Erythrosyte P antigen: cellular receptor for B19 parvovirus. Science 262, 114-117.

Brown, W. J. & Farquhar, M. G. 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. Methods Cell Biol. 31, 553-69.

Brownstein, D. G., Smith, A. L., Johnson, E. A., Pintel, D. J., Naeger, L. K. & Tattersall, P. 1992. The pathogenesis of infection with minute virus of mice depends on expression of the small nonstructural protein NS2 and on the genotype of the allotropic determinants VP1 and VP2. J. Virol. 66, 3118-24.

Brunstein, J., Söderlund-Venermo, M. & Hedman, K. 2000. Identification of a novel RNA splicing pattern as a basis of restricted cell tropism of erythrovirus B19. Virology 274, 284-291.

Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B. & Zerial, M. 1992. The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell 70, 715-728.

Bullough, P. A., Hughson, F. M., Skehel, J. J. & Wiley, D. C. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature 371, 37-43.

Burger, K. N., Demel, R. A., Schmid, S. L. & De Kruijff, B. 2000. Dynamin is membrane-active: lipid insertion is induced by phosphoinositides and phosphatidic acid. Biochemistry 39, 12485-12493.

Caillet-Fauquet, P., Perros, M., Brandenburger, A., Spegelaere, P. & Rommelaere, J. 1990. Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. Embo J. 9, 2989-2995.

Cain, C. C., Sipe, D. M. & Murphy, R. F. 1989. Regulation of endocytic pH by the Na+,K+-ATPase in living cells. Proc. Natl. Acad. Sci. U S A 86, 544-548.

Chang, S.-F., Sgro, J.-Y. & Parrish, C. R. 1992. Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties. J. Virol. 66, 6858-6867.

Chapman, M. S. & Rossmann, M. G. 1993. Structure, sequence, and function correlations among parvoviruses. Virology 94, 491-508.

Chardin, P. & Mccormick, F. 1999. Brefeldin A: the advantage of being uncompetitive. Cell 97, 153-155.

Chatterjee, S., Lu, D., Podsakoff, G. & Wong, K. K., Jr. 1995. Strategies for efficient gene transfer into hematopoietic cells. The use of adeno-associated virus vectors in gene therapy. Annu. Acad. Sci. 770, 79-90.

Chemello, M. E., Aristimuno, O. C., Michelangeli, F. & Ruiz, M. C. 2002. Requirement for vacuolar H+ -ATPase activity and Ca2+ gradient during entry of rotavirus into MA104 cells. J. Virol. 76, 13083-13087.

Chipman, P. R., Agbandje-Mckenna, M., Kajigaya, S., Brown, K. E., Young, N. S., Baker, T. S. & Rossmann, M. G. 1996. Cryo-electron microscopy studies of empty capsids of human parvovirus B19 complexed with its cellular receptor. Proc. Natl. Acad. Sci. U S A 93, 7502-7506.

Christensen, J., Cotmore, S. F. & Tattersall, P. 1997. A novel cellular site-specific DNA-binding protein cooperates with the viral NS1 polypeptide to initiate parvovirus DNA replication. J. Virol. 71, 1405-1416.

Clague, M. J., Urbé, S., Anieto, F. & Gruenberg, J. 1994. Vacuolar ATPase activity is required for endosomal carrier vesicle formation. J. Biol. Chem. 269, 21-24.

Clemens, K. E., Cerutis, D. R., Burger, L. R., Yang, C. Q. & Pintel, D. J. 1990. Cloning of minute virus of mice cDNAs and preliminary analysis of individual viral proteins expressed in murine cells. J. Virol. 64, 3967-3973.

Cortes, E., San Martin, C., Langeveld, J., Meloen, R., Dalsgaard, K., Vela, C. & Casal, I. 1993. Topographical analysis of canine parvovirus virions and recombinant VP2 capsids. J. Gen. Virol. 74, 2005-2010.

Cossons, N., Faust, E. A. & Zannis-Hadjopoulos, M. 1996. DNA polymerase delta-dependent formation of a hairpin structure at the 5' terminal palindrome of the minute virus of mice genome. Virology 216, 258-64.

Cotmore, S. F. & Tattersall, P. 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. 58, 724-732.

Cotmore, S. F. & Tattersall, P. 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Vir. Res. 33, 91-169.

Cotmore, S. F. & Tattersall, P. 1989. A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. J. Virol. 63, 3902-3911.

Cotmore, S. F. & Tattersall, P. 1990. Alternate splicing in a parvoviral nonstructural gene links a common amino-terminal sequence to downstream domains which confer radically different localization and turnover characteristics. Virology 177, 477-487.

Cotmore, S. F. & Tattersall, P. 1992. In vivo resolution of circular plasmids containing concatemer junction fragments from minute virus of mice DNA and their subsequent replication as linear molecules. J. Virol. 66, 420-431.

Cotmore, S. F. & Tattersall, P. 1994. An asymmetric nucleotide in the parvoviral 3' hairpin directs segregation of a single active origin of DNA replication. Embo J. 13, 4145-4152.

Cotmore, S. F., Christensen, J., Nuesch, J. P. & Tattersall, P. 1995. The NS1 polypeptide of the murine parvovirus minute virus of mice binds to DNA sequences containing the motif [ACCA]2-3. J. Virol. 69, 1652-1660.

Cotmore, S. F., D'abramo a, M., Jr., Ticknor, C. M. & Tattersall, P. 1999. Controlled conformational transitions in the MVM virion expose the VP1 N-terminus and viral genome without particle disassembly. Virology 254, 169-181.

Cudmore, S., Reckmann, I. & Way, M. 1997. Viral manipulations of the actin cytoskeleton. Trends. Microbiol. 5, 142-148.

Cullen, B. R. 2001. Journey to the center of the cell. Cell 105, 697-700.

D'arrigo, A., Bucci, C., Toh, B. H. & Stenmark, H. 1997. Microtubules are involved in bafilomycin A1-induced tubulation and Rab5-dependent vacuolation of early endosomes. Eur. J. Cell Biol. 72, 95-103.

Daro, E., Van Der Sluijs, P., Galli, T. & Mellman, I. 1996. Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. Proc. Natl. Acad. Sci. U S A 93, 9559-9564.

Daro, E., Sheff, D., Gomez, M., Kreis, T. & Mellman, I. 1997. Inhibition of endosome function in CHO cells bearing a temperature-sensitive defect in the coatomer (COPI) component epsilon-COP. J. Cell Biol. 139, 1747-1759.

Davis, L. I. 1995. The nuclear pore complex. Annu. Rev. Biochem. 64, 865-896.

De Beeck, A. O., Sobczak-Thepot, J., Sirma, H., Bourgain, F., Brechot, C. & Caillet-Fauquet, P. 2001. NS1- and minute virus of mice-induced cell cycle arrest: involvement of p53 and p21(cip1). J. Virol. 75, 11071-11078.

Dohner, K., Wolfstein, A., Prank, U., Echeverri, C., Dujardin, D., Vallee, R. & Sodeik, B. 2002. Function of dynein and dynactin in herpes simplex virus capsid transport. Mol. Biol. Cell 13, 2795-2809.

Donaldson, J. G., Cassel, D., Kahn, R. A. & Klausner, R. D. 1992. ADP-ribosylation factor, a small GTP-binding protein, is required for binding

of the coatomer protein beta-COP to Golgi membranes. Proc. Natl. Acad. Sci. U S A 89, 6408-6412.

