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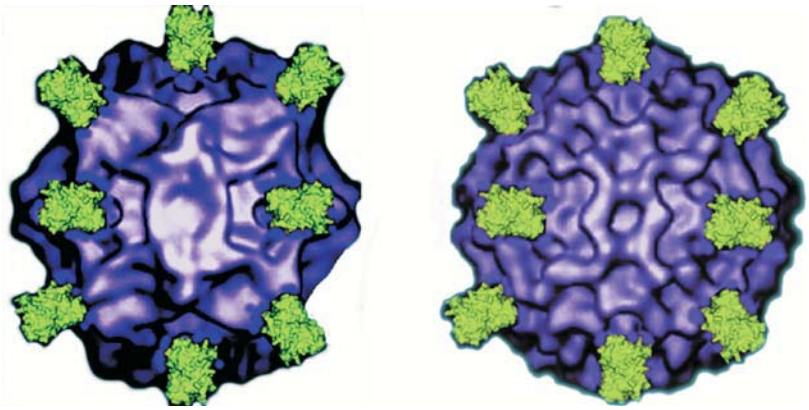
154

Leona Gilbert

Development of Biotehcnological Tools for Studying Infectious Pathways of Canine and Human Parvoviruses



JYVÄSKYLÄN YLIOPISTO

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2005

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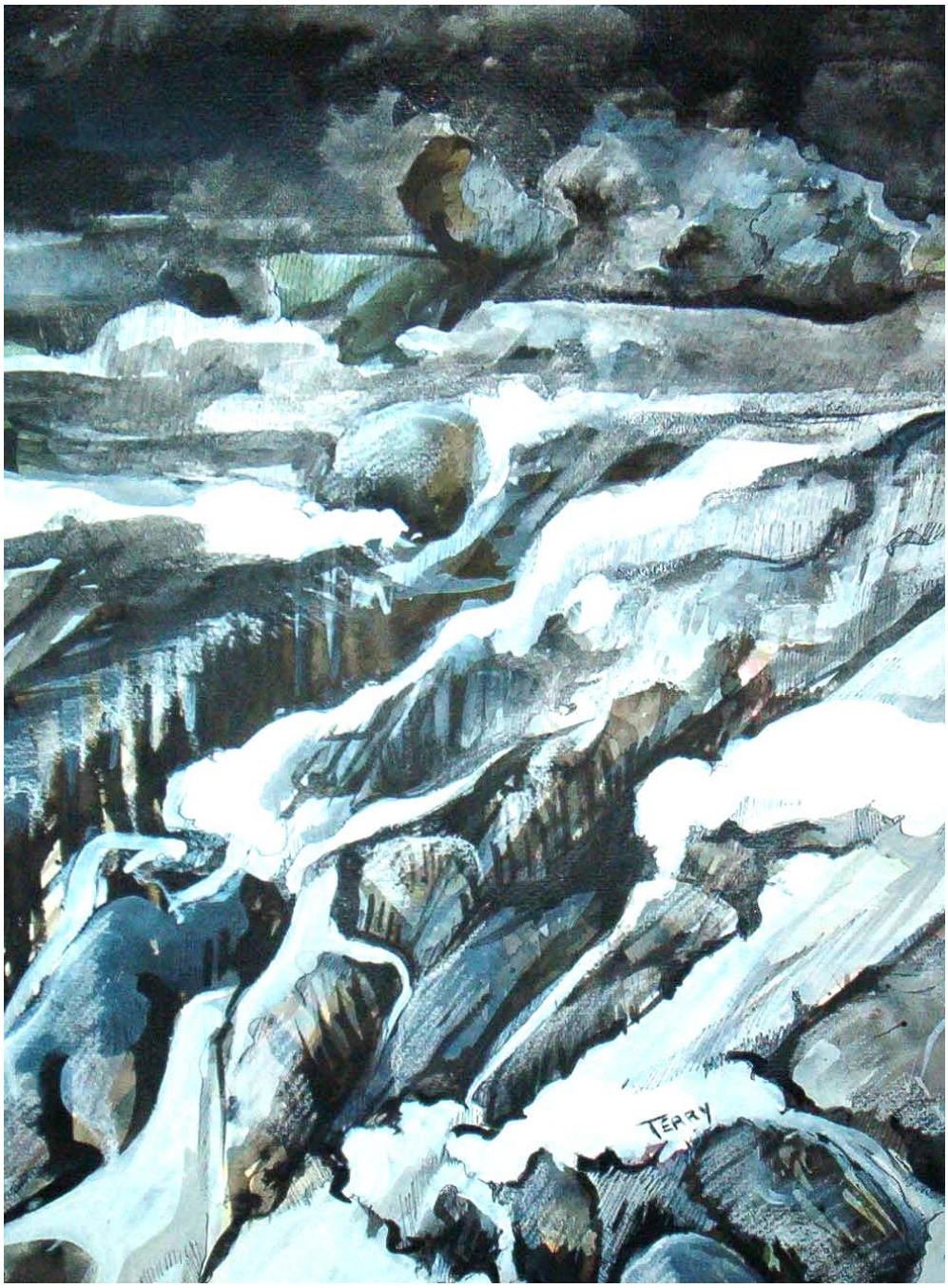
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ABSTRACT

Gilbert, Leona
Development of biotechnological tools for studying infectious pathways of canine and human parvoviruses.
Jyväskylä, University of Jyväskylä, 2005, 104 p.
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Parvoviruses are among the smallest vertebrate DNA viruses known to date. The production of parvovirus-like particles (parvo-VLPs) has been successfully exploited for parvovirus vaccine development. The baculovirus expression vector system (BEVS) has been the popular choice to correctly express and produce VLPs. These multimeric structures are morphologically and structurally identical to the original virus. The capsid proteins can also be produced individually or in combination to monitor structural protein functions in the parvovirus life cycle. Moreover, the ability of baculoviruses to transduce a wide range of mammalian cells has brought about the opportunity to use these viruses as gene vectors to express individual parvoviral structural proteins of interest. The present study was aimed at the development of biotechnological tools that could be used to gain insight into the trafficking events of parvoviral infections. The non-fluorescent and fluorescent fusion proteins, as well as recombinant baculoviruses encoding these constructs were successfully generated and EGFP was incorporated on the surface of the parvo-VLPs. These recombinant baculoviruses (rBVs) were shown to be practical when following parvoviral structural proteins *in vivo*. The chimeric parvo-VLPs were further scrutinized specifically with regard to their assembly capabilities, trafficking events and nuclear targeting. In addition, a baculovirus-mediated *de novo* vector system was created to shed additional light on the synthesis and trafficking of individual parvoviral structural proteins in mammalian cells. Together, these results showed that the surface of parvoviruses could be modified, display a large foreign moiety and assemble correctly. Similarly recombinant baculoviruses could be used as gene transfer vectors to monitor the individual parvoviral proteins in transduced mammalian target cells. The parvovirus display and transduction techniques show promise as potential tools for various scientific applications ranging from basic virological studies, to gene therapy applications, and developments in biomedicine.

Key words: Capsids; display; enhanced green fluorescent protein; parvovirus; virus-like particles; VP1; VP2.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I Gilbert, L., Toivola, J., Lehtomäki, E., Donaldson, L., Käpylä, P., Vuento, M. & Oker-Blom, C. 2004. Assembly of fluorescent chimeric virus-like particles of canine parvovirus in insect cells. Biochem. Biophys. Res. Commun. 313: 878-887.

II Gilbert, L., Toivola, J., White, D., Ihalainen, T., Smith, W., Lindholm, L., Vuento, M. & Oker-Blom, C. 2005. Molecular and structural characterization of fluorescent human parvovirus B19 virus-like particles. Biochem. Biophys. Res. Commun. 331: 527-535.

III Gilbert, L., Välilehto, O., Kirjavainen, S., Tikka, P. J., Mellett, M., Käpylä, P., Oker-Blom, C. & Vuento, M. 2005. Expression and subcellular targeting of canine parvovirus capsid proteins in baculovirus-transduced NLFK cells. FEBS Letter 579: 385-392.

IV Gilbert, L., Välilehto, O., Kirjavainen S., Lundbom, H., Vuento, M. & Oker-Blom, C. 2005. Entry and intracellular targeting of canine parvovirus-like particles in NLFK cells. (submitted)

RESPONSIBILITIES OF LEONA GILBERT IN THE ARTICLES OF THIS THESIS

Article I: I am responsible for the study except for FCS analysis and I also wrote the article. Eija Lehtomäki did her master's thesis on this study under my supervision.

Article II: I am responsible for the study except for FCS analysis and AFM imaging. I wrote the article and Wesley Smith did his master's thesis on this study under my supervision.

Article III: I am responsible for the study and I also wrote the article.

Article IV: I am responsible for the study except for Phospholipase A_2 assays. I also wrote the article.

All these studies were carried out under the joint supervision of Prof. Christian Oker-Blom and Prof. Matti Vuento.

ABBREVIATIONS

aa	amino acid
AAV	adeno-associated virus
AcMNPV	*Autographa californica* M nucleopolyhedrovirus
ADV	Aleutian mink disease parvovirus
AFM	atomic force microscopy
APAR	autonomous parvovirus-associated replication
BEVS	baculovirus expression vector system
BSA	bovine serum albumin
BVP	bovine parvovirus
BV	budded virion
B19	human parvovirus B19
CI-MPR	cation-independent mannose-6-phosphate receptor
CMV	cytomegalovirus immediate early promoter
CPV	canine parvovirus
DIC	differential interference contrast
DMEM	Dulbecco's modified eagle's medium
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EM	electron microscopy
ER	endoplasmic reticulum
FCS	fluorescence correlation spectroscopy
FPV	feline panleukopenia parvovirus
g	normal acceleration of free fall (9.81 m/s^2)
GFP	green fluorescent protein
gp64	major envelope glycoprotein 64
GV	granulovirus
HEK 293	human embryonic kidney cells
HEP G2	human hepatoma cells
HIV-1	human immunodeficiency virus type I
h	hour
HSV	herpes simplex virus
H-1	H-1 virus
Ig	immunoglobulin
kb	kilobase
kDa	kilodalton
LuIII	LuIII parvovirus
MEV	mink enteritis virus
min	minutes
MNPV	M nucleopolyhedrovirus
MOI	multiplicity of infection
MVM	minute virus of mice

NS	nonstructural protein
NLFK	Norden Laboratory Feline Kidney
NLS	nuclear localization signal
NPC	nuclear pore complex
ODV	occlusion derived virion
ORF	open reading frame
parvo-VLP	parvovirus-like particles
p.f.	post feeding
p.i.	post infection
p.t.	post transduction
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline, pH 7.4
PCR	polymerase chain reaction
PFA	paraformaldehyde
pfu	plaque forming unit
PLA2	phospholipase A_2 activity
polH	polyhedrin promoter
PPV	porcine parvovirus
rBV	recombinant baculovirus
RT	room temperature
SDS	sodium dodecyl sulfate
Sf	*Spodoptera frugiperda*
ssDNA	single stranded DNA
TfR	transferrin receptor
TGN	*trans*-Golgi network
VLP	virus-like particle
VP	viral protein
wt	wild-type

1 INTRODUCTION

Viruses are among the most elegant molecular assemblies in nature and parvoviruses make no exception to this. The Latin word *Parvus* meaning "small" describes parvoviruses' small and naked structure. Now epidemic, parvoviruses have the capacity to infect prenatal, neonatal, young and older animals including humans. The main stages of the parvovirus life cycle have been characterized for the most part by scrutiny of infected cells in the presence or absence of drugs. In addition, microinjected viruses have been followed fixation of the injected cells and laborious labeling techniques to view the viruses. Chemical labeling of virions with fluorophores is arduous and time consuming, moreover could disturb the native characteristics of the capsid. Hence, real time viewing of the life cycle of parvoviruses is not currently achievable. A promising tool to aid investigation of parvoviral life cycle is the generation of fluorescent virus-like particles (VLPs) that do not change the inherent characteristics of the capsid.

The main goal of this study was to develop efficient tools that would be suitable for studying the life cycles of canine and human parvoviruses (CPV and B19, respectively). This aim was approached by exploiting the baculovirus expression vector system to produce chimeric parvovirus-like particles (parvo-VLPs; I, II, IV) and to propagate recombinant baculoviruses that housed parvoviral structural proteins (III). Chimeric VLPs and recombinant proteins were analyzed and the recombinant proteins were used to study the life cycles of parvoviruses. These studies are of importance when elucidating the possibilities of parvoviruses or their constituents as one of the future choices for gene therapy applications and biomedical tools.

In addition, baculovirus-mediated transduction of mammalian cells was utilized for the investigation of parvoviral structural proteins. Here, the aim was to utilize baculovirus technology to reveal the intracellular localization of the parvoviral structural proteins during synthesis. This would give insight into the trafficking and assembly of a CPV infection that could be applied in understanding and developing parvoviral gene therapy vectors.

2 REVIEW OF THE LITERATURE

2.1 Parvoviruses

The Parvoviridae family consists of small, icosahedral, non-enveloped viruses that contain linear single-stranded DNA (ssDNA) genomes about 5000 nucleotides long. This family is divided into two subfamilies: the Parvovirinae, that infects vertebrates, and the Densovirinae that infects invertebrates (Siegel et al. 1985, Berns 1996, Muzyczka & Berns 2001, Heegard & Brown 2002). Each subfamily contains three genera and the Parvovirinae subfamily is the main focus of this thesis. The three genera of the Parvovirinae consist of: *Parvovirus,* autonomous parvoviruses; *Dependovirus,* dependent on a helper virus such as adenovirus or herpesvirus for replication; and *Erythrovirus*, autonomous and has high tropism for erythroid progenitor cells (Ozawa et al. 1987, Heegaard & Brown 2002). See Table 1 for selected viruses of the Parvovirinae subfamily.

TABLE 1 Parvovirinea subfamily prototypes with their abbreviations and host species. Modified from Blechacz & Russell 2004.

Genus	Virus (abbreviated name)		Host
Parvovirus	Aleutian mink disease parvovirus	(ADV)	Mink
	Bovine parvovirus	(BPV)	Cattle
	Canine parvovirus	(CPV)	Dog
	Feline panleukopenaea virus	(FPV)	Cat
	H-1 virus	(H-1)	Rodents, human
	LuIII	(LuIII)	Unknown, human
	Mink enteritis virus	(MEV)	Mink
	Minute virus of mice	(MVM)	Mice
	Porcine parvovirus	(PPV)	Pig
Erythrovirus	Human parvovirus B19	(B19)	Human
Dependovirus	Adeno-associated virus serotypes 1-8	(AAV-1-8)	Human

The pathogenicity of autonomous parvoviruses, which mostly concerns fetuses and neonates, is restricted to tissues with a high proliferation index (Cotmore & Tattersall 1987). These viruses require cellular S-phase functions for their DNA replication (Tennant et al. 1969, Brown et al. 1993). These teratogenic agents cause fetal and neonatal abnormalities by destroying specific cell populations and may be pantropic, infecting a wide range of cells in various organs (Fenner et al. 1987). Canine parvovirus (CPV) is a prime example of the pathogenic nature of autonomous parvoviruses. CPV is highly homologous to feline panleukopenia virus (FVP) and mink enteritis virus (MEV), having approximately 98% and 50% sequence identity respectively. It is classified as a host range variant of feline parvovirus with a divergent host range (Parrish 1990, Truyen & Parrish 1992, Chapman & Rossmann 1993). Since the discovery of CPV in 1987 (Appel et al. 1979, Burtonboy et al. 1979), viral infections in dogs have become epidemic. Puppies infected either prior or shortly following birth suffer lethal acute myocarditis, and older dogs endure lymphocytolysis and severe enteritis (Fenner et al. 1987, Parrish 1990, Parrish 1995). Although there is no treatment for parvoviral myocarditis or enteritis, domestic canines are vaccinated and management of symptoms is supportive (Pollock & Carmichael 1982, Saliki et al. 1992).

Human parvovirus B19 (B19) was originally discovered by a virologist that was assaying serum samples for hepatitis B virus and the name came from the blood bank code by which the original positive serum sample was labeled (Cossart et al. 1975, Cherry 1999). B19 replicates only in human erythroid progenitor cells, and cell binding and infection require the erythrocyte P antigen (globoside) (Brown et al. 1993). It is known to be the only parvovirus that has been directly linked to disease in humans (Sabella & Goldfarb 1999, Katta 2002, Vafaie & Schwartz 2004). Although having limited tropism in human tissues (Brunstein et al. 2000, Söderlund-Venermo et al. 2002), B19 DNA or antigen has been found in heart, liver, spleen, kidney, testes, skin, cerebrospinal fluid and synovium of children and adults (Hokynar et al. 2002, Söderlund-Venermo et al. 2002). Three primate parvoviruses that are similar to B19 at the genome level are Rhesus and pig-tailed macaque parvoviruses (Green et al. 2000), simian parvovirus in cynomolgus monkeys (O'Sullivan et al. 1996).

B19 entered the medical curriculum as an agent of human disease when its involvement with erythema infectiosum (childhood fifth disease), hydrops fetalis and transient aplastic anemia was demonstrated in the early 1980s (Anderson et al. 1982, Anderson et al. 1985, Brown et al. 1994, Pamidi et al. 2000). B19 has also been associated with an anthology of clinical manifestations (Pattison et al. 1981, Koch et al. 1990, Trapani et al. 1999) including myocarditis (Malm et al. 1993, Brown et al. 1994, Schowengerdt et al. 1997, Enders et al. 1998, Murry et al. 2001), vasculitis syndromes (Corman & Dolson 1992, Finkel et al. 1994, Trapani et al. 1999, Dingli et al. 2000), hepatitis (Naides et al. 1996, Hillingso et al. 1998, Pardi et al. 1998, Sokal et al. 1998) and neurological disorders (Yoto et al. 1994, Barah et al. 2001). B19 infection is associated with elevated levels of anti-nuclear antibody, anti-double stranded DNA antibody,

anti-neutrophil cytoplasmic antibodies, and anti-cardiolipin antibodies (Chou et al. 2000, Von Landenberg et al. 2003) and suggested to be associated with autoimmune diseases as systemic lupus erythematosus, rheumatoid arthritis, primary biliary cirrhosis, and polymyositis (Naides et al. 1990, Kalish et al. 1992, Takahashi et al. 1998, Trapani et al. 1999, Moore 2000, Stahl 2000, Morita & Sugamura 2002, Lehmann et al. 2003).