Dorsch, S., Liebisch, G., Kaufmann, B., Von Landenberg, P., Hoffmann, J. H., Drobnik, W. & Modrow, S. 2002. The VP1 unique region of parvovirus B19 and its constituent phospholipase A2-like activity. J. Virol. 76, 2014-2018.

Douar, A. M., Poulard, K., Stockholm, D. & Danos, O. 2001. Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. J. Virol. 75, 1824-1833.

Duan, D., Li, Q., Kao, A. W., Yue, Y., Pessin, J. E. & Engelhardt, J. F. 1999. Dynamin is required for recombinant adeno-associated virus type 2 infection. J. Virol. 73, 10371-10376.

Eichwald, V., Daeffler, L., Klein, M., Rommelaere, J. & Salomé, N. 2002 The NS2 proteins of parvovirus minute virus of mice are required for efficient nuclear egress of progeny virions in mouse cells. J. Virol. 76, 10307-10319.

Empig, C. J. & Goldsmith, M. A. 2002. Association of the caveola vesicular system with cellular entry by filoviruses. J. Virol. 76, 5266-5270.

Feldherr, C. M. & Akin, D. 1990. The permeability of the nuclear envelope in dividing and nondividing cell cultures. J. Cell Biol. 111, 1-8.

Feldherr, C. M., Akin, D. & Cohen, R. J. 2001. Regulation of functional nuclear pore size in fibroblasts. J. Cell Sci. 114, 4621-7.

Fenner, F., Bachmann, P. A., Gibbs, E. P. J., Murphy, F. A., Studdert, M. J. & White, D. O. 1987. Parvoviridae In: (ed.) Veterinary virology, 1: 407-419. Academic press, inc., Orlando, Florida.

Fernandez-Puentes, C. & Carrasco, L. 1980. Viral infection permeabilizes mammalian cells to protein toxins. Cell 20, 769-775.

Frischknecht, F., Moreau, V., Rottger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G. & Way, M. 1999. Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. Nature 401, 926-929.

Fuchs, R., Schmid, S. & Mellman, I. 1989. A possible role for Na+,K+-ATPase in regulating ATP-dependent endosome acidification. Proc. Natl. Acad. Sci. U S A 86, 539-543.

Gagescu, R., Demaurex, N., Parton, R. G., Hunziker, W., Huber, L. A. & Gruenberg, J. 2000. The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. Mol. Biol. Cell 11, 2775-2791.

Gahl, W. A. & Tietze, F. 1985. pH effects on cystine transport in lysosome-rich leucocyte granular fractions. Biochem. J. 228, 263-267.

Gardiner, E. M. & Tattersall, P. 1988. Mapping of the fibrotropic and lymphotropic host range determinants of the parvovirus minute virus of mice. J. Virol. 62, 2605-2613.

Ghosh, R. N., Gelman, D. L. & Maxfield, F. R. 1994. Quantification of low density lipoprotein and transferrin endocytic sorting HEp2 cells using confocal microscopy. J. Cell Sci. 107, 2177-2189.

Gillooly, D. J., Morrow, I. C., Lindsay, M., Gould, R., Bryant, N. J., Gaullier, J. M., Parton, R. G. & Stenmark, H. 2000. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. Embo J. 19, 4577-4588.

Girod, A., Wobus, C. E., Zadori, Z., Ried, M., Leike, K., Tijssen, P., Kleinschmidt, J. A. & Hallek, M. 2002. The VP1 capsid protein of adeno-

associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. J. Gen. Virol. 83, 973-978.
Glomb-Reinmund, S. & Kielian, M. 1998. The role of low pH and disulfide shuffling in the entry and fusion of Semliki Forest virus and Sindbis virus. Virology 248, 372-381.
Görlich, D. 1997. Nuclear protein import. Curr. Opin. Cell Biol. 9, 412-419.
Görlich, D. & Kutay, U. 1999. Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. 15, 607-660.
Görlich, D. & Mattaj, W. 1996. Nucleocytoplasmic transport. Science 271, 1513-1519.
Greber, U. F., Willetts, M., Webster, P. & Helenius, A. 1993. Stepwise Dismantling of adenovirus 2 during entry into cells. Cell 75, 477-486.
Greber, U. F., Suomalainen, M., Stidwill, R. P., Boucke, K., Ebersold, M. W. & Helenius, A. 1997. The role of the nuclear pore complex in adenovirus DNA entry. Embo J. 16, 5998-6007.
Grewe, C., Beck, A. & Gelderblom, H. R. 1990. HIV: early virus-cell interactions. J. Acquir. Imm. Defic. Syndr. 3, 965-974.
Griffiths, G. & Gruenberg, J. 1991. The arguments for pre-existing early and late endosomes. Trends. Cell Biol. 1, 5-9.
Griffiths, G., Mcdowall, A., Back, R. & Dubochet, J. 1984. On the preparation of cryosections for immunocytochemistry. J. Ultrastruct. Res. 89, 65-78.
Griffiths, G., Matteoni, R., Back, R. & Hoflack, B. 1990. Characterization of the cation-independent mannose 6-phophate receptorenriched prelysosomal compartment in NRK cells. J. Cell Sci. 95, 441-461.
Gruenberg, J. 2001. The endocytic pathway: a mosaic of domains. Nat. Rev. Mol. Cell Biol. 2, 721-30.
Gruenberg, J. & Howell, K. E. 1989. Membrane traffic in endocytosis: insights from cell-free assays. Annu. Rev. Cell Biol. 5, 453-481.
Gruenberg, J. & Maxfield, F. R. 1995. Membrane transport in the endocytic pathway. Curr. Opin. Cell Biol. 7, 552-563.
Gruenberg, J., Griffiths, G. & Howell, K. E. 1989. Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with assay of vesicle fusion in vitro. J. Cell Biol. 108, 1301-1316.
Gu, F. & Gruenberg, J. 2000. ARF1 regulates pH-dependent COP functions in the early endocytic pathway. J. Biol. Chem. 275, 8154-60.
Guinea, R. & Carrasco, L. 1995. Requirement for vacuolar proton-ATPase activity during entry of influenza virus into cells. J. Virol. 69, 2306-12.
Guo, Q., Vasile, E. & Krieger, M. 1994. Disruptions in Golgi structure and membrane traffic in a conditional lethal mammalian cell mutant are corrected by epsilon-COP. J. Cell Biol. 125, 1213-24.
Hansen, J., Qing, K. & Srivastava, A. 2001. Infection of purified nuclei by adeno-associated virus 2. Mol. Ther. 4, 289-96.
Harada, M., Shakado, S., Sakisaka, S., Tamaki, S., Ohishi, M., Sasatomi, K., Koga, H., Sata, M. & Tanikawa, K. 1997. Bafilomycin A1, a specific inhibitor of V-type H+-ATPases, inhibits the acidification of endocytic structures and inhibits horseradish peroxidase uptake in isolated rat sinusoidal endothelial cells. Liver 17, 244-50.
Hasilik, A. 1992. The early and late processing of lysosomal enzymes: proteolysis and compartmentation. Experientia 48, 130-51.
Helenius, A., Kielian, M., Mellman, I., & Schmid, S. 1989. Entry of enveloped viruses into their host cells. In: Compans, R. W., Helenius A., &

Oldstone, M. B. (eds), Cell Biology of Virus Entry, Replication, and pathogenesis: 145-161. John Wiley & Sons, Inc.