2.1.1 Parvovirus genome and proteins

The low genetic complexity of parvoviruses compels them to strictly be maintained by host cell factors in order to complete their life cycle. Small ssDNA genomes of autonomous parvoviruses are mostly of negative polarity, while the genome of B19 and adeno-associated viruses (AAV; dependovirus) are of both polarities (Berns & Adler 1972, Cotmore & Tattersall 1987, Berns 1996). Characteristically the terminal palindromic sequences at both ends of the ssDNA fold into stable T-, Y- or cruciform hairpin structures that serve as self-priming origins of replication (Lusby et al. 1980, Cotmore & Tattersall 1987, 1994, Cotmore et al. 1999). To maximize coding potential of the short genome, parvoviruses employ partially overlapping transcripts, three reading frames. Fig. 1 outlines a schematic of the CPV genome.

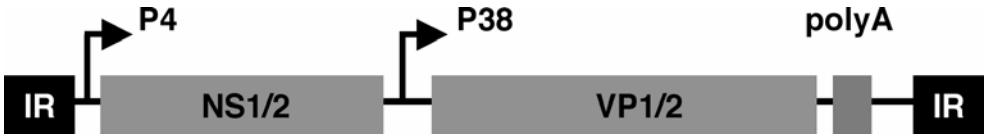

FIGURE 1 Schematic of the CPV genome. The 5.1 kb genome is represented in the 3′→5′ orientation and the inverted repeat (IR) sequences at the termini serve as self-priming origins of replication. Transcripts are in order of left hand open reading frame (ORF) driven by promoter P4 and right hand ORF driven by P39. Modified from Reed et al 1988, Blechacz & Russell 2004.

For CPV these open reading frames (ORFs) are controlled by promoters P4 and P38 (Astell et al. 1983, Pintel et al. 1983, Cotmore et al. 1986, Cotmore & Tattersall 1986, Reed et al. 1988). The left hand (5′) ORF encodes the nonstructural proteins (NS1 and NS2) and is driven by the P4 promoter (Alexandersen et al. 1988, Spegelaere et al. 1991, Cotmore & Tattersall 1986, 1987, Reed et al. 1988, Berns 1996). The right hand (3′) ORF encodes the capsid structural proteins (VP1 and VP2) by alternate splicing driven by P38, which is trans-activated and regulated by the NS1 protein (Cotmore & Tattersall 1986, Jongeneel et al. 1986, Cotmore & Tattersall 1987, Berns 1990, Clemens et al. 1990, Ahn et al. 1992, Christensen et al. 1995, Berns 1996, Lorson & Pintel 1997, Lorson et al. 1998).

CPV VP1 contains the complete sequence of VP2 and a unique 143-residue N-terminal sequence necessary for viral infectivity but not for capsid formation (Tullis et al. 1992; Vihinen-Ranta et al. 2000), while VP3 is formed in full capsids by the cleavage of a peptide from the N-terminus of VP2 exposed outside the

capsid (Clinton & Hayashi 1976, Tattersall et al. 1977, Paradiso et al. 1982, Tsao et al. 1991, Tullis et al. 1992, Langeveld et al. 1994, Casal et al. 1995, Xie et al. 1996, Weichert et al. 1998, Muzyczka & Berns 2001). The 23 amino acids long sequence of the N-terminus of CPV VP2 has a cleavage site for VP3 production and is identical to those of FPV and MEV (Chapmann & Rossmann 1993). This terminus is well conserved in MVM and the H-1 parvovirus, but the homology with other parvoviruses, including PPV lower (Chapmann & Rossmann 1993).

In contrast, B19 has approximately 5,600 nucleotides with 2 large open reading frames and transcription of the genome is controlled by a single promoter, p6, located at map unit 6 that regulates the synthesis of all nine viral transcripts (Blundell et al. 1987, Doerig et al. 1987, St Amand 1991). The 5′ end of the genome encodes a single non-spliced transcript for the production of the nonstructural protein (NS1), and the 3′ end is responsible for eight other transcripts that through a combination of different splicing events encode the two capsid proteins (VP1 and VP2) and two smaller proteins of unknown function (Cotmore et al. 1986, Ozawa et al. 1987, Luo & Astell 1993, Vafaie & Schwartz 2004). B19 VP1 has a unique region composed of 227 amino residues and does not have VP3 (Ozawa et al. 1988).

The NS1 (approximately 83 kDa) of autosomal parvoviruses is a pleiotrophic nuclear phosphoprotein that has several intrinsic enzymatic properties. It has been shown to be absolutely required for viral replication both *in vivo* and *in vitro* (Clemens et al. 1990, Cotmore & Tattersall 1990, 1992, Cotmore et al. 1992, Nüesch et al. 1992, Cotmore et al. 1993, Christensen et al. 1995). Functional analyses have shown NS1 to be a sequence specific DNA binding protein that has intrinsic ATPase, helicase, and site-specific endonuclease activities. It interacts with host cell factors and is needed for viral DNA amplification and transcriptional regulation (Rode SK III 1989, Cotmore & Tattersall 1990, Im Muzyczka 1990, Wilson et al. 1991, Cotmore et al. 1992, Nüesch et al. 1992, Cotmore et al. 1993, McCarty et al. 1994, Cotmore et al. 1995, Krady & Ward 1995, Pujol et al. 1997, Lorson et al. 1998, Christensen & Tattersall 2002). NS1 proteins of autonomous parvoviruses may also fulfill other as yet unknown functions during infection (Cziepluch et al. 1998). In addition, this nonstructural protein is well known for its cytotoxic or cytostatic actions on host cells due to their interference with host cell physiology and morphology and the ability to interact with a variety of other cellular proteins (Ozawa et al. 1988, Hermonat et al. 1989, Caillet-Fauquet et al. 1990, Clemens et al. 1990, Li & Rhode 1990, Vanacker & Rommelaere 1995, Anouja 1997, Corbau 2000).

NS2 is a smaller (approximately 22 kDa) nonstructural protein formed from alternate splicing. It shares a common N-terminal region with NS1 and is also able to influence the cellular environment as in the case for NS1 (Cotmore & Tattersall 1986). Its phosphorylated isoforms are distributed in the cytoplasm and nucleus (Cotmore & Tattersall 1990) and this protein is responsible for multiple functions including capsid assembly (Cotmore et al. 1997), messenger translation (Naeger et al. 1993), DNA replication, virus production (Naeger et al. 1990), and nuclear egression of progeny virions (Bodendorf et al. 1999, Ohshima et al. 1999, Eichwald et al. 2002, Miller & Pintel 2002). NS2 seems to be

dispensable when not coded, as in the case for some CPV infected cells, or not present at all for certain parvoviruses like B19. However, when encoded it seems to be necessary, in a cell-type-specific manner, for efficient genome replication and virus production as in the case for MVM and H-1 (Naeger et al. 1990, Li & Rhode 1991, Brownstein et al. 1992, Wang et al. 1998). Interestingly, cells can tolerate NS2 (Mousset et al. 1994), but suffer great cytotoxicity when NS1 is present (Brandenburger et al. 1990). It is obvious that NS1 and NS2 are relevant determinants of parvovirus fitness in a natural host.

2.1.2 Parvovirus virion structure

It is apparent that the protein shell of the viral capsid functions not only to protect the viral genome from the outside surroundings, but also carries signals that allow systematic intracellular trafficking of the virion during entry and egress from its host cell. Parvoviral capsids of approximately 25 nm in diameter do not carry accessory proteins into the cell that assist in the entry process (Tsao et al. 1991, Xie & Chapmann 1996). As a consequence the parvoviral polypeptides must display signals for entry and facilitate their own trafficking through intracellular compartments, eventually delivering their genomes into the nucleus for replication.

The first parvovirus structure to be determined was the full (DNA containing) capsid of CPV at 3.4 Å (Tsao et al. 1991), followed by its empty capsid refined to 3.0 Å (Wu & Rossmann 1993). There are also atomic models of related parvoviruses resolved by x-ray crystallography (Agbandje et al. 1991, 1993, 1994, Llamas-Saiz et al. 1997, Simpson et al. 1998, McKenna et al. 1999, Hernando et al. 2000, Simpson et al. 2002, Xie et al. 2002). Improved resolution of capsid structures have also been obtained for B19 at 3.5 Å (Kaufmann et al. 2004) and CPV at 2.9 Å (Xie & Chapman 1996). Images of many parvoviruses were improved by the observation that VP2 (the major capsid protein) is able to self-assemble into empty capsids, known as virus-like particles (VLPs) and that the stoichiometry of the VLPs containing both VP1 and VP2 was similar to that previously observed in parvovirus infected cells (Brown 1991, Lopez de Turiso et al. 1992, Saliki et al. 1992, Llamas-Saiz et al. 1997, Agbandje-McKenna et al. 1998, Choi et al. 2000, Hernando et al. 2000,). Interestingly, by far the largest conformational differences between full and empty capsids were found in the region where some ordered DNA has been observed to bind inside full particles (Wu & Rossmann 1993).

From the resolved structures, parvoviruses are seen as non-enveloped viruses approximately 260 Å in diameter (Luo et al. 1998). The icosahedral T = 1 virion of CPV is formed by 60 protein subunits that are contributed by the three non-identical polypeptide chains of VP1, VP2, and VP3 (Cotmore & Tattersall 1987, Tsao et al. 1991, Agbandje et al. 1993, Wu & Rossmann 1993). All structural proteins share a conserved β-barrel core that forms the contiguous parvoviral capsid and similar three-dimensional structures. The core domain (Fig. 2A) contains an eight-stranded, anti-parallel ß-barrel motif consisting of two ß-sheets in the standard BIDG and CHEF arrangements common to many

viral capsid proteins (Rossmann & Johnson 1989). This domain accounts for one-third of the amino acid content in each polypeptide and the other two-thirds of the polypeptide sequence consists of 4 large loop insertions between the strands of the ß-barrel. These loops create the surface of the virion and are the most variable regions in the amino acid sequences amongst parvoviruses. Differences in the loops account for surface variations that control many biological differences, including tissue tropism, pathogenicity, and antigenicity among members as well as between strains of the same virus (Tsao et al. 1991, Ball-Goodrich & Tattersall 1992, Agbandje et al. 1993, Chapman, & Rossmann 1993, Bergeron et al. 1996, Xie & Chapman 1996, Agbandje-McKenna et al. 1998, Simpson et al. 2002, Xie et al. 2002, Hueffer et al. 2003). CPV, FPV and MVM share 50–97% capsid sequence identity for VP1 and VP2 (Hueffer & Parrish 2003), whereas B19 VP2 has only 26% sequence identity to VP3 of AAV (Kaufmann et al. 2004).

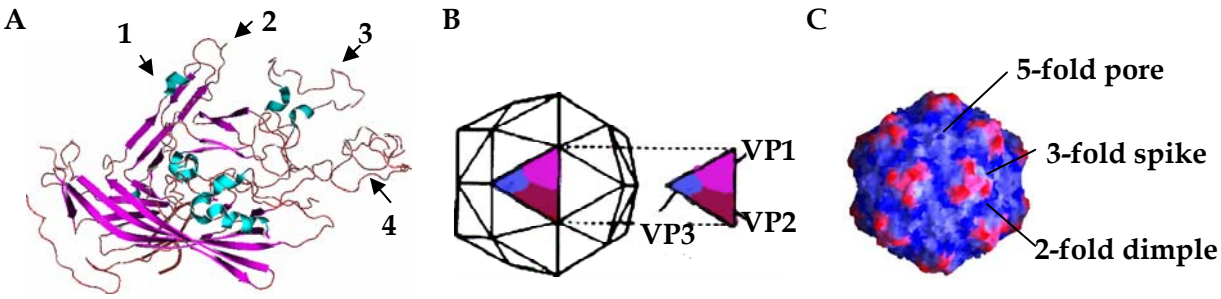

FIGURE 2 CPV capsid structure. (A) Conserved β-barrel core of each subunit that forms the contiguous parvoviral capsid. β-barrel core outlined in dark pink. Loops are numbered. (B) Icosahedral capsid with the position of viral proteins are outlined. (C) Topology and molecular model of the CPV capsid. 5-fold pore, 3-fold spike and 2-fold dimple-like depressions are marked. From www.virology.net/courseware.html; 10 Sept 01, according to Tsao et al. 1991.

Despite dissimilarity in amino acid sequence and low homology between some of the parvoviruses, common prominent structural elements on the capsid surface are seen (Fig. 2); raised regions at the 5-fold axes of symmetry, which in some viruses might form a pore into the capsid, depressed regions (canyons) surrounding the 5-fold axes, one or three protrusions at or surrounding the 3-fold axes of symmetry (3-fold spikes or peaks), and depressed regions (dimples) at the 2-fold axes of symmetry (Tsao et al. 1991, Agbandje et al. 1993, Agbandje et al. 1995, Agbandje-McKenna et al. 1998, Simpson et al. 2000, 2002, Kaufmann et al. 2004). The electron density map shows that approximately 87% of the VP2 capsid proteins have the N-termini on the inside of the capsid, but for approximately 13%, the polypeptide starts on the outside and runs through one of the 8 Å pores at each 5-fold axis (Xie & Chapman 1996, Farr & Tattersall

2004). The structure of empty canine parvovirus capsids shows that residues 37 to the C-terminal residue 584 (VP2 numbering) are ordered in each of the 60 subunits (Wu & Rossmann 1993). In addition, strong electron density corresponding to at least 11 nucleotides of ordered DNA per protein subunit bound to the inner surface of the capsid is seen in full DNA-containing capsids (Reed et al. 1988, Tsao et al. 1991). Another similar property that is well conserved in all autonomous parvoviruses is the glycine-rich region of VP1 (Chapman et al. 1993, 1995). It has been hypothesized, in consistency with electron density distributions, that this N-terminal glycine-rich sequence can be accommodated within the 5-fold pore without excessive steric hindrance on the capsid (Tsao et al. 1991). In addition, this glycine-rich sequence has been shown to have a role in externalizing the unique N-terminal region of VP1 and exposuring part of VP2 on the capsid surface (Tsao et al. 1991, Wu & Rossmann 1993, Xie & Chapman 1996). Full capsids of MVM, CPV, and AAV2 have 20 to 30 nucleotides of the 5' end of the viral genome exposed on the outside of the capsid through a pore at the 5-fold axis, and the NS1 protein is covalently attached to the 5' end (Cotmore & Tattersall 1989, Prasad & Trempe 1995, Xie & Chapman 1996, Wang & Parrish 1999, Xie et al. 2002). Notably the cylindrical channels in the 5-fold axes of CPV, FPV, and MVM, which are surrounded by five symmetry-related loops, are closed in ADV and B19 (Agbandje et al. 1994, McKenna et al. 1999).

Although having a consensus core motif, parvoviruses display large differences in their surface topology. The large loops connecting the strands of the ß-barrel to the surface features differentiate B19 from other parvoviruses. The most striking differences are the lack of prominent spikes on the 3-fold icosahedral axes observed in FPV and CPV and that the 5-fold vertices are closed in B19 (Agbandje et al. 1994, Kaufmann et al. 2004**)**. Similarly, the insect parvovirus has a relatively smooth surface compared to that of the vertebrate parvoviruses due to the fact that the loops are a total of 130 residues shorter (Simpson et al. 1998). In contrast, the spike-like protrusions that decorate the 3-fold axes in ADV are more prominent compared to the other parvoviruses owing to the presence of loop insertions, which create mounds near the 3-fold axes (McKenna et al. 1999). Equally, in AAV with capsid sequence identities of 7-22% with other parvoviruses, it is reasonable that there are substantial differences in surface topology compared to CPV (Kronenberg et al. 2001). In addition, AAV-2 has all characteristics seen in representations for other parvoviruses, but has remarkable differences in the topology of features around the 3-fold axes of symmetry, which form three separate structures rather than the combined structure seen in CPV, MVM and PPV (Xie et al. 2002).

2.1.3 Parvovirus life cycle

Animal viruses employ various strategies for infecting their host cells, but the basic steps are: binding to receptors on the cell surface, penetration into the cytosol, uncoating or release of the viral genome, and targeting of the genome and any required accessory proteins toward the nucleus for replication (Greber

2002, Sieczkarski et al. 2002, Pelkmans & Helenius 2003). Most non-enveloped viruses enter cells by endocytosis and subsequently may undergo pH-dependent structural changes necessary for productive infection (Giranda et al. 1992). This entry route may be clathrin-mediated, caveola-mediated, or achieved through clathrin- and caveola-independent uptake mechanisms that include macropinocytosis and other less well defined processes (Nichols et al. 2001, Nichols 2002, Sieczkarski & Whittaker 2002, Conner & Schmid 2003).

The Parvoviridae life cycle is outlined in Fig. 3. In short parvoviruses bind to their cellular receptor and penetrate into the cell. This is followed by intracellular trafficking towards the nucleus where replication, as well as transcription of nonstructural (NS) and structural genes (VP) occurs. Translation of VPs occurs in the cytoplasm and newly synthesized proteins are translocated into the nucleus where virus production and DNA packaging occurs. Finally, the cell lyse, releasing releasing intracellular viral particles.

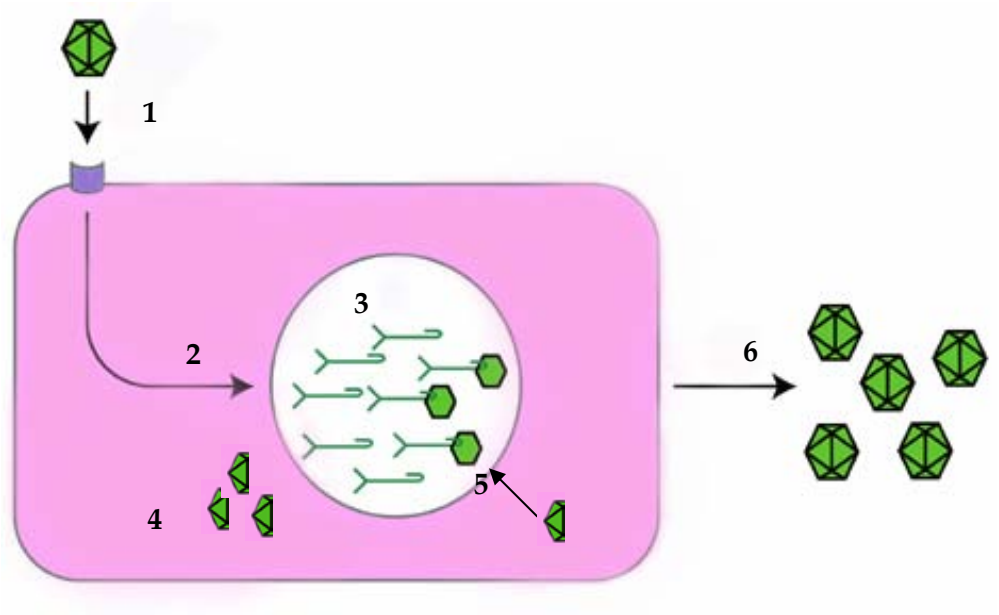

FIGURE 3 Parvoviridae life cycle. (1) Virus binding and penetration into the cell; (2) intracellular trafficking (endocytosis) and nuclear translocation; (3) replication and transcription; (4) translation and VP production; (5) virus production and DNA packaging; and (6) particle release and cell death. Modified from Blechacz & Russell (2004).

Autonomous parvoviruses require receptor-mediated endocytosis for cell infection (Parker & Parrish 2000, Ros et al. 2002, Suikkanen et al. 2002, 2003). Cellular receptors for various parvoviruses have been reported: AAVs [heparin sulphate proteoglycan, human fibroblast growth factor receptor 1, αvß5 integrin and sialic acid (Summerford & Sumulski 1998, Quing et al. 1999, Kaludov 2001)]; B19 [erythrocyte P antigen and α5ß1 integrin (Brown et al. 1993, Weigel-Kelley et al. 2001, 2003)]; CPV and FPV [transferrin receptor (Parker et al. 2001, Govindasamy et al. 2003, Hueffer et al. 2003)]; H-1 and MVM [N-acetylneuraminic acid (sialyl-containing glycoproteins (Cotmore & Tattersall 1987)]. Moreover, it has been verified in certain cell lines that some

parvoviruses such as CPV and MVM can use carbohydrates for cellular attachment (Chapman & Rossmann 1993).

Internalization of parvoviruses into cells is rapid and most likely involves entry through clathrin-mediated endocytosis where the viruses are then transported to the nucleus along the microtubules (Linser et al. 1979, Duan et al. 1999, Barlett et al. 2000, Parker & Parrish 2000, Suikkanen et al. 2002, 2003). Shortly after uptake, CPV capsids colocalize with transferrin in the perinuclear endosomes (Parker & Parrish 2000, Suikkanen et al. 2002). Endosomal entry triggers for membrane penetration can include viral protein structural changes induced by receptor binding (Rossmann et al. 2000, Tsang et al. 2001), changes due to the endosome's low pH (Skehel et al. 1982, Bullough 1994), or cleavage of viral proteins by the activities of low-pH-dependent endosomal proteases (Ebert et al. 2002). For parvoviruses some of these changes have been seen (Cotmore et al. 1999, Maroto et al. 2000). Parvoviral infections can be inhibited by treatment of cells with lysosomotropic agents, indicating that low endosomal pH is required for infection or for endosomal trafficking (Basak & Turner 1992, Barlett et al. 2000, Parker & Parrish 2000, Ros et al. 2002, Suikkanen et al. 2002, Morón et al. 2003). Disruption of the endosomal network by Brefeldin A or NH_4Cl interferes with infection, indicating that parvoviral particles are routed farther than the early endocytic compartment (Basak & Turner 1992, Bartlett et al. 2000, Ros et al. 2002, Suikkanen et al. 2003). During endosomal transport, partial decapsidation does occur through acidification-dependent proteolytic cleavage of VP2, which consequently causes conformational alterations in the capsid (Ros et al. 2002, Ros & Kempf 2004). The unique VP1 N-terminal domain becomes exposed on the outside of the capsid and is essential for infectivity since deletions of this area lead to noninfectious viruses (Tullis et al. 1993, Cotmore et al. 1999, Vihinen-Ranta et al. 2002). Exposure of the unique region of VP1 exhibits a novel viral phospholipase A_2 (PLA2) activity that plays a role in capsid release from vesicles (Zodori et al. 2001, Dorsch et al. 2002, Suikkanen et al. 2003, Bleker et al. 2005). In addition, this unique region of VP1 contains clusters of basic residues, a nuclear localization sequence (NLS), which could contribute to nuclear import of virions (Vihinen-Ranta et al. 1997, 2000, Lombardo et al. 2002).

Viral particles are routed toward the late compartments in the endosomal pathway and remain in the endosomal compartment for several hours (up to 8 h) before release into the cytosol (Ros et al. 2002, Parker & Parrish 2000, Vihinen-Ranta et al. 2000, Suikkanen et al. 2002). Capsids are most likely released into the cytoplasm from a lysosomal vesicle in a perinuclear location (Suikkanen et al. 2003), but the mechanism of escape from endocytic vesicles into the cytosol is still unknown; however, PLA2 activity and other factors do aid in this step (Parker & Parrish 2000, Suikkanen et al. 2003). Nuclear-replicating viruses may use cytoskeleton-driven transport of the capsid or viral components mediated by either microtubules or actin or, in some cases, by both (Luby-Phelps et al. 2000, Sodeik et al. 2000). Indeed, intercellular movement of parvoviruses is seen to depend on a functional cytoskeleton with the requirement of both microtubules and microfilaments (Ros et al. 2002,

Suikkanen et al. 2002, 2003, Nüesch et al. 2005). Active transport mechanisms are also likely to be required for capsids to reach the nuclear pore since the cytoplasm contains a lattice-like mesh of microtubules, actin microfilaments, and intermediate filaments which restrict the diffusion of macromolecular complexes (Seksek et al. 1997, Luby-Phelps et al. 2000). There is probably further processing in the cytoplasm before transportation to the nucleus. It has been recognized that CPV and MVM interact with proteasomes after escape from vesicles at 9 h post-infection (p.i.) and most of the viral particles have interacted with cellular proteasomes (Ros et al. 2002, Ros & Kempf 2004). This suggests that for some parvoviruses the ubiquitin-proteasome pathway plays an essential role in their life cycle, probably assisting at the stages of capsid disassembly and/or nuclear translocation.

Transport through the nuclear envelope is another barrier parvoviruses must circumvent in order to have a productive infection. To surmount this parvoviruses could employ multiple strategies for nuclear entry, for example; passive diffusion through the nuclear pore complex (NPC) due to their small size (Pante & Kann 2002); enter as an intact or partially disassembled capsid (Whittaker & Helenius 1998, Greber & Fassati 2003, Smith & Helenius 2004); passage through during mitosis when the nuclear envelope is momentarily lost (Truyen & Parrish 1992, Sieczkarski & Whittaker 2002); or guidance through classical or non-classical import pathways involving NLSs, importins and energy (Nigg 1997, Mattaj & Englmeier 1998, Görlich & Kutay 1999). Although nuclear entry strategy/strategies exploited for parvoviruses are poorly understood, there is evidence to support that guidance through the NPC as intact or partially disassembled virions is the path taken. This hypothesis is supported by the fact that the VP1-specific regions contain nuclear localization signals (Vihinen-Ranta et al. 1997, Lombardo et al. 2002,) that could be potentially recognized by importins (Nigg 1997, Mattaj & Englmeier 1998, Görlich & Kutay 1999). Correspondingly, a basic amino acid rich sequence of MVM in VP2 has been reported to be associated with nuclear import in a conformation-dependent manner (Lombardo et al. 2000). In other studies it was observed that there is no cell cycle-dependent disruption of nuclear lamina in cells infected with parvoviruses indicating that the newly produced viral components had to be transported actively through the NPC. This was probably mediated through direct interaction with NS2 and nuclear export factor CRM1 (Eichwald et al. 2002, Nüesch et al. 2005). In addition, as seen in microinjection studies when using antibodies for intact capsids, microinjected capsids were able to enter the nucleus intact without apparent deformation, although the VP1 N-terminal region was exposed, (Vihinen-Ranta 1997, Suikkanen et al. 2003,). However, at comparable time periods in other microinjection studies, few capsids were imported to the nucleus while most remained in endosomal vesicles or in the cytoplasm (Suikkanen et al. 2003).

All further steps of the parvovirus life cycle, including DNA-amplification, capsid protein production, and packaging are dependent on the presence of the multifunctional viral phosphoproteins NS1 and NS2 (Heegaard & Brown 2002), but are otherwise still unclear. In addition to the nonstructural

proteins, different phosphorylated VP forms (Santarén et al. 1993, Lang et al. 2005) seem to play a role in the late stages of viral life cycle such as in viral egression from the nucleus in conjunction with NS2 (Maroto et al. 2000, Miller & Pintel 2002). Similarly, due to the fact that VP2-termini are only exposed in packaged virions their phosphorylation may function to selectively signal export of virus from the nucleus upon packaging (Maroto et al. 2000). In fact, NS1 and NS2 are observed to concentrate at focal sites in the nucleus, replication compartments, that contained ADV viral DNA, but structural proteins did not colocalize with the NS proteins (Oleksiewicz et al. 1996, 1998). In infected cells, a novel subnuclear compartment has been identified for MVM and H-1 termed autonomous parvovirus-associated replication (APAR) bodies at early time points p.i. up to 15 h (Singer 1975, Singer & Toolan 1975, Richards et al. 1979, Cziepluch et al. 2000, Bashir et al. 2001, Stracker et al. 2004). These APAR bodies are distinct in morphology from most of the classically described nuclear bodies, including Cajal bodies, promyelocytic leukemia oncogenic domains and interachromatin granules or speckles (Young et al. 2002).

The first location in which viral capsid assembly has been detected is the nucleus (Richards et al. 1977, Singer & Toolen 1975). The virus assembly, capsid processing (8 h p.i.) and nuclear release do not appear to be specifically coupled to the host-cell S phase (Richards et al. 1977). The parvovirus life cycle concludes with cell death and virus release into the environment.

2.2 Parvovirus applications

The production of parvovirus-like particles (parvo-VLPs) has been extremely successful in the past. These multimeric structures are morphologically and structurally identical to the original virus and are safe immunological reagents for virus or antibody detection in enzyme immunoassays, as vaccines, as an antigen delivery system, and as gene delivery systems for gene therapy (Brown et al. 1991, Touze & Coursaget 1998, Casal 1999, Maxwell et al. 2002, Blechacz & Russell 2004, Cornelis et al. 2004b). These VLPs and gene therapy vectors based on parvoviruses are the focus of this section. Pilot gene therapy vectors have exploited the innate ability of parvoviruses to selectively kill infected tumor cells, to suppress tumors, and to activate the immune system (Cornelis et al. 2004a). However these VLPs or vectors may have factors limiting their success, which is based on their correct assembly of the chimera.

Due to the convoluted oligomeric disposition of capsids subunits, even minimal insertions or deletions could have a negative effect on assembly and/or decrease capsid stability (Ladurner & Fersht 1997). Taking this into consideration, rational manipulations in order to understand the function of the structural proteins in parvoviruses have been undertaken extensively. Combined theoretical and experimental approaches to address problems in structural and antigenic properties of parvo-VLPs have included: deletions in

the 4 large loops (Fig. 2A; Hurtado et al. 1996), epitope insertions in the second loop of VP2 (Rueda et al. 1999), N-terminal fusion of foreign moieties to full or truncated versions of VP1 (Miyamura et al. 1994, Wong et al. 1994), N-terminal insertion and fusions (Sedlik et al. 1995) or deletions of VP2 (Hurtado et al. 1996, Kawase et al. 1995), C-terminal fusion to VP2 (Sedlik et al. 1995, Rueda et al. 1999), and N-terminal fusion of the Flag epitope tag to VP3 (Hoque et al. 1999).

A summary of manipulations of the capsid follows Large deletions in three exposed loops (1, 3, 4; Fig. 2) on the surface of the VLPs impaired expression and assembly of the capsid but changes in loop 2 were without effect (Hurtado et al. 1996). Insertion in this loop (loop 2) of a short amino acid sequence without any deletions allowed chimeric VLPs formation (Rueda et al. 1999). However, in these studies reduced amounts of VLP formation occurred or formation did not occur at all when fusions were placed at the C-terminus of VP2. Similarly when epitopes were fused to VP2 at the C-terminus or deletions at this site, there was slower expression of proteins and a quick degradation of the fusion (Sedlik et al. 1995, Hurtado et al. 1996). On the other hand Miyamura and colleagues (1994) demonstrated that hen white lysozyme (2.5 kb) fused to VP1 was displayed on the outside of the B19 chimera, but capsid assembly only occurred when the VP1 fusion was co-expressed with VP2. When truncated (39, 77, 115, 153, 191, 228 amino acids) versions of VP1 were fused to poly-his-tags the N-terminal portion of the VP1 protein remained in the interior of the capsid (Wong et al. 1994). In this study, assembly occurred in the presence of VP2 with the longer versions of VP1, but was progressively less efficient with shorter VP1 and the capsids showed marked dysmorphic appearance as the VP1-unique region was shortened. Insertions up to 17 amino acids within the N-terminus of VP2 of PPV have been shown not to alter capsid formation (Sedlik et al. 1995). This study also demonstrated the internal position of the N-terminus of VP2 in empty capsids. Mutants of CPV VP2 with deletions (9, 14 or 24 amino acids) at the N-terminus showed that VLP formation did not occur with deletions beyond 14 amino acids suggesting that the amino acid residues beyond this point are essential for capsid formation (Hurtado et al. 1996). In contrast, Kawase and coworkers (1995) demonstrated that most of the N-terminus of B19 VP2 up to 25 amino acids, including the polyglycine region, could be removed without affecting capsid self-assembly. This study also indicated that a single amino acid deletion at position 25 or truncations beyond amino acid 30 were incompatible with either self-assembly or coassembly with normal VP2. To test whether VP2 can self-assemble into VLPs and to avoid the internalization of its N-terminus, hybrid CPV VLPs were created by inserting epitopes into the VP2 loop 2 (Rueda et al. 1999, 2000). In this study, foreign epitopes were displayed successfully on the surface of the particle and there were 3 copies of each epitope clustered in each threefold axis. In addition, combined theoretical and experimental approaches have also been extended to AAV with various types of approaches to modify AAV2 in order to alter specificity and infectivity: random mutagenesis (Rabinowitz et al. 1999), alanine scanning mutagenesis

(Wu et al. 2000), site-directed insertions (Girod et al. 1999, Grifman et al. 2001), and bispecific antibody targeting (Bartlett et al. 1999).

As well as success in producing chimeric VLPs, there have also been failures. There has been repeated reference to reduced titers, fitness or low production of chimeric particles (Rueda et al. 1999, Wu et al. 2000). What has been learned from the success and failures is that the N-terminus of VP2 can be used to present epitopes correctly, loop 2 can accommodate linear epitopes on the surface of the capsid, deletions in loops 1, 3, 4, and C-terminus abolish capsid assembly, and that limited deletions at the N-terminus of VP1 can permit capsid formation. The key in developing parvo-VLPs is not to alter the regions of the VP2 in the 8-stranded anti-parallel β barrel or residues on the interior of the capsid near the 5-fold axis of symmetry (reviewed in Casal 1999, Casal et al. 1999, Rueda et al. 2000, 2004).

2.2.1 Vaccines

A promise of biotechnology in developing new approaches for the design of safer vaccines is the engineering of non-replicative, but highly immunogenic VLPs. This involves the identification of the viral proteins containing the protective determinants and systems capable of synthesizing such proteins at high levels. Synthetic peptides and VLPs offer safer vaccine alternatives to the more conventional use of attenuated viruses because there is no risk of these recombinant vaccines reverting back to being pathogenic. The success in parvo-VLPs used as vaccines is considerable. The first peptide vaccine for CPV was developed in 1994 (Langeveld et al. 1994), which protected dogs and mink (Langeveld et al. 1995) from a lethal CPV challenge. This was followed by a series of chimeric synthetic peptides with differing lengths (Casal et al. 1995). These vaccines were constructed and optimized from the sequence responsible for the N-terminal region of VP2. The first 37 residues of VP2 are disordered and the spatial structure remains unknown (Tsao et al. 1991, Agbandje et al. 1993), but this region has an epitope that is recognized by neutralizing antibodies (López de Turiso et al. 1991, Langeveld et al. 1993).

With the advent of producing a renewable source of self-assembling viral proteins (section 2.3.3 BEVS) with the ambition to enhance induction of the immune system, use of VLPs as vaccines began. Empty capsids from a wt infection and CPV VLPs were able to elicit a good protective response and protect dogs from CPV infection better than inactivated vaccine (López de Turiso et al. 1992, Saliki et al. 1992). Similarly recombinant empty capsids enriched for the VP1 protein should serve as the basis for a human B19 parvovirus vaccine (Bansal et al. 1993). Here the developed capsids consisting of at least 25% VP1 efficiently and consistently provoked vigorous B19 virus neutralizing responses. More recently, the common property of eliciting a response toward VP1 was exploited again with having a defined vaccine with 25% of VP1 and 75% of VP2 proteins incorporated into the B19 VLP (Ballou et al. 2003).