Hendrickson, H. S., Hendrickson, E. K. & Dybvig, R. H. 1983. Chiral synthesis of a dithiolester analog of phosphatidylcholine as a substrate for the assay of phospholipase A2. J. Lipid Res. 24, 1532-1537.

Hilden, S. A., Ghoshroy, K. B. & Madias, N. E. 1990. Na(+)-H+ exchange, but not Na(+)-K(+)-ATPase, is present in endosome-enriched microsomes from rabbit renal cortex. Am. J. Physiol. 258, 1311-1319.

Hirokawa, N., Noda, Y. & Okada, Y. 1998. Kinesin and dynein superfamily proteins in organelle transport and cell division. Curr. Opin. Cell Biol. 10, 60-73.

Hobbie, L., Fisher, A. S., Lee, S., Flint, A. & Krieger, M. 1994. Isolation of three classes of conditional lethal Chinese hamster ovary cell mutants with temperature-dependent defects in low density lipoprotein receptor stability and intracellular membrane transport. J. Biol. Chem. 269, 20958-20970.

Holleran, E. A., Karki, S. & Holzbaur, E. L. 1998. The role of the dynactin complex in intracellular motility. Int. Rev. Cytol. 182, 69-109.

Holzbaur, E. L. & Vallee, R. B. 1994. DYNEINS: molecular structure and cellular function. Annu. Rev. Cell Biol.10, 339-372.

Höltta-Vuori, M., Tanhuanpaa, K., Mobius, W., Somerharju, P. & Ikonen, E. 2002. Modulation of cellular cholesterol transport and homeostasis by Rab11. Mol. Biol. Cell. 13, 3107-3122.

Hopkins, C. R. & Trowbridge, I. S. 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. J. Cell Biol. 97, 508-521.

Hoque, M., Ishizu, K., Matsumoto, A., Han, S. I., Arisaka, F., Takayama, M., Suzuki, K., Kato, K., Kanda, T., Watanabe, H. & Handa, H. 1999. Nuclear transport of the major capsid protein is essential for adeno-associated virus capsid formation. J. Virol. 73, 7912-7915.

Horiuchi, M., Goto, H., Ishiguro, N. & Shinagawa, M. 1994. Mapping of determinants of the host range for canine cells in the genome of canine parvovirus using canine parvovirus/mink enteritis virus chimeric viruses. J. Gen. Virol. 75, 1319-1328.

Ireton, K. & Cossart, P. 1997. Host-pathogen interactions during entry and actin-based movement of Listeria monocytogenes. Annu. Rev. Genet. 31, 113-138.

Jahraus, A., Storrie, B., Griffiths, G., & Desjardins, M. 1994. Evidence for retrograde traffic between terminal lysosomes and the prelysosomal/late endosome compartment. J. Cell Sci. 107, 145-157.

Janik, J. E., Huston, M. M. & Rose, J. A. 1981. Locations of adenovirus genes required for the replication of adenovirus-associated virus. Proc. Natl. Acad. Sci. U S A 78, 1925-9.

Johnson, L. S., Dunn, K. W., Pytowski, B. & Mcgraw, T. E. 1993. Endosome acidification and receptor trafficking: bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. Mol. Biol. Cell 4, 1251-1266.

Jongeneel, C. V., Sahli, R., Mcmaster, G. K. & Hirt, B. 1986. A precise map of splice junctions in the mRNAs of minute virus of mice, an autonomous parvovirus. J. Virol. 59, 564-573.

Jost, M., Simpson, F., Kavran, J. M., Lemmon, M. A. & Schmid, S. L. 1998. Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. Curr. Biol. 8, 1399-1402.

Juuti-Uusitalo, K., Airenne, K. J., Laukkanen, A., Punnonen, E. L., Olkkonen, V. M., Gruenberg, J., Kulomaa, M. & Marjomäki, V. 2000. Selective targeting of avidin/mannose 6-phosphate receptor chimeras to early or late endosomes. Eur. J. Cell Biol. 79, 458-468.

Kaludov, N., Brown, K. E., Walters, R. W., Zabner, J. & Chiorini, J. A. 2001. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. J. Virol. 75, 6884-6893.

Kielian, M. 1995. Membrane fusion and the alphavirus life cycle. Adv. Virus Res. 45, 113-151.

Kielian, M. & Helenius, A. 1985. pH-induced alterations in the fusogenic spije protein of semliki forest virus. J. Cell Biol. 101, 2284-2291.

Kikkawa, M., Sablin, E. P., Okada, Y., Yajima, H., Fletterick, R. J. & Hirokawa, N. 2001. Switch-based mechanism of kinesin motors. Nature 411, 439-445.

King, S. J. & Schroer. T. A. 2000. Dynactin increases the processivity of the cytoplasmic dynein motor. Nat. Cell Biol. 2, 20-24.

Knipe, D. M. 1996. Virus-host cell interactions. In: (Fields, B. N. Knipe, D. M. & Howley, (eds), Virology: 273-299. Lippincott-Raven Publishers, Philadelphia.

Kobayashi, T., Stang, E., Fang, K. S., De Moerloose, P., Parton, R. G. & Gruenberg, J. 1998. A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. Nature 392, 193-197.

Kobayashi, T., Beuchat, M. H., Chevallier, J., Makino, A., Mayran, N., Escola, J. M., Lebrand, C., Cosson, P. & Gruenberg, J. 2002. Separation and characterization of late endosomal membrane domains. J. Biol. Chem. 277, 32157-32164.

Kornfeld, S. & Mellman, I. 1989. The biogenesis of lysosomes. Annu. Rev. Cell Biol. 5, 483-525.

Kotin, R. M., Siniscalco, M., Samulski, R. J., Zhu, X. D., Hunter, L., Laughlin, C. A., Mclaughlin, S., Muzyczka, N., Rocchi, M. & Berns, K. I. 1990. Site-specific integration by adeno-associated virus. Proc. Natl. Acad. Sci. U S A 87, 2211-2215.

Kotin, R. M., Menninger, J. C., Ward, D. C. & Berns, K. I. 1991. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. Genomics 10, 831-834.

Kreis, T. E., Lowe, M. & Pepperkok, R. 1995. COPs regulating membrane traffic. Annu. Rev. Cell. Dev. Biol.11, 677-706.

Langeveld, J. P., Kamstrup, S., Uttenthal, A., Strandbygaard, B., Vela, C., Dalsgaard, K., Beekman, N. J., Meloen, R. H. & Casal, J. I. 1995. Full protection in mink against mink enteritis virus with new generation canine parvovirus vaccines based on synthetic peptide or recombinant protein. Vaccine 13, 1033-1037.

Legendre, D. & Rommelaere, J. 1992. Terminal regions of the NS1 protein of the parvovirus minute virus of mice are involved in cytotoxicity and promoter transinhibition. J. Virol. 66,5705-5713

Legrand, C., Rommelaere, J. & Caillet-Fauquet, P. 1993. MVM(p) NS-2 protein expression is required with NS-1 for maximal cytotoxicity in human transformed cells. Virology 195, 149-55.

Leopold, P. L., Kreitzer, G., Miyazawa, N., Rempel, S., Pfister, K. K., Rodriguez, B. E. & Crystal, R. G. 2000. Dynein- and microtubule-mediated translocation of adenovirus serotype 5 occurs after endosomal lysis. Hum. Gene Ther. 11, 151-165.

Li, X. & Rhode, S. L., 3rd. 1990. Mutation of lysine 405 to serine in the parvovirus H-1 NS1 abolishes itd function for viral DNA replication, late promoter transactivation, and cytotoxicity. J. Virol. 64, 4654-4660.