Recent strategies have been employed to improve induction efficiency of the immune system by creating chimeric parvo-VLPs that carry heterologous epitopes derived from diverse pathogens (Casal 1996, 1999). Neutralizing antibodies against the donor pathogen recognize these new vaccines, elicit immune responses specific to the epitopes inserted, and induce protection in animals against infection with the donor virus. These new vaccines include chimeric VLPs from human parvovirus B19 (Brown et al. 1994, Miyamura et al. 1994), porcine parvovirus (Sedlik et al. 1995, Rueda et al. 1999), or CPV (Rueda et al. 1999, Rueda et al. 2004). These chimeric VLPs with new immunogenic properties have been employed to induce strong B-cell (Sedlik et al. 1994, Palmer et al. 2004), Th-cell (Sedlik et al. 1994, Lo-Man et al. 1998, Palmer et al. 2004), and cytolytic T-lymphocyte responses (Sedlik et al. 1997) or a combination thereof (Rueda et al. 2000, 2004). In addition, displayed epitopes were enzymatically active and properly displayed (Brown et al. 1994, Miyamura et al. 1994, Rueda et al. 1999, 2000, 2004) and were able to protect against lethal challenge infection of corresponding viruses or pathogen.

Further exploitation of the unique ability of plant viruses to present epitopes is the *in planta* production and isolation of large quantities of chimeric virus containing epitopes from the N-terminal VP2 of MEV. The chimeric plant viruses were propagated at high levels easily purified and conferred protection against clinical disease in mink (Dalsgaard et al. 1997). Additionally, a vaccine platform was created that induced priming and continual boosting of a protective immune response by a single inoculation when a crippled parvovirus vector was constructed, based on a chimera between minute virus of mice (MVM) and LuIII, which expressed *Borrelia burgdorferi* outer surface protein A instead of its coat protein (Palmer et al. 2004). Specific antibody production was stimulated and provided lifetime protection from live spirochete challenge while inducing humoral and Th1 cell-mediated immune response. Similarly in some AAV vector studies, humoral or cell-mediated immune responses have been observed, and this gene delivery system has also been developed as a vaccine strategy (During et al. 2000, Lui et al. 2000, Lui et al. 2001, Ponnazhagan et al. 2001, Xin et al. 2002). Foreign epitopes from human papillomavirus and human immunodeficiency virus were genetically engineered in the genome of the AAV and consequently this antigen gene delivery system for human cells was used to elicit virus-specific immune responses.

2.2.2 Gene therapy applications

Practical therapeutic applications for parvoviruses have now been expanded to gene therapy applications. Gene therapy is the treatment for a wide range of diseases for which a genetic root or an inherited predisposition exists and involves the introduction of novel genetic material to amend the abnormality, disease-causing gene (Bunnell & Morgan 1998). Parvovirus-based vectors are gaining importance, as an alternative to the more commonly used retroviral, lentiviral or adenoviral vectors in human gene therapy (Srivastava 1994, Somia & Verma 2000, Buchschacher & Wong-Staal 2001, Palmer & Ng 2005), and have

been derived from AAV, H-1, LuIII and MVM, which have been successfully tested in many preclinical models of human diseases, including cancer (Ponnazhagan 2004). The strategy of exploiting these viruses is based on efficient integration and mechanisms that allow long-term expression of transgenes by AAV (Büeler 1999, Synder 1999) or transient expression by non-integrating viral vectors by autonomous parvoviruses (Maxwell et al. 2002, Cornelis et al. 2004b). As witnessed already, parvo-VLPs could be used as drug delivery systems and as vaccines by changing the facade of the VLPs, but an alternative way to exploit the inherent apathogenic (AAV) and oncotropic (H-1, LuIII and MVM) nature of parvoviruses is in gene therapy.

The natural host of some serotypes of AAV is humans and 80% of the population is seropositive because of natural exposure to AAV (Erles et al. 1999, Moskalenki et al. 2000); while endemic, AAV is apathogenic to humans (Pattison 1990). What makes AAV-based vectors ideal for gene-therapy application is that AAV can transduce dividing and non-dividing cells, has anti-tumor and anti-proliferative properties, has an extremely wide host and tissue range, and some serotypes do not induce an immune response (Kotin et al. 1990, Shaughnessy et al. 1996, Kay et al. 1997, Snoeck et al. 1997, Somia & Verma 2000). Another apparent advantage of this system is the possibility of long-term gene therapy, because AAV is the only known parvovirus that integrates at a specific site in the human genome (Kotin et al. 1990, Samulski et al. 1991, Di Pasquale & Stacey 1998). Other studies have shown that even with this potential of integration and stable expression, this does not cause oncogenicity or mutagenesis (Di Pasquale & Stacey 1998, Maxwell et al. 1996). AAV-based vectors have emerged as a leading contender (Pfeifer & Verma 2001), in which the therapeutic DNA replaces much of the viral genome that is contained within the protein capsid shell and the gene for the replication protein that encodes site specificity. There are copious testimonies of AAV-mediated gene transfer in which the immune response to the transgenes was examined, and recombinant AAV elicited low or undetectable innate or adaptive immune responses to the expressed transgene (Conrad et al. 1996, Fields et al. 2000, Monahan & Samulski 2000, Zaiss et al. 2002, Carter & Samulski 2000). Consequently, many trials have been initiated due to these qualities.

Clinical trials of AAV-based vectors are underway for treatment of several diseases including cystic fibrosis (Flotte & Carter 1998) and hemophilia (High 2000), whereas achievement in animal models is encouraging with the development of treatments for high blood pressure (Phillips 1997), melanoma (Bantel-Schaal 2001), muscular dystrophy (Greelish et al. 1999, Wang & Xiao 2000), Parkinson's disease (Mandel et al. 1999, Mochizuki et al. 2002), and many other afflictions (Xiao et al. 1998, Waller & Ernstoff 2003). In cancer gene therapy, restriction of transgene expression to one specific tissue target is the goal and to increase the efficiency and safety of AAV-based vectors, re-targeting strategies have been attempted. In particular, manipulation of the AAV capsid by displaying a targeting peptide that does not interfere with packaging or indirect re-targeting by displaying a molecule on the surface of the capsid that

binds specifically and stably to target cells have been conducted to increase transduction (Blechacz & Russell 2004). These studies have included genetic capsid modification, incorporation of tumor-targeting peptides, insertional mutagenesis of the capsid gene, or random peptides to modify the tropism of the AAV vector (Girod et al. 1999, Rabinowitz et al. 1999, Grifman et al. 2001, Muller et al. 2003, Perabo et al. 2003, Shi & Bartlett 2003). Similarly bispecific antibodies and avidin-linked ligands have been used as a coupling agent to firmly bind transduced cells (Bartlett et al. 1999, Ponnazhagan 2004). Another strategy to increase transgene expression of AAV-based vectors is transcriptional targeting in a tissue-specific manner controlled by promoters and regulatory elements encoded in the vector (Gow et al. 1992, Chen et al. 1998, Xiao 1998, Cucchiarini et al. 2003).

However, there are problems in using AAV as a gene therapy agent. Tribulations include cytotoxicity of REP protein encoding for replication and integration functions, lack of efficiency, as well as, restriction of the 4.5 kb size for the transgene (Saudan et al. 2000). Moreover, it seems that recombinant AAVs integrate less specifically into chromosome 19 than previously thought (Mulligan 1993, Balague et al. 1997, Robbins & Ghivizzani 1998, Mountain 2000). In addition, due to the need of a helper virus in manufacturing of the virion, cross-contamination of the helper virus might be present in purified samples. These dilemmas could be circumvented with stable packaging cells lines for the *rep* and *cap* genes (Clark et al. 1995, Gao et al. 1998, Gao 2002), use of a heterologous inducible promoter to drive *rep* gene expression (Qiao et al. 2002) and with improved purification methods or with helper-free production systems (Mountain 2000, Wu & Ataai 2000, Toublanc et al. 2004).

MVM, LuIII and H-1 based-vectors have also shown promise as gene therapy vectors as these parvoviruses can also infect human cells (Dupont et al. 1994, Berns 1996, Rommelaere & Cornelis 2001). The first autonomous parvovirus vectors based on LuIII were described about a decade ago (Russell et al. 1992, Maxwell et al. 1993) and now have been extensively developed (Palmer & Tattersall 2000, Rommelaere & Cornelis 2001, Maxwell et al. 2002, Raykov et al. 2002, Cornelis et al. 2004a, b). H-1, LuIII and MVM-based vectors are interesting candidates for gene therapy because they are inherently oncotropic and oncolytic, have relatively small genomes, do not integrate into cellular chromosomes, and are apathogenic to humans. Anti-cancer gene therapies of autonomous parvoviruses exploit their inherent oncosuppressive, oncotropic and oncotoxic properties. These properties appear to be determined by their enhanced replication capacity in transformed human cells, oncogene- and cell cycle-dependent activation of the viral early promoter, and the oncogene-induced cytotoxic activity of the NS1 protein (Blechacz & Russell 2004).

Tumor-suppressing properties and preferential killing of transformed and tumor cells have been continually documented for parvoviruses (Toolan 1967, Kimsey et al. 1986, Cornelis et al. 1988, Rommelaere & Cornelis 1991, McKisic et al. 1996, Ball-Goodrich 1998, Faisst et al. 1998, Dupont et al. 2000). Murine and human tumor cells implanted in mice were also shown to be targets for

parvovirus infections (Guetta et al. 1986, Dupressoir et al. 1989, Rommelaere & Cornelis 1991, Faisst et al. 1998, Haag et al. 2000, Giese et al. 2002). Autonomous parvovirus may act not only by killing tumor cells, but also by activating the immune system to induce in animals a dynamic process that promotes the generation of anti-tumor cytotoxic T lymphocytes, which may result in tumor cure (Melcher et al. 1998, Ronchetti et al. 1999, Wretzel et al. 2001). Initially, autonomous parvovirus antitumor vectors with a therapeutic transgene in place of the gene encoding the capsid genes were developed with the aim of strengthening the antineoplastic activities (Rommelaere & Cornelis 1991) of the natural parvoviruses (Russell et al. 1992, Dupont et al. 1994, Brandeburger et al. 1999, Kestler et al. 1999, Palmer & Tattersall 2000). It has been recognized that some of these capsid replacement vectors displayed stronger antineoplastic effects in various mouse and human tumor models than wt parvovirus (Haag et al. 2000, Wetzel et al. 2001, Giese et al. 2002). H-1, LuIII and MVM-based vectors' pathogenicity that mostly concerns fetuses and neonates, is restricted to tissues with a high proliferation index such as cancer cells (Jacoby 1996). These vectors are not by themselves potent enough to suppress tumor formation; subsequently newer vectors are based on exploiting the oncolytic properties of parvoviral NS1 protein or the addition of another gene to kill tumors (Caillet-Fauquet Perros et al. 1990, Cornelis et al. 2001, Olijslagers et al. 2001, Rommelaere & Cornelis 2001). Hyper- and hypotoxic mutant forms of MVM NS1 have also been generated (Corbau et al. 2000). Oncolytic properties of NS1 proteins have also been extended to recombinant adenovirus expression (Raykov et al. 2002).

Additional improvements of developing an efficient parvoviral gene therapy vector are in enhanced transduction and selective expression of a potent therapeutic gene. Transductional re-targeting of FPV with the aim of modifying it to target human tumor cells had been attempted that enabled transduction of a human rhabdomyosarcoma cell line (Maxwell et al. 2001). Transcriptional targeting was used to achieve cell-type specific transgene expression under the control of a chimeric promoter containing a liver-specific enhancer, or the use of a transcription factor that is constitutively activated in colon carcinoma (Maxwell et al. 1996, Maxwell & Maxwell 1999, Malerba et al. 2003). Although promising, a main disadvantage in using MVM, LuIII and H-1 based-vectors in gene therapy is the limitation of the transgene size due to the unproductive encapsidation beyond a size limit of 106% of the wt virus (Brandenburger et al. 1999, Kestler et al. 1999). This size limitation has been meanwhile expanded for AAV by using strategies based on heterodimerisation of separate AAV vectors in head-to-tail orientation during concatemer formation (Sun et al. 2000, Duan et al. 2001). However, some crucial differences between MVM, LuIII and H-1 based-vectors and AAV-based vectors should be distinguished. AAV-based vectors induce constitutive expression of the encoded transgene, driven by strong, heterologous promoters, in every transduced cell, whether or not it is mitotically dividing, without intrinsically harming the cell. Transgene expression by MVM, LuIII and H-1 based-vectors is under viral control, and non-dividing cells do not express the transgene until

they start to proliferate following, which they rapidly die given that the products of the vector are cytolytic once activated (Blechacz & Russell 2004).

2.3 Baculoviruses

The Baculoviridae are a family of large, rod-shaped, enveloped arthropod viruses that contain large circular double-stranded DNA genomes (Blissard 1996). Baculoviruses have a restricted host range, but a vast majority of permissive species falls within one Order, the Lepidoptera of the Class Insecta. Due to the biphasic life cycle of baculoviruses, two distinct morphological forms of infectious baculovirus particles are seen (Miller 1988). Firstly, occluded derived viruses (ODVs) comprise of enveloped virions enclosed within a matrix of protein, polyhedrin or granulin. Secondly, budded viruses (BVs) comprise of a single virion enveloped by a membrane obtained from the infected cell. The Baculoviridae family is however divided into two genera: nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) (Volkman et al. 1997). The larger NPVs contain typically several nucleocapsids per envelope encased in the polyhedrin matrix and hence are called multicapsid, M nucleopolyhedroviruses (MNPVs). On the other hand GVs have only one virion embedded in each ovicylindrical occluded virus particle that appear "granula" under the light microscope.

2.3.1 Baculovirus life cycle

The two distinct morphological forms of the infectious baculovirus particles in the biphasic life cycle are responsible for different roles. Primary infection of lepidopteran hosts occurs when ODVs matrix bodies have been ingested from contaminated food sources. The alkaline environment of the host's gut dissolves the matrix of proteins, releasing the ODVs. The columnar epithelial cells of the midgut are then infected by direct fusion of the ODVs with the plasma membrane (Summers & Smith 1987, Grabherr et al. 2001). Nucleocapsids are then released into the cytoplasm and transported to the nucleus via actin filaments (Charlton & Volkman 1993). The secondary infection occurs when the BVs enter the surrounding cells by receptor-mediated endocytosis.

The baculovirus life cycle is divided into several phases: early, late, and very late (Miller 1988). Early, 6 to 9 h p.i., transcription accounts for regulatory proteins for virus production (Friesen & Miller 1987). Late genes, 6- 24 h p.i., code for structural proteins of BVs, whereas ODVs are produced from the polyhedra during the very late phase (18 to 72 h p.i.) of the virus life cycle (Summers & Smith 1987). Glycoprotein 64 (gp64) is an important membrane protein for BVs and is expressed late and very late in the cycle. Budded viruses are then transported out of the nucleus during the late phase of infection and acquire its gp64 composite envelope when budding from the basolateral side of

the cell. At this point BVs are now able to infect surrounding cells. The ODVs are retained in the nucleus enclosed with the crystalline matrix until the cell lyses whereby acquiring components of the modified intranuclear membrane (Friesen & Miller 1987, Blissard et al. 1996, Volkman et al. 1997). Fig. 4 recounts the budded baculovirus life cycle.

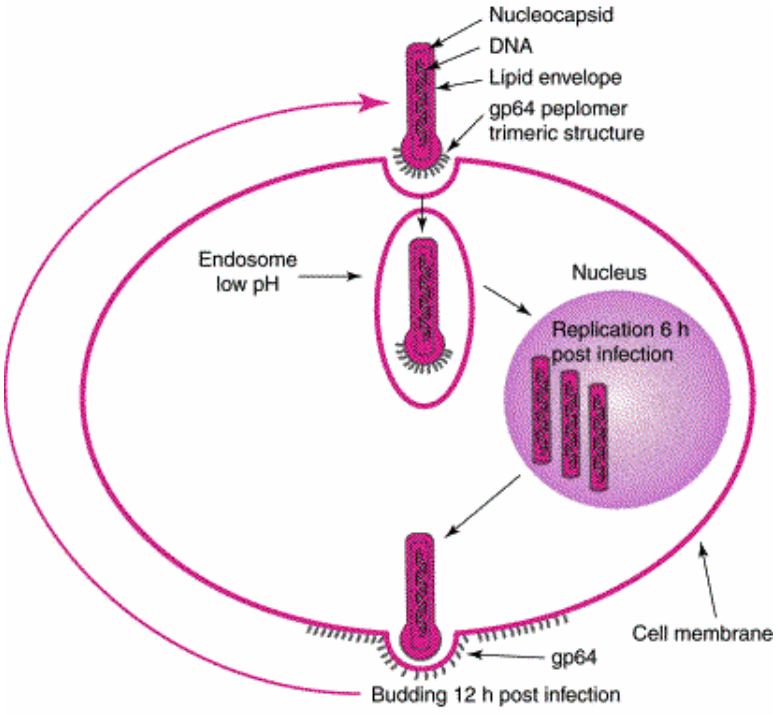

FIGURE 4 Budded baculovirus life cycle as found in insect cells and BEVS.. The budded virion binds the cell via gp64 trimers. The virus is transported by the endosomes and nucleocapsids are released into the nucleus, where replication takes place. The assembled virus buds from the cell while picking up gp64. Virus egression is at approximately 12 h p.i. Modified from Grabherr et al. 2001.

2.3.2 Baculovirus applications

Baculoviruses are an integral part of nature's ecosystem playing an important role in regulating the size of insect populations. This quality made baculoviruses a central biological control agent of insect pests (Valk 1993, Miller 1995, Bonning & Hammock 1996, Thiem 1997). Baculoviruses are safe for humans and other vertebrates since vertebrates lack the capability to employ insect promoters (Carbonell et al. 1985, Kool et al. 1994). Prototype viruses of the family Baculoviridae are *Autograph californica* multiple nucleopolyhedrovirus (*Ac*MNPV) and *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) (O'Reilly et al. 1994). *Ac*MNPV, an archetype of baculoviruses, has been widely utilized in molecular biology, biotechnology, and medical research purposes, as well as, being characteristically used in the baculovirus expression vector system (BEVS) (Possee 1997, Grabherr et al. 2001).

2.3.3 Baculovirus expression vector system

The BEVS has routinely been the choice system to produce recombinant eukaryotic proteins mediated by baculoviruses over other alternative bacterial and eukaryotic systems. Reason for this are because insects cells possess posttranslational modifications similar to mammalian cells, a feature that prokaryotic expression systems lack, and cost of producing proteins is low (Summer & Smith 1987, Crossen & Gruenwald 1988, Luckow & Summer 1988, O'Reilly et al. 1994). These modifications include signal sequencing processing, proper protein localization within the insect cell, and functional glycosylation of glycoproteins. In addition proteins are usually soluble, correctly folded and biologically active (King & Possee 1992). The BEVS is easy to use, can accommodate large shuttle cassettes of foreign DNA, and hetero-oligomeric protein complexes are attained by co-expression of several genes. Since its introduction in the 1980s proteins from all types of organisms (animals, plants, bacteria, viruses) have been expressed with this system, and has been extensively used for the production of viral proteins (Smith et al. 1983, Summer & Smith 1987, Roy 1990, O'Reilly et al. 1994, Casal et al. 1996, Posee 1997, Casal et al. 2001, Maranga et al. 2002a).

A central method in generating recombinant baculoviruses (rBVs) is by utilizing the Bac-To-Bac® System (Luckow 1993; Instruction Manual: Bac-to-Bac® Baculovirus Expression Systems, Life Technologies). The life cycle of rBVs has been illustrated in Fig. 4. The Bac-to-Bac® generates rBVs by site-specific transposition in order to insert foreign DNA by homologous recombination into a bacmid propagated in *Escherichia coli* (*E. coli*). This bacmid contains a kanamycin resistance marker, a segment of DNA encoding the β-galactosidase gene (lacZα) with a short segment containing the attachment site for the bacterial transposon Tn7 (mini-attTn7). The mini-attTn7 does not interrupt the reading frame of the lacZα peptide and when propagated in DH10BAC™ *E. coli*, the bacmid complements a lacZ deletion present on the chromosome, and consequently, blue colonies are formed in the presence of 5-bromo-3-indolyl-β-D-galactopyronoside and the inducer isopropyl-β-D-thiogalactopyranoside. Plasmid pFastbac1 (Anderson et al. 1995) is used to house shuttle cassettes or a single gene for the transposition into the bacmid. The mini-Tn7 cassette from pFastbac1 consists of a gentamicin resistant gene, the polyhedrin promoter, a multiple cloning site, and an SV40 poly-adenylation signal inserted between the left and right arms of Tn7. The transposing mini-Tn7 element from pFastbac1 to the mini-attTn7 attachment site on the bacmid occurs when the Tn7 transposition functions are provided *in trans* by a helper plasmid. The helper plasmid confers resistance to tetracycline, and encodes transposase. Once transposition occurs white colonies are formed, the composite bacmid DNA can be removed and accordingly used to transfect insect cells. Finally rBVs are harvested and further amplified by infection of insect cells. This system provides a mean of propagating rBVs within 10 days. The end result of the Bac-to-Bac® is the generation of rBVs to be used in BEVS to produce recombinant

proteins of interest or rBVs exploited in mammalian transduction experiments, such as in gene therapy.

Production of recombinant proteins of interest fabricated with BEVS have been effective for decades, as well as, various VLPs of numerous viruses used as candidate vaccine or in diagnostics have been made (Jones & Morikawa 1996, Possee 1997, Casal 2001, Maranga et al. 2002b). Interestingly this system provides the means to study for example: capsid assembly of viruses (Wong et al. 1994, Kawase et al. 1995, Hurtado et al. 1996, Newcomb et al. 1999), proteins stoichiometry of bluetongue virus, Norwalk virus, parvovirus, and rotavirus VLPs (French et al. 1990, Crawford et al. 1994, 1999, Tsao et al. 1991, White et al. 1997, Jiange et al. 1998, Palomares et al. 2002), production processes of viral proteins in birnavirus, hepatitis C virus, human immunodeficiency virus, parvovirus, and rotavirus (Baumert et al. 1998, Cruz et al. 1998, Jiange et al. 1998, Kang et al. 1999, Martínez-Torrecuadrada et al. 2000, Yuan & Parrish 2001, Palomares et al. 2002), and mutagenesis of proteins (Hagensee et al. 1993, Lawton 1997, Girod et al. 1999, Rabinowitz et al. 1999, Wu et al. 2000, Grifman et al. 2001). Similarly, antigen delivery systems, VLP display, have been fashioned via BEVS to be used as a subunit vaccine (Brown et al. 1994, Casal 1999, Sedlik et al. 1999), to alter viral tropism (Langer et al. 1998, Warrington et al. 2004), or to relieve purification steps (Hu et al. 1999, Wang et al. 2000). What is unique in this system is the ability to co-express individual capsid proteins via multi-gene baculovirus vectors and the ability to analyze the assembly of VLPs as with bluetongue virus (French & Roy 1990, Belyaev & Roy 1993, Zheng et al. 1999), infectious Bursal disease virus (Hu & Bentley 1999), human immunodeficiency virus (Cruz et al. 2000), human polyomavirus JC virus (Goldmann et al. 1999), human papillomavirus (Kirnbauer et al. 1993), parvovirus (Tsao et al. 1991, Bansal et al. 1993, Wong et al. 1994), polyomavirus (An et al. 1999), and rotavirus (Redmond et al. 1993, Crawford et al. 1994). Theoretically, these chimeric VLPs created by multi-gene baculovirus vectors are valid systems for broader vaccine production to increase the number of different serotypes it can be effective for. BEVS allows production of safe concoctions of vaccines between closely related viruses, and is useful for producing display VLPs. Additionally, these VLPs made in BEVS have also been used to mimic wild-type virus-host interactions in mammalian cells studies (Liprandi et al. 1997, Rollo et al. 1999, Charpilienne et al. 2002).

A limitation of the BEVS includes its inefficiency at properly processing heterologous proteins that are initially larger inactive precursor proteins but have been improved by the co-expression of prohormone convertase furin (Laprise et al. 1998), chaperones (Hyatt et al. 1993, Hsu et al. 1994), and a bacterial signal peptidase (Ailor et al. 1999). Another way this system has been scrutinized is in its capabilities to propagate recombinant baculoviruses for the purpose of transducing mammalian cells (Kost & Condreay 1999). An extension of this is the role that baculoviruses play as gene therapy vectors.

2.3.4 Baculovirus-mediated mammalian transduction

Baculoviruses do enter mammalian cells and their viral DNA is able to reach the nucleus, nevertheless, they do not replicate in any of the 35 mammalian cell lines studied to date (McIntosh & Shamy 1980, Tjia et al. 1983, Volkman & Goldsmilth 1983, Groner et al. 1984). Even non-hepatocytes can be transduced (Shoji et al. 1997), and when an exogenous promoter such as that derived from Rous sarcoma virus, simian virus 40 (SV40) or cytomegalovirus (CMV) is inserted into the baculovirus genome, gene expression in non-Lepidoptera cell lines can occur (Carbonell et al. 1985, Hofmann et al. 1995, Boyce & Bucher 1996, Song et al. 2003). Cell lines susceptible to baculovirus transduction were again extended by using a chimeric actin-CMV promoter (Shoji et al. 1997, Yap et al. 1997). In addition, an expression cassette from *Ac*MNPV has been stably maintained in mammalian cells (Condreay et al. 1999). A major advantage to using rBVs for mammalian studies, especially for gene therapy, is that there is an absence of preexisting antibodies against baculovirus in animals and little or no cytotoxicity is detected in the context of baculovirus transduction (Hoffman et al. 1995, Sandig et al. 1996, Delaney et al. 1999, Liang et al. 2004). Studies of baculoviruses for gene therapy suggest that baculoviruses could not be utilized *in vivo* due to inactivation of the virus by the serum complement system (Sandig et al. 1996, Hofmann & Strauss 1998). However, baculovirus-mediated gene transfer *in vitro* is feasible and can be used for a wide range of applications. This has led to studies of an anti-C5 antibody, cobra venom factor, or recombinant soluble complement receptor type 1 to treat serum, thus pathways of the complement cascade are inhibited and inactivation of rBVs does not occur (Hofmann & Strauss 1998, Hoffman et al. 1999). Another strategy would be to develop complement-resistant gene transfer vectors (Huser et al. 2001).

Transcription, and transduction efficiencies in baculovirus-mediated transduction of mammalian cell have been improved. Enhancement of foreign gene expression by baculovirus-mediated transduction was demonstrated by constructing a pseudotype virus possessing the vesicular stomatitis virus G glycoproteins (VSV-G) (Barsoum et al. 1997) and by using histone deacetylase inhibitor, which increased the transcription level (Condreay et al. 1999). Addition of sodium butyrate or trichostatin A, a histone deacetylase inhibitor, while transducing mammalian cells has also been shown to significantly enhance transduction by rBVs (Condreay et al. 1999, Airenne et al. 2000, Sarkis et al. 2000, Hu et al. 2003, Leisy et al. 2003). Similarly others have enriched the baculoviral envelope in order to increase the concentration of envelope fusion glycoproteins, gp69, on the virion surface to boost transduction (Tani et al. 2001). Recently there has been an explosion of studies that are directed to augment baculovirus-mediated transduction by means of baculoviral surface display.

Displaying or pseudotyping the viral surface accomplishes specific targeting of baculovirus for transduction enhancement (Grabherr et al. 2001). Foreign protein sequences have been essentially displayed on the viral surface

as a fusion protein to gp64 or to a second copy of gp64 (Boublik et al. 1995, Grabherr et al. 1997, Mottershead et al. 1997, 2000, Lindley et al. 2000, Ojala et al. 2001). These studies that have a second copy of gp64 result in a mixture of native and fusion gp64 on the virus surface. Recently, also indigenous (Mangor et al. 2001, Park et al. 2001, Pieroni et al. 2001, Spenger et al. 2002,) or modified (Chapple & Jones 2002, Ojala et al. 2004) VSV-G has again been used to alter the rBVs' tropism. In these studies VSV-G was fused either with EGFP (Chapple & Jones 2002) or with the IgG-binding ZZ-domains of protein A, expressed on the surface of baculoviruses and the surface distribution of the fusion proteins with gp64 was scattered in both cases. Attempts have also been made in modifying the major baculoviral capsid protein, vp39. This capsid protein is capable of displaying foreign protein molecules i.e. EGFP in large quantities around the capsid (Kukkonen et al. 2003, Oker-Blom et al. 2003).

Furthermore, rBVs have also been used to launch an infection of another foreign virus in mammalian cells. Infection of hepatitis B (Delaney & Isom 1998) and hepatitis C (Fipaldini et al. 1999) viruses have occurred when rBVs acts as a delivery system to deliver the entire genomes of the foreign viruses into mammalian cells. All hepatitis' viral proteins are synthesized, extracellular virions are produced and long-term expression of the transduced baculoviral genes did not perturb the physiological state of the transduced cell. The rabies virus glycoprotein G has also been expressed in BEVS and the recombinant proteins was antigenically similar to authentic G protein (Tuchiya et al. 1992). Also attenuated influenza virus were indeed propagated when baculovirus transduced cells were infected with influenza helper virus (McCormick et al. 2002). Likewise, co-infecting HEK293 cells with two different baculoviruses harboring the AAV vector and rep expression cassette respectively, together with a replication-deficient adenovirus expressing its cap gene, lead to infectious recombinant AAV particles (Sollerbrant et al. 2001). Additionally, an *in vitro* system for studying hepatitis B virus replication and polyprotein expression (Delaney et al. 1999, Abdelhamed et al. 2002, McCormick et al. 2002, 2004), as well as a system to determine replication competence of hepatitis and influenza viral transcripts (Abe et al. 2003, Poomputsa et al. 2003) have also been created by the use of hybrid baculovirus vectors. This hybrid baculovirus vector system could be used to analyze replication, host-cell interactions, protein expression, and be developed for those viruses that lack an efficient or convenient culture system.

2.3.5 Parvoviral proteins produced in BEVS

The BEVS has undoubtedly been a core technology for expression of genes for the development of biochemical reagents, vaccines, therapeutics, and for drug discovery. The following tables (2 and 3) outline recombinant parvovirus proteins produced by BEVS.

TABLE 2 Parvo-VLPs expressed in BEVS.

Virus-like particles	References
AAV	Ruffling et al. 1992; Steinbach et al. 1997; Hoque et al. 1999, Rabinowitz et al. 1999; Warrington et al. 2004, Sollerbrant et al. 2001
ADV	Christensen et al. 1993; Wu et al. 1994
CPV	Saliki et al. 1992; Hurtado et al. 1996; Casal et al. 1999; Rueda et al. 1999; Toivola et al. 2004
DPV	Le Gall-Recule et al. 1996
B19	Agbandje et al. 1991; Brown et al. 1991; Kajigaya et al. 1991; Salimans et al. 1992; Brown et al. 1994; Wong et al. 1994; Kawase et al. 1995; Kock et al. 1995; Kaikkonen et al. 1999; Franssila et al. 2001
MEV	Christensen et al. 1994; Casal 2001
MVM	Hernando et al., 2000; Livingston et al. 2002
MPV	Livingston et al. 2002
PPV	Sedlik et al. 1995, 1997; Casal et al. 1999; Rueda et al. 2000; Maranga et al. 2003

TABLE 3 Recombinant proteins expressed in BEVS. Individual expression of nonstructural and structural proteins of various parvoviruses.

Virus	Recombinant parvovirus protein	References
AAV	Rep	Ni et al. 1994
ADV	NS1	Christensen et al. 1995
B19	VP1	Gray et al. 1993
	NS proteins	Hicks et al. 1996
GPV	VP1	Takehara et al. 1999
MVM	NS1	Wilson et al. 1991; Liu et al. 1994; Riley et al. 1996

Rep: nonstructural protein of AAV.

Interestingly, when rBVs are used to express one or co-express two proteins, structural analysis could be done on the recombinant proteins. Moreover, using these recombinant proteins in mammalian studies allows monitoring of individual or collective trafficking events in mammalian cells. For parvoviruses, the BEVS has been exploited intensively both in the production of parvo-VLPs (Maranga et al. 2002, 2004) and for production of individual recombinant viral proteins. Despite this area of research, rBVs encoding parvovirus structural proteins have not been used in mammalian transduction experiments. This area once elaborated may prove to be a valuable tool to study individual or cooperative trafficking and nuclear entry events of parvovirus structural proteins.

3 AIMS OF THE STUDY

The development of virus-like particles for parvovirus has gained interest for many in the biological and medical industries. The use of VLPs has become a standard safe way for administering vaccines and studying the delivery of the VLPs in mammalian cells. Recently, attempts to modify the viral surface of these VLPs in order to change tropism, selective targeting and to boost the immune system have made meritorious advancements. To aid in developing biotechnological tools for studying parvovirus' life cycle the specific aims of this study were:

1. To create and assess the assembly of recombinant fluorescent parvovirus-like particles of CPV and B19 in BEVS.

2. To observe and compare to wild-type CPV the behavior of recombinant parvo-VLPs and proteins produced from BEVS in mammalian cells.

3. To scrutinize trafficking events and nuclear targeting of CPV structural proteins in rBV mediated-transduction of mammalian cells.

The tools created were ultimately evaluated for their potential in biomedical applications.

4 SUMMARY OF MATERIALS AND METHODS

Materials and methods are best described with additional details in the original publications I-IV.

4.1 Genetic baculovirus constructs

For construction of viral clones of canine parovirus and human parvovirus B19 structural proteins (VP1 and VP2) with or without a fusion to enhanced green fluorescent proteins (EGFP), the following steps were undertaken. Sequences encoding the structural genes for CPV VP1 and VP2 were amplified by PCR using plasmid pBI518 or the infectious clone of CPV (p265) as a template, respectively (kind gift from Colin Parrish, Cornell University, Ithaca, New York) (I, III, IV). Plasmid pBI518 is a clone which has the small splice removed so that it only expresses VP1. EGFP was amplified from pEGFP-C1 (Clontech, Palo Alto, CA) (I, II, IV) and the sequence encoding B19 EGFP-VP2 was amplified using plasmid pEGFP-VP2-C1 (Lindholm 2005) (II). Primers were from TAG Copenhagen A/S, Copenhagen, Denmark. All restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany).

The PCR products (see Table 4) of CPV VP2 (with or without a stop codon) were digested with *Kpn*I and *Eco*RI and cloned into the corresponding restriction sites of plasmid pSP73 (Promega, Madison, WI). The isolated *Bam*HI/*Eco*RI fragment from the preceding plasmid was then cloned into the corresponding restriction sites of pFastBacI (Gibco-BRL, Grand Island, NY). All other amplified viral gene products were digested with respective restriction enzymes (MBI Fermentas, St. Leon-Rot, Germany) and cloned into the corresponding restriction sites of pFastBacI (Gibco-BRL) (I-IV). The PCR products of the EGFP gene were digested with appropriate restriction enzymes and finally cloned into its respective plasmids (CPV pEGFP-VP2Fastbac, CPV pVP2-EGFPFastbac, pEGFPFastbac, and B19 pEGFP-VP2; I-IV). For use in mammalian cells, all CPV plasmid constructs were also further digested with *SnaB*I and *Bam*HI to remove the polyhedrin promoter, which was replaced with

the *Nru*I and *Bam*HI fragment from plasmid pEGFP-C1 (Clontech) representing the cytomegalovirus immediate-early (CMV) promoter (I, III). Table 4 presents the *sense* and *anti-sense* primers used for the corresponding genes with restriction enzymes sites, and start as well as stop codons are indicated.