Li, X. & Rhode, S. L., 3rd. 1991. Nonstructural protein NS2 of parvovirus H-1 is required for efficient viral protein synthesis and virus production in rat cells in vivo and in vitro. Virology 184, 117-30.

Li, Y., Zadori, Z., Bando, H., Dubuc, R., Fediere, G., Szelei, J., & Tijssen, P. 2001. Genome organization of the densovirus from Bombyx mori (BmDNV-1) and enzyme activity of its capsid. J. Gen. Virol. 82, 2821-5.

Lichtman, S. N., Wang, J., Zhang, C. & Lemasters, J. J. 1996. Endocytosis and Ca2+ are required for endotoxin-stimulated TNF-alpha release by rat Kupffer cells. Am. J. Physiol. 271, 920-928.

Lin, H. C., Barylko, B., Achiriloaie, M. & Albanesi, J. P. 1997. Phosphatidylinositol (4,5)-bisphosphate-dependent activation of dynamins I and II lacking the proline/arginine-rich domains. J. Biol. Chem. 272, 25999-6004.

Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L. & Klausner, R. D. 1991. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechnism for regulating organelle structure and membrane traffic. Cell 67, 601-616.

Liprandi, F., Moros, Z., Gerder, M., Ludert, J. E., Pujol, F. H., Ruiz, M. C., Michelangeli, F., Charpilienne, A. & Cohen, J. 1997. Productive penetration of rotavitus in cultured cells induces coentry of the tranlation inhibitor alfa-sarcin. Virology 237, 430-438.

Liu, J. M., Green, S. W., Shimada, T. & Young, N. S. 1992. A block in full-length transcript maturation in cells nonpermissive for B19 parvovirus. J. Virol. 66, 4686-4692.

Lombardo, E., Ramirez, J. C., Agbandje-Mckenna, M., & Almendral, J. M. 2000. A beta-stranded motif drives capsid protein oligomers of the parvovirus minute virus of mice into the nucleus for viral assembly. J. Virol. 74, 3804-3814.

Lombardo, E., Ramirez, J. C., Garcia, J. & Almendral, J. M. 2002. Complementary roles of multiple nuclear targeting signals in the capsid proteins of the parvovirus minute virus of mice during assembly and onset of infection. J. Virol. 76, 7049-7059.

Lowe, M. & Kreis, T. E. 1998. Regulation of membrane traffic in animal cells by COPI. Biochim. Biophys. Acta 1404, 53-66.

Luby-Phelps, K. 1994. Physical properties of cytoplasm. Curr. Opin. Cell Biol. 6, 3-9.

Luby-Phelps, K. 2000. Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. Int. Rev. Cytol. 192, 189-221.

Lukashov, V. V. & Goudsmit, J. 2001. Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate

parvoviruses and links between adeno-associated and avian parvoviruses. J. Virol. 75, 2729-2740.
MaCartney, L., Mccandlish, A. P., Thompson, H., & Cornwell, H. J. C. 1984. Canine parvovirus enteritis 2: pathogenesis. The Veterinary Record 3, 453-460.
Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B. & Johannes, L. 1998. Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment. J. Cell Biol. 143, 973-990.
Mandelkow, E. & Mandelkow, E. M. 1995. Microtubules and microtubule-associated proteins. Curr. Opin. Cell Biol. 7, 72-81.
Marjomäki, V., Pietiäinen, V., Matilainen, H., Upla, P., Ivaska, J., Nissinen, L., Reunanen, H., Huttunen, P., Hyypiä, T. & Heino, J. 2002. Internalization of echovirus 1 in caveolae. J. Virol. 76, 1856-1865.
Maroto, B., Ramirez, J. C. & Almendral, J. M. 2000. Phosphorylation status of the parvovirus minute virus of mice particle: mapping and biological relevance of the major phosphorylation sites. J. Virol. 74, 10892-10902.
Marsh, E. W., Leopold, P. L., Jones, N. L. & Maxfield, F. R. 1995. Oligomerized transferrin receptors are selectively retained by a lumenal sorting signal in a long-lived endocytic recycling compartment. J. Cell Biol. 129, 1509-1522.
Marsh, M. 2001. Endocytosis. Frontiers in Molecular Biology M. Marsh (ed.) Oxford University Press, Oxford.
Marsh, M. 1984. The entry of enveloped viruses into cells by endocytosis. Biochem. J. 218, 1-10.
Marsh, M. & Helenius, A. 1989. Virus entry into animal cells. In: Maramorosch, K. Murphy, F. A. & Shatkin, A. J. (eds), Advances in virus research, 36: 107-151. Academic Press inc., London.
Marsh, M. & Pelchen-Matthews, A. 2000. Endocytosis in viral replication. Traffic 1, 525-532.
Marsh, M. A. & Pelchen-Matthews, A. 1993. Entry of animal viruses into cells. Med. Virol. 3, 173-185.
Martinez, C. G., Guinea, R., Benavente, J. & Carrasco, L. 1996. The entry of reovirus into L cells is dependent on vacuolar proton-ATPase activity. J. Virol. 70, 576-579.
Matlin, K. S., Reggio, H., Helenius, A. & Simons, K. 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J. Cell Biol. 91(3 Pt 1), 601-13.
Matteoni, R. & Kreis, T. E. 1987. Tranlocation and clustering of endosomes and lysosomes depends on microtubules. Eur. J. Cell Biol. 105, 1253-1265.
Maxwell, I. H., Terrell, K. L. & Maxwell, F. 2002. Autonomous parvovirus vectors. Methods 28, 168-181.
Mayor, S., Presley, J. F. & Maxfield, F. R. 1999. Sorting of membrane components from endosomes and subsequent recycling to the cell surfase occurs by a bulk flow process. J. Cell Biol .121, 1257-1269.
McClure, M. O., Marsh, M. & Weiss, R. A. 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. Embo J. 7, 513-518.
McGraw, T. E., Dunn, K. W. & Maxfield, F. R. 1993. Isolation of a temperature-sensitive variant Chinese hamster ovary cell line with a morphologically altered endocytic recycling compartment. J. Cell Physiol. 155, 579-594.

McKenna, R., Olson, N. H., Chipman, P. R., Baker, T. S., Booth, T. F., Christensen, J., Aasted, B., Fox, J. M., Bloom, M. E., Wolfinbarger, J. B. & Agbandje-Mckenna, M. 1999. Three-dimensional structure of Aleutian mink disease parvovirus: implications for disease pathogenicity. J. Virol. 73, 6882-6891.

Meier, O., Boucke, K., Hammer, S. V., Keller, S., Stidwill, R. P., Hemmi, S. & Greber, U. F. 2002. Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. J. Cell Biol. 158, 1119-1131.

Mellman, I. 1996. Endocytosis and molecular sorting. Annu. Rev. Cell Dev. Biol. 12, 575-625.

Mellman, I. & Plutner, H. 1984. Internalization and degradation of macrophage Fc receptors bound to polyvalent immune complexes. J. Cell Biol 98, 1170-1177.

Mermall, V., Post, P. L. & Mooseker, M. S. 1998. Unconventional myosins in cell movement, membrane traffic, and signal transduction. Science 279, 527-533.

Merrifield, C. J., Moss, S. E., Ballestrem, C., Imhof, B. A., Giese, G., Wunderlich, I. & Almers, W. 1999. Endocytic vesicles move at the tips of actin tails in cultured mast cells. Nat. Cell Biol. 1, 72-74.