TABLE 4 Primers for PCR amplification of CPV $VP1_{s/wo}$, $VP2_{s/wo}$, $EGFP_{s/wo}$, and B19 EGFP-VP2.

Publication	Gene	Sense Primer (5' end): restriction enzyme sites are in **purple.** Anti-sense Primer (3' end): restriction enzyme sites are in **blue.** Start sequence is in **green.** Stop sequence is in **red.**
III, IV	CPV $VP1_s$	5′-TA CAT **GGA TCC ATG** GCA CCT CCG-3′ ***Bam*HI** 5'-CGA GGC **GAA TTC TTA** ATA TAA TTT TCT AGGTGC-3' ***Eco*RI**
III	CPV $VP1_{wo}$	5′-TA CAT **GGA TCC ATG** GCA CCT CCG-3′ ***Bam*HI** 5′-CCG A GGC **GAA TTC** ATA TAA TTT TCT AGG TGC-3′ ***Eco*RI**
I, III, IV	CPV $VP2_s$	5'-TT **GGT ACC ATG** AGT GAT GGA GCA GTT CAA-3' ***Kpn*I** 5'-CGA GGC **GAA TTC TTA** ATA TAA TTT TCT AGGTGC-3' ***Eco*RI**
I, III, IV	CPV $VP2_{wo}$	5'-TT **GGT ACC ATG** AGT GAT GGA GCA GTT CAA-3' ***Kpn*I** 5′-CCG A GGC **GAA TTC** ATA TAA TTT TCT AGG TG C-3′ ***Eco*RI**
I, III	$EGFP_s$	5'-GTC **GAA TTC ATG** GTG AGC AAG GGC GAG GAG-3' ***Eco*RI** 5'-TCT **TCT AGA TTA** CTT GTA CAG CTC GTC CAT GCC-3' ***Xba*I**
II	$EGFP_{wo}$	5'-GTC **GAA TTC ATG** GTG AGC AAG GGC G-3' ***Bam*HI** 5'-TAA **AGA TCT** CTT GTA CAG CTC GTC CA-3' ***Bgl*II**
II	B19 EGFP-VP2	5'-G TCC GAA **GCG CGC ATG** GTG AGC AAG GGC-3' ***Not*I** 5'-C GGC ACA **CGT GGG TAA** CCG CCG GCG GA-3' ***Pau*I**

s = with a stop codon; wo = without a stop codon.

The following diagram (Fig. 5) depicts all genetic constructs cloned and then used to propagate recombinant baculoviruses (rBVs) for this thesis. The CPV constructs under the polyhedrin promoter were named pVP1Fastbac, pVP2Fastbac, pVP2-EGFPFastbac, and pEGFP-VP2Fastbac. For EGFP the construct was named pEGFPFastbac. The B19 construct was named pEGFP-VP2. For mammalian transduction experiments the following constructs were named pCMVVP1Fastbac, pCMVVP2Fastbac, pCMVVP2-EGFPFastbac for CPV proteins and pCMVEGFPFastbac.

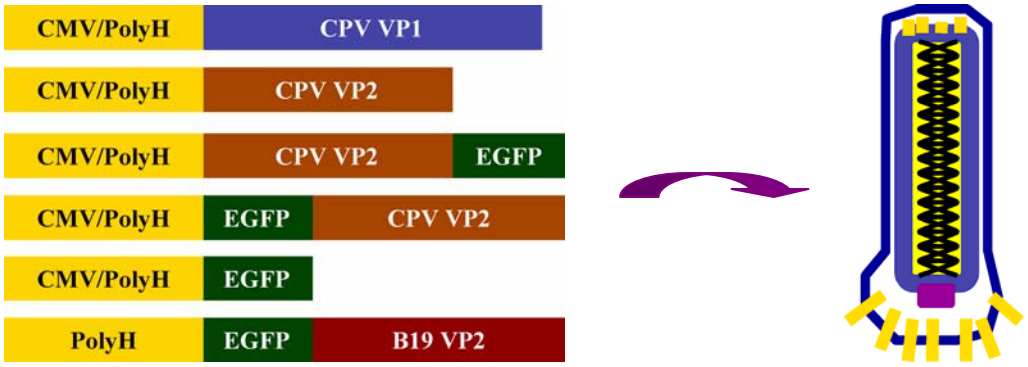

FIGURE 5 Summary of rBV constructs. CPV constructs were placed in pFastbacI and then each were used to generate recombinant baculoviruses (right image). Either CMV or PolyH promoter was used to direct expression of the CPV constructs. CMV = cytomegalovirus intermediate-early promoter; PolyH = polyhedron promoter.

All essential plasmids were sequenced according to protocols from Epicentre Technologies (Madison, WI) that used the SequiTherm EXCEL II Long-Read DNA Sequencing Kit-LC with a LI-COR automated DNA sequencer and two labeled primers (TAG Copenhagen A/S) for pFastbacI. The following two primers were used to sequence the plasmids; *sense* primer A413 5'-AAT GAT AAC CAT CTC GCA-3' IRD-700 and *antisense* primer A414 5'-CTA CAA ATG TGG TAT GGC TC-3'. Alignment of the amplified sequence with its original template was used to confirm the proper sequence of the cloned genes. The constructs were then used to propagate rBVs using the Bac-to Bac® system (Gibco-BRL).

4.2 Cell lines and viruses

For cloning of plasmids and recombinant baculoviral genomes, *E. coli* JM 109 cells (I-III) and *E. coli* DH10Bac cells (I-III) were employed. *Spodoptera frugiperda* 9 (*Sf*9) cells were exploited to propagate recombinant baculoviruses (I-IV) and protein production of recombinant proteins (I, II, IV). In feeding and transduction experiments, Norden Feline Laboratory Kidney (NFLK; provided by Colin Parrish) cells (I, III, IV) and human hepatoma (Hep G2) cells (II) were utilized. Bacterial cell lines were grown in Luria-Bertani medium at 37 °C with appropriate antibodies for the selection of desired clones (I, II, III). Insect cells were grown on monolayer or in suspension at 28 °C in HyQ SFX-Insect cell culture medium (HyClone Inc., Logan, UT, USA). NLFK and Hep G2 cells were maintained as monolayers in Dulbecco's modified eagle's medium (DMEM; Gibco-BRL) containing 2 mM L-glutamine supplemented with 10% fetal calf serum, 1% non-essential amino acids, and 1% penicillin/1% streptomycin mixture at 37 °C in a 5% CO_2 atmosphere.

Creation of biotech tools for studying parvovirus infections required propagation of rBVs that housed structural proteins of CPV and B19 parvoviruses. The Bac-to-Bac™ system (Gibco-BRL) uses site-specific transposition to insert foreign DNA by homologous recombination into a bacmid propagated in *E. coli* DH10Bac cells. Together with pFastBacI (Anderson et al. 1995), shuttle cassettes or a single gene are then transposed into the baculovirus genome and generation of rBVs is accomplished. Table 5 presents the rBVs constructed and other viruses adopted within this thesis.

TABLE 5 Viruses employed within this thesis. Recombinant baculovirus (rBV) under the polyhedrin promoter served for protein production. Mammalian transduction vectors were under the CMV promoter and used to transform mammalian cells.

Virus	Gene of interest	Type or Vector	Publication and Reference
***Ac*VP1**	CPV VP1	rBV	IV
CPV *Ac*VP2	CPV VP2	rBV	I, IV
***Ac*EGFP**	EGFP	rBV	I
CPV *Ac*EGFP-VP2	CPV VP2 N-terminus fusion to EGFP	rBV	I
***Ac*CMVEGFP,**	EGFP under the CMV-IE promoter	Mammalian transduction	II
***Ac*CMVVP1**	CPV VP1 under the CMV-IE promoter	Mammalian Transduction	II
***Ac*CMVVP2**	CPV VP2 under the CMV-IE promoter	Mammalian Transduction	II
***Ac*CMVVP2-EGFP**	CPV VP2 C-terminus fusion to EGFP	Mammalian transduction	II
B19 *Ac*EGFP-VP2	B19 VP2 N-terminus fusion to EGFP	rBV	III
B19 AcVP2	B19 VP2	rBV	III, Kaikkonen et al. 1999; Franssila et al. 2001
B19 *Ac*VP1/VP2	B19 VP1/VP2	rBV	III, Kaikkonen et al. 1999; Franssila et al. 2001
wild-type CPV-d	ALL	CPV	II, III, IV, Parrish 1991; Parker et al. 2001

To further analyze the mammalian transduction vectors and to serve as a control, wt canine parvovirus type 2, CPV-d (Parrish et al. 1982) was propagated from the infectious clone p265 (Parrish 1991, Parker et al. 2001) (I, III, IV).

4.3 Purification of recombinant proteins and wild-type viruses

To purify recombinant proteins for characterization experiments, *Sf*9 at 2 x 10^6/mL were grown in suspension in HyQ SFX medium (HyClone Inc.). At log phase, cells were infected with rBVs, or a combination thereof (IV), at a multiplicity of infection (MOI) of 10 and incubated at 27 °C for 72 h (I, II, IV). Infected cells were pelleted by low-speed centrifugation (268 x *g*, 15 min, 4 °C) and resuspended in 4 ml of ice-cold TENT buffer (50 mM Tris/HCl, 10 mM EDTA, 150 mM NaCl, 2 mM phenylmethylsulfonylfluoride, pH 7.4) that contained 10 µg/ml each of aprotinin, leupeptin and pepstatin (Sigma, St. Louis, MO). After an optional three freeze/thaw repeats in liquid nitrogen and water bath (25 °C) respectively, suspensions were treated with 0.2% Triton X-100, 15 min, 4 °C. Clarification of cell lysates continued (10,000 x *g*, 1 h, 4 °C), supernatants were collected, then loaded on 37 ml sucrose gradients (10% to 40%) in TENT buffer and finally ultracentrifuged (102,000 x *g*, 4 h, 4 °C). Opalescent bands or fluorescent band, 1 ml for each sample, were extracted with a syringe and transferred to ultracentrifuge tubes (Beckman Instruments, San Diego, CA). Samples were diluted in 7 ml PBS, ultracentrifuged (200,000 x *g*, 1 h, 4 °C) and pellets were gently resuspended in 200 - 500 µl of ice-cold TENT buffer. Alternatively, CPV VP1 (IV) and B19 EGFP-VP2 VLPs (II), for atomic force microscopy experiments, were purified as above with the exception of using a 45% $CsCl_2$ gradient instead of the sucrose gradient (Suikkanen et al. 2002). Purification of wt CPV was followed as described elsewhere (Suikkanen et al. 2002) (I, III). In short, CPV type 2 (CPV-d) was grown in NLFK cells for 5 days. The culture medium (300 ml) from infected cells was centrifuged and concentrated by ultrafiltration (30-kDa filter; Millipore Corp., Bedford, Mass.). CPV-d was pelleted (173,000 x *g*, 1 h), resuspended in 0.9 mL PBS and sonicated at low power. Heavy and light capsids were purified by isopycnic centrifugation in a 45% $CsCl_2$ gradient. The opalescent band containing full capsids was collected with a syringe and pelleted (245,000 x *g*, 2 h). Capsids were then resuspended in 200 µl PBS and stored at 4 °C. For purification of EGFP (I, II), cells infected with *Ac*EGFPVP2 were pelleted by low-speed centrifugation (268 x *g*, 15 min, 4 °C) and resuspended in 4 ml of ice-cold H_2O. Cells were then lysed by sonication (30%, 10 sec) and cell lysates were heated to 65 °C for 10 min after which they were transferred on ice. Samples were further ultracentrifuged (200,000 x *g*, 1 h, 4 °C) and the supernatants removed and stored at 4 °C until use.

4.4 Antibodies

Antibodies were utilized within this thesis for Western blotting (I, II, IV), immunofluorescence microscopy (I-IV), immunoelectron microscopy (I), and immunoreactivity assays (II). Table 6 depicts the variety of antibodies and

targets within each publication described. In addition the source and providers are given.

TABLE 6 Antibodies used in this thesis.

Primary Antibodies	Target	Source	Provider and Reference	Article
anti-VP1 Elmer	unique N-terminal of CPV VP1	rabbit	Laboratory of Peter Tattersall and Susan Cotmor Cotmore et al. 1997	III, IV
anti-VP2 Cornell #2	all forms of CPV structural proteins	rabbit	Colin Parrish (Cornell University, Ithaca, NY)	I, III, IV
anti-capsid A3B10	Intact CPV capsids	mouse	Strassheim et al. 1994; Wikoff et al. 1994	I, III, IV
anti-NS1	CPV nonstructural protein 1	mouse	Colin Parrish	III
anti-VP	B19 structural proteins VP1 and VP2	mouse	Yaegashi et al. 1989; Morita et al. 2001	II
Acute-phase, past-immunity and negative serum samples	B19 virus	human	Söderlund et al. 1995	II
anti-GFP	GFP	rabbit	Promega, Madison, WI	I, II
anti-α-tubulin	α-tubules	mouse	Amersham Biosciences, Uppsala, Sweden	II
anti-human transferrin	transferrin	mouse	Molecular Probes, Eugene, OR	I
anti-tubulin	tubules	mouse	Amersham, Buckinghamshire, UK	I
Ci-MPR	cation independent mannose-6-phosphate receptor	rabbit	Varpu Marjomäki (University of Jyväakylä, Jyväskylä, Finland) Juuti-Uusitalo et al. 2000	II
EEA1	early endosomes	mouse	Varpu Marjomäki	IV
LAMP-2	Lysosomal glycoproteins LAMP-2	mouse	Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA	I, IV
anti-mouse IgG	mouse antibodies target	rabbit	Promega, Madison, WI	I
Secondary Antibodies	**Conjugate**	**Source**	**Provider**	**Article**
anti-rabbit IgG	alkaline phosphatase	goat	Promega, Madison, WI	I, II
anti-mouse IgG	alkaline phosphatase	goat	Promega, Madison, WI	II, IV
anti-rabbit/ mouse antibody	Alexa-488 Red	goat	Molecular Probes, Eugene, OR	IV

TABLE 6 Continued.

Secondary Antibodies	Conjugate	Source	Provider	Article
anti-rabbit antibody	Alexa-546 Red	goat	Molecular Probes, Eugene, OR	IV
anti-mouse antibody	Alexa-546 Red	goat	Molecular Probes, Eugene, OR	II, III, IV
anti-rabbit antibody	Alexa-633 violet	goat	Molecular Probes, Eugene, OR	III, IV
anti-mouse antibody	Alexa-633 violet	goat	Molecular Probes, Eugene, OR	I, II, IV

4.5 Western Blotting

Infected insect cell samples (I, II) or purified proteins (I) were boiled for 5 min in reducing Laemmli sample buffer (Laemmli et al. 1970). Molecular weight markers (BioRad, Richmond, IL) and prepared samples were separated on the 10% SDS-PAGE slab gels and stained with Coomassie blue (I; Bio-Safe™ Coomassie, BioRad) or transferred to nitrocellulose sheets for immunoblot analysis (I, II, IV; Schleicher & Schuell BioScience, Inc., Keene, NH). For Dot Blot analysis (IV), CPV purified proteins (VP1, VP2 and co-expressed VP1 and VP2) with or without heat treatment (15 min, 65 °C) were transferred to nitrocellulose sheet for 1 h. Nitrocellulose sheets were blocked with 2% solution of milk powder in PBS and probing of the proteins was conducted with corresponding primary antibodies (Table 6). This was followed by secondary AP-conjugated antibodies (Table 6) and signals developed with NBT (nitro blue tetrazolium; Sigma) and BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma).

4.6 Feeding and transduction of mammalian cells

Approximately 1 x 10^6 NFLK (I, III, IV) or Hep G2 (II) cells per ml were maintained in 10 ml DMEM supplemented with 10% FCS (DMEM-FCS) and grown overnight on coverslips (diameter 13 mm) at 37 ^{o}C. The growth medium was then replaced with a mixture of 40 µl DMEM-FCS and 40 µl (0.750 - 1.31 mg/ml) of purified proteins CPV VP1 (IV), CPV VP2 (IV), CPV EGFP-VP2 (I), CPV VP1/VP2 (IV), B19 VP2 (II), B19 VP1/VP2 (II), B19 EGFP-VP2 (I, II) and EGFP (I, II). After binding for 1 hour on ice, cells were washed with PBS and 5 ml DMEM was added to the coverslips. The cells were incubated at 37 ^{o}C for 3 – 6 h, fixed with 4% paraformaldehyde (PFA/PBS) in PBS (15 min, RT) and left in 1% PFA/PBS at 4 ^{o}C until prepared for confocal microscopy.

For transduction experiments (III), the growth medium was replaced with a mixture of 30 µl DMEM-FCS and 30 µl of virus inoculum (*Ac*CMVVP1, *Ac*CMVVP2, *Ac*CMVVP1EGFP, *Ac*CMVVP2EGFP, *Ac*CMVEGFP and *Ac*CMVEGFPVP2 or combinations thereof) containing 1 x 10^8 plaque forming units (PFUs) per ml for each coverslip. After binding (30 min, 37 °C), 5 ml DMEM was added to the coverslips and incubated at 37 °C for an additional 48 h. Transduced cells were fixed (100% methanol, 6 min, -20 °C) and left in PBS at 4 °C until immunostaining (Section 4.7). For infection of initially transduced (24 h) NLFK cells with *Ac*CMVVP2EGFP (III), infection with wt CPV (Parrish et al. 1991) was initiated and allowed to progress for 24 h then fixed as above. Wild-type CPV infected NLFK cells served as controls for this particular infection (III).

4.7 Immunofluorescence microscopy

Immunolabeling of rBVs infected *Sf*9 cells (I, II) began with low speed centrifugation (800 x *g*, 1 min, RT) then the cell pellet was washed twice with PBS prior to fixation in 50 µl of 4% PFA-PBS (20 min, RT). Concentrated (10,000 x *g*, 1 min, RT) cells were rinsed twice with 50 µl of 0.15% glycine in PBS with a centrifugation step (10,000 x *g*, 1 min, RT) between each wash and the following labeling steps. Fixed cells from rBV infections (I, II), cells fed with fluorescent VLPs (I, II) or cells fed with recombinant CPV proteins (IV), and transduced NLFK cells (III) were permeabilized (1% BSA, 1% Triton X-100 and 0.01% sodium azide in PBS) for 20 min, RT. After incubation (10,000 x *g*, 1 min, RT) with the corresponding primary antibodies (Table 6), cells were washed several times in 50 µl PBS and finally recognized with the fluorescence labeled secondary antibody (Table 6). Cells were then rinsed with permeabilization buffer and embedded with 2 - 7 µl of MOWIOL-DABCO (30 mg/ml; Sigma). Coverslips were left at 4 °C before examination with a laser scanning fluorescence microscope (equipped with Nomarski DIC; Carl Zeiss Laser Scanning Microscope, Axiovert 100M, LSM510, Jena, Germany) with excitation and emission settings appropriate for the dyes used.

4.8 Electron microscopy

Sucrose purified VLPs (I, II, IV) were used as specimens for electron microscopy and immunoelectron microscopy studies. Carbon coated nickel or copper grids were incubated with 10 µl of the purified VLPs. After excess liquid was blotted away, samples were negatively stained with 2% potassium phosphotungstate, pH 6. Grids were left to dry and examined at 60 kV with a JEOL JEM-1200 EX transmission electron microscope (Jeol Ltd. Tokio, Japan). For double

immunogold labeling (I), CPV VLPs on grids were treated with 0.15% glycine in PBS solution and free protein binding sites were blocked using PBS with 10% fetal bovine serum. CPV VLP specimens (I) were labeled with anti-capsid antibodies (Table 6) and identified using rabbit anti-mouse antibodies (Table 6). The immune complexes were detected with protein-A-gold (5 or 10 nm) (kindly provided by G. Posthuma, University of Utrecht, Netherlands), rinsed several times with 0.15% glycine in PBS, and finally blocked with 10% protein A solution in PBS. Additionally, anti-GFP antibodies (Table 6) were used to detect the EGFP fusion protein and this was followed by incubation with protein-A-gold (10 nm). Specimens were contrasted with 0.4% uranyl acetate pH 7.4 (Griffiths et al. 1984) and left to dry before viewing as above. Alternatively to the labeling procedures described above, the DAKO EnVision™+ system, HRP (DAB) (DAKO Corporation, Glostrup, Denmark) was used to visualize the immune complexes after labeling with anti-capsid antibodies.

4.9 Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) was carried out using a ConfoCor 2 fluorescence correlation microscope (Carl Zeiss, Jena, Germany) (I, II). An Ar-ion laser at an excitation wavelength of 488 nm was utilized. The emission photons were adjusted using a 530-600 nm bandpass filter with the pinhole attuned by using Rhodamine 6G dye (Molecular Probes). All samples were diluted in PBS (1:100) and directly used for the FCS experiments (I, II). LabTek II 8-well chambered borosilicate glass plates (Nalge Nunc International, Naperville, IL, USA) were used as carriers and the measuring time was 20-seconds (40 repeats). Diffusion times and the normalized autocorrelations G (t) were calculated using software provided by the manufacturer (Carl Zeiss). Diffusion coefficients and hydrodynamic radii for the particles were calculated from the measured diffusion times using 10 autocorrelation measurements with detailed description as previously described (Yoshida et al. 2001, Toivola et al. 2004). For computation of the number of EGFP domains present in the B19 EGFP-VP2 VLPs (II), the fluorescent VLPs were diluted (1:200) and then treated with 3 mM SDS**.** Wild-type canine parvovirus (CPV-d, 200 μg; 1.34 mg/ml) was labeled with Oregon Green 488 succinimidylester (Molecular Probes) and prepared as previously described (Suikkanen et al. 2002).

4.10 Immunoprecipitation of fluorescent VLPs

To assess if EGFP moieties were displayed on the outside of B19 fluorescent VLPs (III), purified VLPs were incubated (4 h, 4 ^{o}C) with anti-GFP antibodies (Table 6) and then immunoprecipitated as described below. B19 VP2 VLPs were

used as a control in this immunoreactivity assay (III). Lysates from infected *Sf*9 cells with rBVs encoding B19 EGFP-VP2, B19 VP2, and EGFP were incubated (4 h, 4 °C) with B19 acute-phase, past-immunity, and negative serum samples (III; Söderlund et al. 1995). Protein A sepharose beads (Amersham Biosciences, Uppsala, Sweden) were added and the mixture was incubated for 1 h, 4 °C. After a series of washes, immunocomplexes were pelleted and the fluorescence monitored in microtiter plates (Victor I, LKG, Turku, Finland). The fluorescence of EGFP was excited at 488 nm and detected at 543 nm.

4.11 Atomic force microscopy

Atomic force microscopy (AFM) was used to obtain topological images of the fluorescent VLPs (II). Cesium chloride gradient purified B19 EGFP-VP2 fVLPs (1.31 μg/ml in PBS) were incubated (40 min, RT) on freshly cleaved mica (SPI Supplies, West Chester, PA). Samples were thoroughly washed with PBS and dried in a desiccator for 1 – 3 h. Further, AFM samples were rinsed with distilled water and dried with a He_2 stream prior to imaging with a Dimension 3100 Atomic Force Microscope (Veeco Instruments, Santa Barbara, CA) operating in tapping mode. RTESP tips (Veeco Instruments) with a resonant frequency of approximately 300 kHz, and a scanning speed of 1 – 5 Hz were used along with NANOSCOPE 6.11 to perform height analyses of all AFM data collected. The surface area from the imaged VLPs allowed the size of the fluorescent VLPs to be calculated. The surface area was estimated from half of the oblate of the VLP attached to the mica. The final size of the fluorescent B19 VLP was calculated based on the surface area of the oblate with the following equations I & II (Beyer et al. 1987):

$$S = 3\pi a^2 + \pi \frac{c^2}{e} \ln \frac{1+e}{1-e} \qquad \text{[Equation I]}$$

S is the surface area of the oblate; *a* and *c* are the radius and the height of the imaged VLP, respectively; *e* is the ellipticity of the imaged VLP as defined below:

$$e = \sqrt{1 - \frac{c^2}{a^2}} \qquad \text{[Equation II]}$$

4.12 Molecular modeling

A schematic representation of the B19 EGFP-VP2 VLPs was created from PDB file 1S58 (Kaufmann et al. 2004) for B19 VP2 and EGFP domains were modeled against PDB file 1S6Z (Rosenow et al. 2004), with the linker region arbitrarily modeled as an α-helix. Based on the knowledge that EGFP is displayed on the outside of the VLP, the EGFP domain was connected to the internal N-terminus of VP2 and the linker region was extended out through the pore in the 5-fold axis cannon structure, similar to the CPV structure PDB entry 4DPV (Xie & Chapman 1996). Models were fabricated and molecular schemas were rendered using BODIL (Lehtonen et al. 2004) and PYMOL (DeLano 2002).

4.13 Phospholipase A_2 Activity

CPV recombinant proteins VP1, VP2 VLPs, and as a control wt CPV, were investigated to deduce if they possessed phospholipase A_2 activity (PLA2; IV). Samples (5 - 10 μg) were assayed for activity with a commercial kit from Cayman Chemical (MI, USA) as described previously (Suikkanen et al. 2003). Free thiols from hydrolysis of the thio-ester bond at the sn-2 position by PLA2 were detected by using DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid). The recombinant proteins and VLPs were tested for PLA2 activity with and without heat treatment (2 min in 65°C). Heat treatment has been shown to expose VP1 N-terminal ends and to trigger PLA2 activity of native capsids (Vihinen-Ranta et al. 2002, Suikkanen et al. 2003).

5 REVIEW OF THE RESULTS

Production of recombinant proteins in BEVS is a standard protocol and used routinely in molecular biology laboratories. This system allows generation of viral components to assess the assembly of novel VLPs and antigen delivery vehicles, as well as allowing one to follow trafficking of the proteins in permissive and non-permissive cell lines. In addition, rBVs can be further exploited to analyze maturation and localization of foreign viral proteins in mammalian cells. This thesis was conducted to provide a variety of biotechnological tools to study the parvovirus life cycle and was accomplished by using two themes transpiring from BEVS: the use of VLPs/recombinant proteins (I, II, IV) and the usage of rBVs for mammalian transduction experiments (III).

5.1 Assembly of parvo-VLPs

To demonstrate that the generated recombinant baculoviruses were properly produced in infected insect cells, the cells (I, II) and/or purified recombinant proteins (I, II, IV) were analyzed by SDS-PAGE, immunoblotting, confocal imaging, fluorescence correlation spectroscopy, electron microscopy, immunoreactivity, atomic force microscopy and phospholipase A_2 activity assay.

5.1.1 Characterization of recombinant CPV proteins

Analysis of the CPV recombinant proteins began with the exposure of infected insect cells 48 h p.i. to immunoblot analysis and with immunofluorescence microscopy (I). Polyclonal rabbit anti-VP2 antibodies identified CPV viral proteins VP2 and EGFP-VP2 with correct sizes (64.7 kDa and 93.7 kDa, respectively).

Moreover, EGFP protein and EGFP-VP2 fusion protein with correct sizes (29 kDa and 93.7 kDa, respectively) produced in insect cells were recognized with polyclonal rabbit anti-GFP antibodies. Immunofluorescence studies allowed viewing of expressed proteins in these infected insect cells (I). Mouse monoclonal anti-capsid antibody (A3B10) was used to identify CPV capsid epitopes in cells expressing CPV VP2 and CPV EGFP-VP2 VLPs. Mock-infected cells were used as controls. Capsid epitopes from the CPV VP2 protein were seen mainly in the cytosol. In contrast to CPV VP2 proteins, EGFP-VP2 proteins were seen both in the cytosol and in the nucleus suggesting that EGFP might have influenced the structure of the VP2 VLPs so that nuclear entry could occur. Due to the fluorescence of EGFP, direct viewing of cells infected with *Ac*EGFP or CPV *Ac*EGFP-VP2 was straightforward. Again, recombinant EGFP and CPV EGFP-VP2 proteins appeared to be distributed throughout the cell both in the cytoplasm and nucleus. There was a clear localization of EGFP fusion to CPV VP2 VLPs. The fact that anti-capsid antibodies identified CPV EGFP-VP2 fusion proteins suggested that these fusion proteins were capable of forming capsid-like structures (I; Table 6).

CPV VP2 and CPV EGFP-VP2 proteins were purified by differential sucrose gradient centrifugation (I) and analyzed further. Distinct bands sedimenting similarly as compared to native CPV (Tsao et al. 1991, Wu & Rossmann 1993) were obtained for both CPV VP2 VLPs and CPV EGFP-VP2 VLPs. In addition, the fluorescent band corresponding to CPV EGFP-VP2 VLPs was easily seen in the sucrose gradient. The purified CPV VLPs were also exposed to Western blot analysis using polyclonal rabbit antibodies directed against VP2 and GFP (I). Immunoblot and Coomassie blue staining of purified CPV VP2 and CPV EGFP-VP2 VLPs indicated that the protein complexes were of correct size in size to native CPV suggesting that these VLPs were assembled in insect cells.

The assembly of CPV VP2 VLPs and CPV EGFP-VP2 VLPs was further verified with electron microscopy and fluorescence correlation spectroscopy (FCS) (I). Sucrose gradient purified CPV VLPs were negatively stained and portrayed virus-like structures with shape and sizes similar to those of wt CPV (Tsao et al. 1991, Wu & Rossmann 1993). Double immunolabeling of the capsid complexes using anti-capsid and anti-GFP antibodies with 5 and 10 nm protein A-conjugate gold particles allowed visual detection of EGFP associated with VP2. The use of DAKO Envision System also allowed viewing of the incorporated EGFP onto the CPV VP2 VLPs. The fact that gold particles were seen on the capsid in these studies suggests that EGFP is displayed on the outside of the VLP. To characterize the incorporation of CPV EGFP-VP2 into fluorescent VLPs FCS analysis was conducted (I). Recombinant EGFP and Oregon Green 488 labeled CPV were used as controls. From the autocorrelation curves the corresponding translational diffusion times were 90 ± 10, 570 ± 100, 630 ± 30 µs with the calculated hydrodynamic radii being 2, 14 ± 3, 16 nm for EGFP, CPV EGFP-VP2, and Oregon Green 488 labeled CPV, respectively. The hydrodynamic radii of CPV VP2 and CPV EGFP-VP2 corresponded to the size previously determined for wt CPV (Tsao et al. 1991, Wu & Rossmann 1993). The

diffusion coefficient for CPV has not been reported previously and it is note worthy that CPV EGFP-VP2 has a similar diffusion coefficient (1.7 $x10^{-7}$ cm^2s^{-1}) to wt CPV (1.5 ± 0.1 $x10^{-7}$ cm^2s^{-1}).

Another aspect in this thesis was to produce and further characterize CPV VP1 proteins, CPV VP2 VLPs and CPV VP1/VP2 VLPs for feeding experiments (IV). Samples of $CsCl_2$ purified CPV VP1 and sucrose gradient purified CPV VP2 VLPs and CPV VP1/VP2 VLPs were studied by using dot blot analysis, electron microscopy and tested for phospholipase A_2 activity. In the dot blot analysis, a polyclonal rabbit anti-VP1 antibody was used to detect CPV VP1 and a mixture of co-expressed CPV VP1 and VP2. The monoclonal anti-capsid antibody (A3B10) was used for detection of intact CPV capsid epitopes. The preparations for VP1 detection were heat treated prior to loading on the nitrocellulose membrane in order to have the VP1 N-terminal exposed. The anti-VP1 antibody did recognize VP1 both in preparation of VP1 and in the co-expressed mixture of VP1 and VP2, but it did not recognize the preparation of VP2. Similarly, the anti-capsid antibody recognized non-heated VP2 proteins in both purified VP2 and non-heated mixture of VP1 and VP2 but it did not recognize VP1 or heated VP2. Electron micrographs of negatively stained VP2 and co-expressed VP1 and VP2 displayed the presence of VLPs similar to those previously described for VP2 (Tsao et al. 1991, Wu & Rossmann 1993) and seen before in this thesis (I). To further characterize the recombinant CPV proteins, investigation of phospholipase A_2 activity was conducted on recombinant VP1 and VP2 VLPs (IV). CPV VP2 VLPs did not show activity before of after heat treatment. When recombinant VP1 was tested, the activity of VP1 was almost 10 times higher than that of heated native capsids based on similar proteins concentrations.

Together these findings indicated that recombinant CPV VP1, as well as, CPV VLPs from VP2, VP1/VP2 and EGFP-VP2 were obtained when produced in BEVS (I, IV). In addition, these results indicated that the CPV EGFP-VP2 assembled correctly and has EGFP displayed on the outside of the VLP (I). Also, the method of purification excludes the possibility that CPV VP2 VLPs, CPV VP1/VP2 VLPs, and CPV EGFP-VP2 VLPs purified from baculovirus-infected *Sf*9 cells would exist as non-specific aggregates (I, IV). This method of sucrose gradient purification has shown to exclude aggregates and to prove that the final samples do represent VLPs (Brown st al. 1991, Tullis et al. 1993, Hernando et al. 2000, Simpson et al. 2002, Toivola et al. 2004).

5.1.2 B19 fluorescent VLPs

After evaluating the recombinant CPV VLPs and confirming that CPV EGFP-VP2 could assemble into VLPs, the human parvovirus B19 counter part was tested (II). The B19 VP2 structural protein was fused to EGFP and thoroughly analyzed to demonstrate if this fusion protein could assemble into VLPs and be useful as a biotechnological tool. The B19 VP2, VP1/VP2 and EGFP-VP2 infected insects cells underwent Western blotting, and immunofluorescence

analyses to discover if the recombinant proteins were being produced (II). Monoclonal mouse antibodies raised against B19 structural proteins (anti-VP) were able to identify proteins with molecular weights of 58, 83, and 84 kDa corresponding to recombinant B19 proteins VP2, VP1 and EGFP-VP2, respectively. The polyclonal rabbit anti-GFP antibodies identified the B19 EGFP-VP2 fusion proteins and soluble EGFP with appropriate molecular weights of 84 kDa and 26 kDa, respectively. As seen with the fluorescent fusion of EGFP to VP2 for CPV (I), immunoblots of B19 EGFP-VP2 fusion proteins indicated that the fusion proteins are slightly being degraded in this expression system (II). Additionally, immunofluorescence imaging of *Sf*9 cells infected with rBVs B19 *Ac*VP2, *Ac*VP1/VP2 and *Ac*EGFP-VP2 showed that the viral proteins were expressed at high level when probed for B19 structural proteins or viewed directly in the case of EGFP-VP2 (II). Mid-section images of B19 EGFP-VP2 showed that the VP2 proteins were colocalized with EGFP and that these proteins were localized in globular-shaped cellular structures. Investigation of these structures with polyclonal rabbit antibody to Ci-MPR (a marker for cation independent mannose-6-phosphate receptor) suggested that these fusion proteins of B19 EGFP-VP2 accumulated into large vesicles of the late endosomal compartments. Collectively, recombinant proteins of VP2, VP1/VP2, and EGFP-VP2 for B19 were being produced when BEVS was used as an expression system and assembly of these B19 fluorescent proteins was then analyzed (II).