Molitor, T. W., Joo, H. S. & Collett, M. S. 1985. Identification and characterization of a porcine parvovirus nonstructural polypeptide. J. Virol. 55, 554-559.

Morita, E., Nakashima, A., Asao, H., Sato, H. & Sugamura, K. 2003. Human parvovirus B19 nonstructural protein (NS1) induces cell cycle arrest at G(1) phase. J. Virol. 77, 2915-21.

Mukherjee, S., Ghost, R. N. & Maxfield, R. 1997. Endocytosis. Physiol. Rev. 77, 759-803.

Murray, J. W., Bananis, E. & Wolkoff, A. W. 2000. Reconstitution of ATP-dependent movement of endocytic vesicles along microtubules in vitro: an oscillatory bidirectional process. Mol. Biol. Cell 11, 419-33.

Naeger, L. K., Cater, J., & Pintel, D. J. 1990. The small nonstructural protein (NS2) of the parvovirus minute virus of mice is required for efficient DNA replication and infectious virus production in a cell-type-specific manner. J. Virol. 64, 6166-6175.

Naides, S. J., Scharosch, L. L. Foto, F. & Howard, E. J. 1990. Rheumatologic manifestations of human parvovirus B19 infection in adults. Initial two-year clinical experience Arthritis Rheum. 33, 1297-1309.

Nigg, E. A. 1997. Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature 386, 779-787

Nuesch, J. P., Cotmore, S. F. & Tattersall, P. 1995. Sequence motifs in the replicator protein of parvovirus MVM essential for nicking and covalent attachment to the viral origin: identification of the linking tyrosine. Virology .209, 122-135.

Nuesch, J. P., Corbau, R., Tattersall, P. & Rommelaere, J. 1998. Biochemical activities of minute virus of mice nonstructural protein NS1 are modulated In vitro by the phosphorylation state of the polypeptide. J. Virol. 72, 8002-8012.

Nussbaum, O., Roop, A. & Anderson, W. F. 1993. Sequences determining the pH dependence of viral entry are distinct from the host range-

determining region of the murine ecotropic and amphotropic retrovirus envelope proteins. J. Virol. 67, 7402-7405.

Oda, H., Stockert, R. J., Collins, C., Wang, H., Novikoff, P. M., Satir, P. & Wolkoff, A. W. 1995. Interaction of the microtubule cytoskeleton with endocytic vesicles and cytoplasmic dynein in cultured rat hepatocytes. J. Biol. Chem. 270, 15242-15249.

Op De Beeck, A. & Caillet-Fauquet, P. 1997. The NS1 protein of the autonomous parvovirus minute virus of mice blocks cellular DNA replication: a consequence of lesions to the chromatin? J. Virol. 71, 5323-5329.

Ozawa, K. J., Ayub, S., Kajigaya, T., Shimada & Young, N. 1988. The gene encoding the nonstructural protein of B19 human parvovirus may be lethal in transfected cells. J. Virol. 62, 2884-2889.

Paradiso, P. R. 1981. Infectious process of the parvovirus H-1: correlation of protein content, particle density, and viral infectivity. J. Virol 39, 800-807.

Paradiso, P. R., Rhode, S. L., 3rd. & Singer, I. 1982. Canine parvovirus: a biochemical and ultrastructural characterization. J. Gen. Virol. 62, 113-125.

Parker, J. S. & Parrish, C. R. 2000. Cellular uptake and infection by canine parvovirus involves rapid dynamin-regulated clathrin-mediated endocytosis, followed by slower intracellular trafficking. J. Virol. 74, 1919-1930.

Parker, J. S., Murphy, W. J., Wang, D., O'brien, S. J. & Parrish, C. R. 2001. Canine and feline parvoviruses can use human or feline transferrin receptors to bind, enter, and infect cells. J. Virol. 75, 3896-3902.

Parrish, C. R. 1990. Emergence, natural history, and variation of canine, mink, and feline parvoviruses. Adv. Virus Res. 38, 403-450.

Parrish, C. R. 1991. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones Virology 183,195-205.

Parrish, C. R. & Carmichael, L. E. 1986. Characterization and recombination mapping of an antigenic and host range mutation of canine parvovirus. Virology 148, 121-132.

Parrish, C. R., Carmichael, L. E. & Antczak, D. F. 1982. Antigenic relationships between canine parvovirus type-2, feline panleukopenia virus and mink enteritis virus using conventional antisera and monoclonal antibodies. Arch. Virol. 72, 267-278.

Parrish, C. R., Aquadro, C. F., Strassheim, M. L., Evermann, J. F., Sgro, J.-Y. & Mohammed, H. O. 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J. Virol. 65, 6544-6552.

Pérez, L. & Carrasco, L. 1993. Entry of poliovirus into cells does not require a low-pH step. J. Virol. 67, 4543-4548.

Perez, L. & Carrasco, L. 1994. Involvement of the vacuolar H(+)-ATPase in animal virus entry. J. Gen. Virol. 75, 2595-2606.

Pillay, C. S., Elliott, E. & Dennison, C. 2002. Endolysosomal proteolysis and its regulation. Biochem. J. 363, 417-429.

Pisoni, R. L. & Thoene, J. G. 1991. The transport systems of mammalian lysosomes. Biochim. Biophys. Acta. 1071, 351-373.

Plank, C., Zauner, W. & Wagner, E. 1998. Application of membrane-active peptides for drug and gene delivery across cellular membranes. Adv. Drug Deliv. Rev. 34, 21-35.

Pless, D. D. & Wellner, R. B. 1996. In vitro fusion of endocytic vesicles: effect of reagents that alter endosomal pH. J. Cell Biochem. 62, 27-39.

Pollock, R. V. H. & Carmichael, L. E. 1982. Dog response to inactivated canine parvovirus and feline panleukopenia virus vaccines. Cornell Vet. 72, 16-35.

Pollock, R. V. H. & Carmichael, L. E. 1990. The canine parvoviruses. In: P. Tijssen (ed.). CRC handbook of parvoviruses. CRC Press, Inc., Boca Raton.

Prchla, E., Kuechler, E., Blaas, D. & Fuchs, R. 1994. Uncoating of human rhinovirus serotype 2 from late endosomes. J. Virol. 68, 3713-3723.

Prchla, E., Plank, C., Wagner, E., Blaas, D. & Fuchs, R. 1995. Virus-mediated release of endosomal content in vitro: different behavior of adenovirus and rhinovirus serotype 2. J. Cell Biol. 131, 111-123.

Prekeris, R., Klumperman, J., Chen, Y. A. & Scheller, R. H. 1998. Syntaxin 13 mediates cycling of plasma membrane proteins via tubulovesicular recycling endosomes. J. Cell Biol. 143, 957-971.

Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J. & Lippincott, S. J. 1997a. ER-to-Golgi transport visualized in living cells. Nature 389, 81-5.

Presley, J. F., Mayor, S., Mcgraw, T. E., Dunn, K. W. & Maxfield, F. R. 1997b. Bafilomycin A1 treatment retards transferrin receptor recycling more than bulk membrane recycling. J. Biol. Chem. 272, 13929-13936.

Pressman, B. C. 1976. Biological applications of ionophores. Annu. Rev. Biochem. 45, 501-530.

Rabinowitz, J. E. & Samulski, R. J. 2000. Building a better vector: the manipulation of AAV virions. Virology 278, 301-308.

Randazzo, P. A., Yang, Y. C., Rulka, C. & Kahn, R. A. 1993. Activation of ADP-ribosylation factor by Golgi membranes. Evidence for a brefeldin A- and protease-sensitive activating factor on Golgi membranes. J. Biol. Chem. 268, 9555-9563.

Reed, A. P., Jones, E. V. & Miller, T. J. 1988. Nucleotide sequence and genome organization of Canine Parvovirus. J. Virol. 62, 266-276.

Ren, M., Xu, G., Zeng, J., De Lemos-Chiarandini, C., Adesnik, M. & Sabatini, D. D. 1998. Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. Proc. Natl. Acad. Sci .U S A 95, 6187-6192.

Rhode, S. L., 3rd. 1974. Replication process of the parvovirus H-1. III. Factors affecting H-1 RF DNA synthesis. J. Virol. 14, 791-801.

Richards, R., Linser, P. & Armentrout, R. W. 1977. Kinetics of assembly of a parvovirus , minute virus of mice, in syncronized rat brain cells. J. Virol. 22, 778-793

Richterova, Z., Liebl, D., Horak, M., Palkova, Z., Stokrova, J., Hozak, P., Korb, J. & Forstova, J. 2001. Caveolae are involved in the trafficking of mouse polyomavirus virions and artificial VP1 pseudocapsids toward cell nuclei. J. Virol. 75, 10880-10891.

Riezman, H., Woodman, P. G., Van Meer, G. & Marsh, M. 1997. Molecular mechanisms of endocytosis. Cell 91, 731-738.

Robinson, M. K. & Pearse, B. M. F. 1986. Immunofluorescent localisation of 100K coated vesicle proteins . J. Cell Biol. 102, 48-54

Ros, C., Burckhardt, C. J. & Kempf, C. 2002. Cytoplasmic trafficking of minute virus of mice: low-pH requirement, routing to late endosomes, and proteasome interaction. J. Virol. 76, 12634-45.

Rossmann, M. G. & Johnson, J. E. 1989. Icosahedral RNA virus structure. Annu. Rev. Biochem. 58, 533-573.

Rozelle, A. L., Machesky, L. M., Yamamoto, M., Driessens, M. H., Insall, R. H., Roth, M. G., Luby-Phelps, K., Marriott, G., Hall, A. & Yin, H. L. 2000. Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. Curr. Biol. 10, 311-320.

Rubino, M., Miaczynska, M., Lippe, R. & Zerial, M. 2000. Selective membrane recruitment of EEA1 suggests a role in directional transport of clathrin-coated vesicles to early endosomes. J. Biol. Chem. 275, 3745-3748.

Ruiz, M. C., Alonso-Torre, S. R., Charpilienne, A., Vasseur, M., Michelangeli, F., Cohen, J. & Alvarado, F. 1994. Rotavirus interaction with isolated membrane vesicles. J. Virol. 68, 4009-4016.

Sabolic, I. & Brown, D. 1990. Na+(Li+)-H+ exchange in rat renal cortical vesicles with endosomal characteristics. Am. J. Physiol. 258, F1245-1253.

Saib, A., Neves, M., Giron, M. L., Guillemin, M. C., Valla, J., Peries, J. & Canivet, M. 1997. Long-term persistent infection of domestic rabbits by the human foamy virus. Virology 228, 263-268.

Sakai, T., Yamashina, S. & Ohnishi, S. 1991. Microtubule-disrupting drugs blocked delivery of endocytosed transferrin to the cytocenter, but did not affect return of transferrin to plasma membrane. J. Biochem. 109, 528-533.

Saliki, J. T., Mizak, B., Flore, H. P., Gettig, R. R., Burand, J. P., Carmichael, L. E., Wood, H. A. & Parrish, C. R. 1992. Canine parvovirus empty capsids produced by expression in a baculovirus vector: use in analysis of viral properties and immunization of dogs. J. Gen. Virol. 73, 369-374.

Samulski, R. J., Zhu, X., Xiao, X., Brook, J. D., Housman, D. E., Epstein, N. & Hunter, L. A. 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. Embo J. 10, 3941-3950.

Sanlioglu, S., Benson, P. K., Yang, J., Atkinson, E. M., Reynolds, T. & Engelhardt, J. F. 2000. Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. J. Virol. 74, 9184-9196.

Sawada, H. & Esaki, M. 1994. Use of nanogold followed by silver enhancement and gold toning for preembedding immunolocalization in osmium-fixed, Epon-embedded tissues. J. Electron. Microsc. 43, 361-366.

Sawada, H. & Esaki, M. 2000. A practical technique to postfix nanogold-immunolabeled specimens with osmium and to embed them in Epon for electron microscopy. J. Histochem. Cytochem. 48, 493-498.

Seksek, O., Biwersi, J. & Verkman, A. S. 1997. Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. J. Cell Biol. 138, 131-142.

Sheetz, M. P. 1999. Motor and cargo interactions. Eur. J. Biochem. 262, 19-25.

Shi, W., Arnold, G. S. & Bartlett, J. S. 2001. Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. Hum. Gene Ther. 12, 1697-1711.

Shisheva, A., Rusin, B., Ikonomov, O. C., Demarco, C. & Sbrissa, D. 2001. Localization and insulin-regulated relocation of phosphoinositide 5-kinase PIKfyve in 3T3-L1 adipocytes. J. Biol. Chem. 276, 11859-11869.

Siegel, G. 1984. The parvoviruses. Berns, K. I. (ed.). Plenum New York, New York.

Siegel, G., Bates, R. C., Berns, K. I., Carter, B. J., Kelly, D. C., Kurstak, E. & Tattersall, P. 1985. Characteristics and taxonomy of parvoviridae. Intervirology 23, 61-73.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M. & Stenmark, H. 1998. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature 394, 494-498.

Simonsen, A., Wurmser, A. E., Emr, S. D. & Stenmark, H. 2001. The role of phosphoinositides in membrane transport. Curr. Opin. Cell Biol. 13, 485-492.

Simpson, A. A., Chandrasekar, V., Hebert, B., Sullivan, G. M., Rossmann, M. G. & Parrish, C. R. 2000. Host range and variability of calcium binding by surface loops in the capsids of canine and feline parvoviruses. J. Mol. Biol. 300, 597-610.

Sipe, D. M. & Murphy, R. F. 1987. High-resolution kinetics of transferrin acidification in BALB/c 3T3 cells: exposure to pH 6 followed by temperature-sensitive alkalinization during recycling. Proc. Natl. Acad. Sci. U S A 84, 7119-7123.

Sodeik, B. 2000. Mechanisms of viral transport in the cytoplasm. Trends Microbiol. 8, 465-72.

Sodeik, B., Ebersold, M. W. & Helenius, A. 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J. Cell Biol. 136, 1007-1021.

Sojakka, K., Punnonen, E. L. & Marjomäki, V. S. 1999. Isoproterenol inhibits fluid-phase endocytosis from early to late endosomes. Eur. J. Cell Biol. 78, 161-169.

Sol, N., Le Junter, J., Vassias, I., Freyssinier, J. M., Thomas, A., Prigent, A. F., Rudkin, B. B., Fichelson, S. & Morinet, F. 1999. Possible iteractions between the NS-1 protein and tumor necrosis factor alpha pathways in erythroid cell apoptosis induced by human parvovirus B19. J. Virol. 73, 8762-8770.

Somsel Rodman, J. & Wandinger-Ness, A. 2000. Rab GTPases coordinate endocytosis. J. Cell Sci. 113, 183-192.

Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J., & Zerial, M. 2000. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J. Cell Biol. 149, 901-14.

Srivastava, A. 1994. Parvovirus-based vectors for human gene therapy. Blood Cells 20, 531-6; discussion 536-8.

Stein, B. S., Bensch, K. G. & Sussman, H. H. 1984. Complete inhibition of transferrin recycling by monensin in K562 cells. J. Biol. Chem. 259, 14762-14772.

Stein, B. S. & Sussman, H. H. 1986. Demonstration of two distinct transferrin receptor recycling pathways and transferrin-independent receptor internalization in K562 cell. J. Biol. Chem. 261, 10319-10331.

Steinman, R. M., Mellman, I. S., Muller, W. A., & Cohn, Z. A. 1983. Endocytosis and the recycling of plasma membrane. J. Cell Biol. 96, 1-27.

Strassheim, M. L., Gruenberg, A., Veijalainen, P., Sgro, J.-Y. & Parrish, C. R. 1994. Two dominant neutralizing antigenic determinants of canine

parvovirus are found on the threefold spike of the virus capsid. Virology 198, 175-184.
Sturzenbecker, L. J., Nibert, M., Furlong, D. & Fields, B. N. 1987. Intracellular digestion of reovirus particles requires a low pH and is an essential step in the viral infectious cycle. J. Virol. 61, 2351-61.
Summerford, C. & Samulski, R. J. 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J. Virol. 72, 1438-1445.
Suomalainen, M., Nakano, M. Y., Keller, S., Boucke, K., Stidwill, R. P., & Greber, U. F. 1999. Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus. J. Cell Biol. 144, 657-72.
Suomalainen, M., Nakano, M. Y., Boucke, K., Keller, S. & Greber, U. F. 2001. Adenovirus-activated PKA and p38/MAPK pathways boost microtubule-mediated nuclear targeting of virus. Embo J. 20, 1310-1319.
Tabas, I., Lim, S., Xu, X. X. & Maxfield, F. R. 1990. Endocytosed beta-VLDL and LDL are delivered to different intracellular vesicles in mouse peritoneal macrophages. J. Cell Biol. 111, 929-940.
Tabas, I., Myers, J. N., Innerarity, T. L., Xu, X. X., Arnold, K., Boyles, J. & Maxfield, F. R. 1991. The influence of particle size and multiple apoprotein E-receptor interactions on the endocytic targeting of beta-VLDL in mouse peritoneal macrophages. J. Cell Biol. 115, 1547-1560.
Tartakoff, A. M. 1983. Perturbation of vesicular traffic with the carboxylic ionophore monensin. Cell 32, 1026-1028.
Tattersall, P. & Cotmore, S. F. 1988. The nature of parvoviruses. In: Pattison, J. R. (ed.), Parvoviruses and human disease : 5-41. CRC Press, Boca Raton.
Tattersall, P., Cawte, P. J., Shatkin, A. J. & Ward, D. C. 1976. Three structural polypeptides coded for by minite virus of mice, a parvovirus. J. Virol. 20, 273-289.
Taunton, J., Rowning, B. A., Coughlin, M. L., Wu, M., Moon, R. T., Mitchison, T. J. & Larabell, C. A. 2000. Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. J. Cell Biol. 148, 519-530.
Thacker, T. C. & Johnson, F. B. 1998. Binding of bovine parvovirus to erythrocyte membrane sialylglycoproteins. J. Gen. Virol. 79, 2163-2169.
Tjelle, T. E., Brech, A., Juvet, L. K., Griffiths, G. & Berg, T. 1996. Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: their role in protein degradation. J. Cell Sci. 109, 2905-2914.
Tokuyasu, K. T. 1973. A technique for ultracryotomy of cell suspensions and tissues. J. Cell Biol. 57, 551-565.
Tokuyasu, K. T. 1978. A study of positive staining of ultrathin frozen sections. J. Ultrastruct. Res. 63, 287-307.
Tomko, R. P., Xu, R. & Philipson, L. 1997. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. U S A 94, 3352-3356.
Tosteson, M. T. & Chow, M. 1997. Characterization of the ion channels formed by poliovirus in planar lipid membranes. J. Virol. 71, 507-511.
Towbin, H., Staehelin, T. & Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U S A 76, 4350-4354.
Tran, D., Carpentier, J. L., Sawano, F., Gorden, P. & Orci, L. 1987. Ligands internalized through coated or noncoated invaginations follow a

common intracellular pathway. Proc. Natl. Acad. Sci. U S A 84, 7957-7961.

Trowbridge, I. S., Collawn, J. F. & Hopkins, C. R. 1993. Signal-dependent membrane protein trafficing in tha endocitic pathway. Annu. Rev. Cell Biol. 9, 129-161.

Truyen, U. A. P. & Parrish, C. R. 1992. Canine and feline host ranges of canine parvovirus and feline panleukopenia virus: distinct host cell tropisms of each virus In vitro and In vivo. J. Virol. 66, 5399-5408.

Truyen, U., Evermann, J. F., Vieler, E. & Parrish, C. R. 1996. Evolution of canine parvovirus envolved loss and gain of feline host range. Virology 215, 186-189.

Tsao, J., Chapman, S., Agbandje, M., Keller, W., Smith, K., Wu, H., Luo, M., Smith, T. J., Rossman, M. G., Compans, R. W. & Parrish, C. R. 1991. The three- dimensional structure of canine parvovirus and its functional implications. Science 251, 1456-1464.

Tullis, G. E., Burger, L. R. & Pintel, D. J. 1992. The trypsin-sensitive RVER domain in the capsid proteins of minute virus of mice is required for efficient cell binding and viral infection but not for proteolytic processing in vivo. Virology 191, 846-857.

Tullis, G. E., Burger, L. R. & Pintel, D. J. 1993. The minor capsid protein VP1 of autonomous parvovirus minute virus of mice is dispensable for encapsidation of progeny sinle-stranded DNA but is required for infectivity. J. Virol. 67, 131-141.

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M. & Parton, R. G. 1996. Rab11 regulates recycling through the pericentriolar recycling endosome. J. Cell Biol. 135, 913-924.

Umata, T., Moriyama, Y., Futai, M. & Mekada, E. 1990. The cytotoxic action of diphtheria toxin and its degradation in intact Vero cells are inhibited by bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase. J. Biol. Chem. 265, 21940-21945.

Vale, R. D. 1996. Switches, latches, and amplifiers: common themes of G proteins and molecular motors. J. Cell Biol. 135, 291-302.

Vale, R. D. & Fletterick, R. J. 1997. The design plan of kinesin motors. Annu. Rev. Cell Dev. Biol. 13, 745-777.

Vale, R. D., Reese, T. S. & Sheetz, M. P. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. Cell 42, 39-50.

Valetti, C., Wetzel, D. M., Schrader, M., Hasbani, M. J., Gill, S. R., Kreis, T. E. & Schroer, T. A. 1999. Role of dynactin in endocytic traffic: effects of dynamitin overexpression and colocalization with CLIP-170. Mol. Biol. Cell 10, 4107-4120.

Van Dam, E. M. & Stoorvogel, W. 2002. Dynamin-dependent transferrin receptor recycling by endosome-derived clathrin-coated vesicles. Mol. Biol. Cell 13, 169-182.

Van Deurs, B., Holm, P. K. & Sandvig, K. 1996. Inhibition of the vacuolar H^+-ATPase with bafilomycin reduces delivery of internalized molecules from mature multivesicular endosomes to lysosomes in hep-2 cells. Eur. J. Cell Biol. 69, 343-350.

Van Kuppeveld, F. J., Galama, J. M., Zoll, J., Van Den Hurk, P. J. & Melchers, W. J. 1996. Coxsackie B3 virus protein 2B contains cationic amphipathic helix that is required for viral RNA replication. J. Virol. 70, 3876-3886.

Van Weert, A. W., Dunn, K. W., Gueze, H. J., Maxfield, F. R. & Stoorvogel, W. 1995. Transport from late endosomes to lysosomes, but not sorting of integral membrane proteins in endosomes, depends on the vacuolar proton pump. J. Cell Biol. 130, 821-834.

Varga, M. J., Weibull, C. & Everitt, E. 1991. Infectious entry pathway of adenovirus type 2. J. Virol. 65, 6061-6070.

Vaughan, P. S., Leszyk, J. D. & Vaughan, K. T. 2001. Cytoplasmic dynein intermediate chain phosphorylation regulates binding to dynactin. J. Biol. Chem. 276, 26171-26179.

Vihinen-Ranta, M., Kakkola, L., Kalela, A., Vilja, P. & Vuento, M. 1997. Characterization of a nuclear localization signal of canine parvovirus capsid proteins. Eur. J. Biochem. 1, 389-394.

Vihinen- Ranta, M., Kalela, A., Mäkinen, P., Kakkola, L., Marjomäki, V. & Vuento, M. 1998. Intracellular route of canine parvovirus entry. J. Virol. 72, 802-806.

Vihinen-Ranta, M., Yuan, W., & Parrish, C. R. 2000. Cytoplasmic trafficking of the canine parvovirus capsid and its role in infection and nuclear transport. J. Virol. 74, 4853-9.

Vihinen-Ranta, M., Wang, D., Weichert, W. S., & Parrish, C. R. 2002. The VP1 N-terminal sequence of canine parvovirus affects nuclear transport of capsids and efficient cell infection. J. Virol. 76, 1884-91.

Vilhardt, F., Nielsen, M., Sandvig, K. & Van Deurs, B. 1999. Urokinase-type plasminogen activator receptor is internalized by different mechanisms in polarized and nonpolarized Madin-Darby canine kidney epithelial cells. Mol. Biol. Cell 10, 179-195.

Walters, R. W., Yi, S. M., Keshavjee, S., Brown, K. E., Welsh, M. J., Chiorini, J. A. & Zabner, J. 2001. Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. J. Biol. Chem. 276, 20610-20616.

Wang, D. & Parrish, C. R. 1999. A heterogeneous nuclear ribonucleoprotein A/B-related protein binds to single-stranded DNA near the 5' end or within the genome of feline parvovirus and can modify virus replication. J. Virol. 73, 7761-7768.

Wang, D., Yuan, W., Davis, I. & Parrish, C. R. 1998. Nonstructural protein-2 and the replication of canine parvovirus. Virology 240, 273-281.

Wang, E., Pennington, J. G., Goldenring, J. R., Hunziker, W. & Dunn, K. W. 2001. Brefeldin A rapidly disrupts plasma membrane polarity by blocking polar sorting in common endosomes of MDCK cells. J. Cell Sci. 114, 3309-3321.

Ward, P. & Berns, K. I. 1995. Minimum origin requirements for linear duplex AAV DNA replication in vitro. Virology 209, 692-695.

Weichert, W. S., Parker, J. S. L., Wahid, A. T. M., Chang, S.-F., Meier, E. & Parrish, C. R. 1998. Assaying for structural variation in the parvovirus capsid and its role in infection. Virology 250, 106-117.

Weisz, O. A., Swift, A. M. & Machamer, C. E. 1993. Oligomerization of a membrane protein correlates with its retention in the Golgi complex. J. Cell Biol. 122, 1185-1196.

Welch, M. D., Iwamatsu, A. & Mitchison, T. J. 1997. Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. Nature 385, 265-269.

Werling, D., Hope, J. C., Chaplin, P., Collins, R. A., Taylor, G. & Howard, C. J. 1999. Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells. J. Leukoc. Biol. 66, 50-58.

Werner, G., Hagenmaier, H., Drautz, H., Baumgartner, A. & Zahner, H. 1984. Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity. J. Antibiot. 37, 110-117.

White, J., Kielian, M. & Helenius, A. 1983. Membrane fusion proteins of enveloped animal viruses. Q. Rev. Biophys. 16, 151-195.

Whitney, J. A., Gomez, M., Sheff, D., Kreis, T. E., & Mellman, I. 1995. Cytoplasmic coat proteins involved in endosome function. Cell. 83, 703-713.

Whittaker, G. R. & Helenius, A. 1998. Nuclear import and export of viruses and virus genomes. Virology 246, 1-23.

Whittaker, G. R., Kann, M. & Helenius, A. 2000. Viral entry into the nucleus. Annu. Rev. Cell Dev. Biol. 16, 627-651

Wickham, T. J., Mathias, P., Cheresh, D. A. & Nemerow, G. R. 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73, 309-319.

Wikoff, W. R., Wang, G., Parrish, C. R., Cheng, R. H., Strassheim, M. L., Baker, T. S. & Rossmann, M. G. 1994. The structure of a neutralized virus: canine parvovirus complexed with neutralizing antibody fragment. Structure 2, 595-607.

Wistuba, A., Weger, S., Kern, A. & Kleinschmidt, J. A. 1995. Intermediates of adeno-associated virus type 2 assembly: identification of soluble complexes containing Rep and Cap proteins. J. Virol. 69, 5311-9.

Wu, H. & Rossmann, M. G. 1993. The canine parvovirus empty capsid structure. J. Mol. Biol. 233, 231-244.

Xie, Q. & Chapman, M. S. 1996. Canine parvovirus capsid structure, analyzed at 2.9 A resolution. J. Mol. Biol. 264, 497-520.

Yamashiro, D. J., Tycko, B., Fluss, S. R. & Maxfield, F. R. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. Cell 37, 789-800.

Yan, Z., Zak, R., Luxton, G. W., Ritchie, T. C., Bantel-Schaal, U. & Engelhardt, J. F. 2002. Ubiquitination of both adeno-associated virus type 2 and 5 capsid proteins affects the transduction efficiency of recombinant vectors. J. Virol. 76, 2043-2053.

Ye, G. J., Vaughan, K. T., Vallee, R. B. & Roizman, B. 2000. The herpes simplex virus 1 U(L)34 protein interacts with a cytoplasmic dynein intermediate chain and targets nuclear membrane. J. Virol. 74, 1355-1363.

Yeung, D. E., Brown, G. W., Tam, P., Russnak, R. H., Wilson, G., Clark-Lewis, I. & Astell, C. R. 1991. Monoclonal antibodies to the major nonstructural nuclear protein of mitute virus of mice. Virology 181, 35-45.

Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M. & Tashiro, Y. 1991. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. J. Biol. Chem. 266, 17707-17712.

Yuan, W. & Parrish, C. R. 2000. Comparison of two single-chain antibodies that neutralize canine parvovirus: analysis of an antibody-combining site and mechanisms of neutralization. Virology 269, 471-480.

Zádori, Z., Szelei, J., Lacoste, M.-C., Li, Y., Gariépy, S., Raymond, P., Allaire, M., Nabi, I. R. & Tijssen, P. 2001. A viral phospholipase A_2 is required for parvovirus infectivity. Devel. Cell 1, 291-302.