Evaluation of the assembly of B19 EGFP-VP2 commenced with separation of of cytoplasm from infected cells by sucrose gradient centrifugation, fluorescence correlation spectroscopy, electron microscopy, and atomic force microscopy (II). Accordingly, cytoplasmic components of infected cells expressing B19 VP2, B19 VP1/VP2, and B19 EGFP-VP2 were separated by sucrose gradient centrifugation. Opalescent bands for B19 VP1 VLPs and B19 VP1/VP2 VLPs, as well as, fluorescent bands corresponding to B19 EGFP-VP2 VLPs were easily seen in the sucrose gradient. Assembly of VLPs for B19 EGFP-VP2 was further analyzed by electron microscopy (II). Negatively stained samples of sucrose gradient purified proteins of B19 indicated that EGFP-VP2 assembled into VLPs with diameters and similar appearance compared to those assembled from VP2 VLPs and VP1/VP2 VLPs. Additionally, FCS was further employed to study the level of incorporation of EGFP into the B19 fluorescent VLPs (II). In the presence and absence of 3 mM SDS, changes in particle brightness, number of the fluorescent molecules in the observation volume, and diffusion time of the B19 EGFP-VP2 VLPs were noted. In the absence of SDS, autocorrelation analysis suggested hydrodynamic radii of approximately 14 nm for B19 EGFP-VP2 VLPs. It is interesting that FCS analysis was able to distinguish aggregates from single VLP. When the fluorescent VLPs were treated with 3 mM SDS, the number of fluorescent units increased 9-fold and the hydrodynamic radii decreased 3-fold to 5 nm. This demonstrated that an average of 9 EGFPs were seen to be associated with B19 VP2 VLP. Atomic force microscopy (AFM) was used to confirm the presence of EGFP on the B19 VP2 VLPs (II). Topological imaging and height analysis of the AFM data collected

were used to calculate the diameter of 28.4 nm for the fluorescent VLP. In addition several EGFP domains were incorporated with the VLP. FCS and AFM data support the idea that the EGFP protein was displayed on the outside of the B19 VP2 VLP.

To further support the FCS and AFM studies, the fluorescence intensity of the immunoprecipitated complexes with anti-GFP antibodies was recorded to explore if EGFP was displayed on the outside of the B19 EGFP-VP2 VLPs (II). Fluorescent readings of 10,429 and 100 units were recorded for EGFP-VP2 VLPs and VP2 VLPs, respectively, suggesting that the anti-GFP were able to bind the EGFP fusion on the fluorescent VLP (II). Immunoreactivity of human sera with EGFP-VP2 protein complexes was further investigated to reveal if these fluorescent VLPs are useful in distinguishing between acute-phase, past-immunity and negative human serum samples from a B19 infection (II). Both acute-phase serum and past-immunity serum immunoprecipitated the EGFP-VP2 complexes, but past immunity serum was less effective within this study. In addition, negligible reactivity with the fluorescent complexes was shown when B19 EGFP-VP2 VLPs were immunoprecipitated with negative sera. In summary, there seems to be a higher binding capacity of acute-phase sera antibodies to EGFP-VP2 complexes compared to past-immunity sera and negative sera samples.

Together these findings indicated that recombinant B19 EGFP-VP2 (II) assembled correctly and has EGFP displayed on the outside of the VLP as in the case for CPV EGFP-VP2 VLPs (I). In addition, the B19 EGFP-VP2 VLPs resembled similar size when compared to B19 VP2 VLPs and B19 VP1/VP2 VLPs (II).

5.2 Behavior of recombinant parvovirus proteins in mammalian cells

Following a detailed portrayal of the CPV and B19 recombinant proteins, characterization of the purified proteins when fed to mammalian cells was also scrutinized. The behavior of the produced CPV proteins was examined in regards to wt CPV on their ability to enter and traffick through the cells into the nucleus (I, IV). The B19 EGFP-VP2 VLPs were analyzed to see if they bind, enter and travel to the nucleus (II). This information allowed us to evaluate if the recombinant proteins behaved similarly or differently to that of their wt part and to verify if these proteins could be used as biotechnological tools to study the parvovirus life cycle (I, II, IV).

5.2.1 Trafficking of CPV recombinant proteins.

Evaluation of CPV EGFP-VP2 VLPs as a tool to study CPV trafficking events commenced with NLFK cells being fed (I). At 3 h p.f. CPV EGFP-VP2 VLPs

were immunostained with monoclonal anti-capsid antibodies and immunofluorescent imaging showed that the VLPs colocalized with its fusion partner EGFP. Direct viewing of CPV EGFP-VP2 (EGFP fluorescence) in fed cells that were immunolabeled for various subcellular structures showed that these VLPs were able to bind and enter cells. In addition, colocalization of the fluorescent CPV VLPs with microtubules, lysosomal vesicles and transferrin were to some extent observed. Interestingly, when soluble EGFP was used as a control in the feeding experiments, no binding, entry or subsequent localization of similar subcellular structures was seen.

Feeding of NLFK cells with purified CPV VP2 and CPV VP1/VP2 VLPs was also carried out for analysis of their entry and trafficking in mammalian cells (IV). With times of 4 h, 12 and 24 h post-feeding, cells were immunostained with anti-VP2 and anti-tubulin antibodies, which were visualized by alexa-633 and alexa-543 conjugated secondary antibodies, respectively. When VP2 VLPs were fed alone or in the form of VP1/VP2 VLPs, VLPs were able to enter the NLFK cells and were seen associated with microtubules. When fed alone, the VP2 was essentially found in a discrete vacuolar-like pattern at a perinuclear location in the cytosol at all time points (4 h, 12 h, and 24 h) post-feeding, whereas when VP1/VP2 VLPs were fed they were clearly targeted towards the nucleus after 4 h post-feeding.

The entry routes of recombinant CPV VP1 and the above CPV VLPs were observed at 5.5 h p.f. with regards to localization with subcellular organelles and further entry into the nucleus (IV). The fed NLFK cells were immunostained with anti-VP1 (elmer) and anti-VP (cornell) antibodies for VP1 and VP2, respectively. In addition, subcellular compartments (microtubules, early endosomes, and lysosomes) were stained with anti-tubulin, EEA1, and Lamp-2, respectively. When recombinant VP1 was fed alone, the proteins were able to enter these cells, use microtubules and enter the nucleus. There was also some localization of VP1 with EEA1 and LAMP-2, but as seen with all subcellular stains, the VP1 was distributed throughout the cell outlined in DIC images. VP2 VLPs were then seen at 5.5 h post-feeding with anti-VP antibodies and colocalization with Lamp-2 and EEA1 is quite evident as seen in the merged images (IV). Images of microtubules with VP2 were seen to be similar in distribution and localization even when the time point p.f. increased to 24 h. When comparing entry route of VP1 at this time point with VP2, one could see that little VP1 protein was seen in the early endosomes (EEA1) and lysosomes (Lamp-2) as compared to merged images of VP2 VLPs. It seems that VP2 VLPs were localized within the endocytotic subcellular compartments and not noticeable in the nucleus as outlined DIC images. This suggests that VP2 has poor nuclear trafficking as compared to VP1.

Similar feeding experiments for CPV VP1/VP2 VLPs were conducted (IV). Staining for VP1 illustrated similar results when VP1 was fed alone in that there was some colocalized with EEA1 and Lamp-2 as seen in the merged images. Similarly VP1 was noticed to colocalize with microtubules and was present in the nucleus. When viewing V1/V2 VLPs with anti-VP antibodies, the viral proteins now have different staining patterns as seen with VP2 VLPs. The viral

proteins from VP1/VP2 VLPs colocalized with microtubules, EEA1, and Lamp-2 but are now seen to be able to localize in the nucleus as depicted with merged and DIC images. It would be interesting to investigate if VP1 alone could direct VP2 into the nucleus.

To investigate if the presence of recombinant VP1 was sufficient to allow rerouting of proteins into the nucleus, feeding experiments were conducted with purified proteins of VP1 and VP2 VLPs (IV). At 5.5 h post-feeding, cells were stained with anti-VP1 (elmer) or anti-VP (cornell) and with similar subcellular compartments as in above. Viewing of VP1 (images not shown) in this experiment displayed equivalent entry route as when VP1 was fed alone or in the assembled VLP. Viewing of VP2 VLPs in the presence of recombinant VP1 on the other hand illustrated similar results as in VP1/VP2 VLP feeding experiments when labeled for anti-VP (cornell) antibodies. Colocalization is seen with microtubules, EEA1, and Lamp-2 as displayed in merged images, but these VP2 VLPs in the presence of VP1 were able to enter the nucleus. These results suggest that VP1 has the capacity to reroute proteins out of the lysosomes. A final feeding experiment was conducted to see if VP2 VLPs, VP1/VP2 VLPs and VP2 VLPs with recombinant VP1 were seen to colocalize with late endosomes (IV). At 5.5 h post-feeding, cells were stained with anti-capsid antibody and the polyclonal antibody to cation-independent mannose-6-phosphate receptor (MPR). Images of VP1/VP2 VLPs and VP2 VLPs with recombinant VP1 indicated that these VLPs colocalized with the late endosomes and nuclear entry occurred.

5.2.2 Trafficking of B19 recombinant proteins.

Entry and trafficking of B19 fluorescent VLPs in Hep G2 cells were also evaluated (II). Cells of this non-permissive cell line were fed with B19 EGFP-VLPs. At 4 or 6 h p.f., cells were immunolabeled with anti-VP antibodies. These VLPs were seen to bind and enter the cells. Additionally, when viewed directly (from EGFP fluorescence) there was a clear colocalization of the viral proteins with its fusion partner EGFP. These B19 fluorescent VLPs were able to enter the nucleus. When microtubules were identified with antibodies, there was colocalization of the intracellular structure and the B19 fluorescent VLPs. Again, when EGFP was used as a control in this cell line, no binding or entry into the cells was seen.

Collectively, these CPV and B19 recombinant proteins and VLPs were used as tools to broaden the understanding of the life cycle of CPV and B19. Distinctively, the fluorescent CPV and B19 VLPs behave similar to CPV VLPs or B19, respectively and can be used *in vivo* for experiments. In addition, these findings suggest that CPV VP2 VLPs have poor nuclear trafficking as compared to VP1, but could be rerouted to the nucleus in the presence of CPV VP1.

5.3 rBVs as tools for transducing mammalian cells

Mammalian transduction experiments were performed to evaluate if these rBVs could be used as tools to investigate the intracellular localization of CPV structural proteins with respect to nuclear targeting (III). This *de novo* system allowed individual evaluation of CPV VP1 and VP2 roles in these effects while having wt CPV as a control. Likewise cooperate roles of both CPV VP1 and VP2 were also scrutinized. Nuclear targeting and structural proteins were viewed in the transduced and infected cells. Baculovirus constructs controlled by the CMV promoter for mammalian transduction studies that expressed EGFP, CPV VP1, CPV VP2 and CPV VP2-EGFP fusion proteins were used (III).

To begin with, immunofluorescence images of wt CPV infection at 24 h p.i indicated a clear nuclear targeting, as well as, some cytosolic distribution for VP1 proteins and capsids when identified with anti-VP1 and anti-capsid antibodies, respectively (III). VP2 antigens were seen evenly distributed throughout the infected cell when probed with anti-VP2 antibodies (III). Phenotypes observed quantitatively from the confocal images of the structural proteins of CPV infected cells also supported these findings (III). The rBVs housing the individual CPV structural proteins, VP1 and VP2, as well as EGFP and its C-terminal fusion to VP2 were then used to evaluate individual expression and subcellular targeting of expressed proteins. NLFK cells expressing recombinant VP1 revealed both nuclear and cytoplasmic localization. EGFP when expressed in these cells also showed even distribution of the fluorescent protein throughout the cells. In contrast to wt infection, recombinant VP2 when probed with anti-VP2 antibodies had comparable nuclear and cytoplasmic distribution and when probed with anti-capsid antibodies the antigens were mostly cytoplasmic. Similarly, VP2-EGFP expression portrayed mainly cytosolic distribution for the capsid antigens (III, Fig. 4J, N) and an even nuclear and cytoplasmic distribution when probed with anti-VP2 antibodies or when viewed directly. It is noteworthy that capsid antigens of VP2 alone or fused with EGFP had difficulty in nuclear targeting.

Co-expression of VP1 and VP2 in rBV transduced NLFK cells was conducted to elucidate the interaction of VP1 and VP2 proteins in regards to subcellular targeting (III). When probing for VP1 proteins, cells displayed an increase targeting of the proteins to the nucleus as compared to VP1 when expressed alone. Confocal imaging of recombinant VP2 when probed for capsid antigens revealed a shift of these proteins from cytosolic to nuclear distribution when VP1 protein is present. Likewise, VP2 proteins when identified with anti-VP2 antibodies displayed increased nuclear targeting in the presence of VP1 when judged against VP2 alone. The presence of both VP1 and VP2 displayed an enhanced nuclear transport of viral proteins (III). To confirm these results, transduced NLFK cells with rBV harboring the VP2-EGFP fusion were further infected with wt CPV. Evaluation of the subcellular targeting of VP2-EGFP in the presence of wt VP1 revealed that this fluorescent fusion protein had

pronounced nuclear targeting when viewed directly as compared to cytosolic distribution when VP2-EGFP is expressed alone. Again a cooperative interaction of VP1 with VP2 proteins is a prerequisite for efficient nuclear targeting of CPV viral proteins; however, other viral components may also aid in this theme. Overall, the use of rBVs allowed individual or collective assessments of CPV structural proteins in nuclear trafficking.

6 DISCUSSION

One protein, VP2, is required for formation of parvo-VLPs independent of the presence of other viral proteins and nucleic acid. Development of chimeric parvovirus virus-like particles (parvo-VLPs) has been routinely conducted for the purpose of elucidating its structure, assembly processes, change of tropism, diagnostics, and development of vaccines. A main factor that contributed to the development of these tools was the use of the baculovirus expression vector system (BEVS). It has been repeatedly reported that the BEVS is the choice system for the production of various core and virus-like particles. Moreover BEVS for the production of parvo-VLP is consistently being used to produce reoccurring supplies of VLPs (Maranga et al. 2002a, 2002b, 2004). It is only recently (Ojala et al. 2004), however, that BEVS has been exploited to transduce mammalian cells monitor individually expressed or co-expresed viral proteins for the purpose of investigating the protein's responsibilities in viral entry and trafficking events of the candidate virus.

The main goal of the study presented in this thesis was to investigate the development and assembly of fluorescent and non-fluorescent VLPs from CPV and B19 structural protein VP2. In addition, production of structural protein VP1 was examined and its role in trafficking events were unraveled. Further, rBVs were constructed to allow mammalian expression of structural chimer of VP1 and VP2 for CPV. These rBVs were examined for their potential in studying synthesis and trafficking of CPV parvoviral proteins in transduced cells.

6.1 Creation of parvoviral biotechnological tools

Construction of reagentless fluorescent VLPs relies on the identification of sites that can facilitate fusion with a fluorophore and accommodate local

conformational changes without disrupting the inherent nature of the virion. Conventional fluorescently labeled VLPs or viruses labeled by chemical means have many disadvantages. Location of the dye is usually impossible to regulate, and in immunolabeling, unspecific binding of antibodies causes background thus diminishing resolution (Heim et al. 1994, Wang & Hazelrigg 1994). These labeling procedures are laborious and time consuming, since the tagged proteins have to be purified from the extra dye prior to further analysis (Heim et al. 1994, Toivola et al. 2004). In addition, surface-bound labels may influence important and functional properties like receptor binding ability. Fluorescent fusion proteins have been used by others to create fluorescent VLPs of bursal disease virus (Chevalier et al. 2002), rotavirus (Charpilienne et al. 2001), hepatitis B virus (Kratz et al. 1999), and adenovirus (Leopold et al. 1998). Their strategy included insertions, C-terminal and N-terminal fusions to structural proteins, as well as insertions in external loops of the virion. These studies confirmed assembly of fluorescent viruses, but fluorescent parvo-VLPs have not been conducted so far. Other fluorescent viral constituents with proper behavior in transport and targeting of the recombinant fluorescent fusion proteins were observed for moloney murine leukemia virus envelope surface protein (Kizhatil et al. 2001), epstein-barr virus latent membrane protein 2B (Lynch et al. 2002), and rubella virus envelope proteins 1 and 2 (Ojala et al. 2004). Again the EGFP fusion did not change the behavior of the viral components.

Creation of chimeric parvo-VLPs has been carried out to develop more potent viral vaccines and for structural studies (Brown et al. 1994, Lo-Man et al. 1998, Casal et al. 1999, Rueda et al. 1999). These studies and others have suggested that C-terminal fusions to VP2 inhibits VLP formation, where as, N-terminal fusion could be accomplished and deletions up to a certain point at the N-terminus of VP2 could be tolerated (Miyamura et al. 1994, Kawase et al. 1995, Hurtado et al. 1996, Hoque et al. 1999). Additionally, green fluorescent proteins and derivatives thereof have proven useful in a number of different applications as fusion partners for structure/function studies, epitope presentation or antigen delivery (Girod et al. 1999, Sadeyen et al. 2003), construction of different display viruses (Charpilienne et al. 2001, Mottershead et al. 1997) and identification of the nuclear localization signal (NLS) of B19 (Pillet et al. 2003).

Previous construction of chimeric parvovirus capsid-like structures provided the rational for fusing EGFP to the N-terminus of VP2 for both CPV and B19 in the current studies (I, II). Assembly of these fluorescent VLPs was monitored using the BEVS (I, II, IV). Here we saw insect cells expressing these VLPs in large amounts and the integrity of VLP formation was seen through immunoblotting, immunofluorescence and electron microscopy, FCS, and AFM (I, II, IV). Initially, differential purification by sucrose gradient allowed the VLPs to be separated from unassembled forms. This method has been employed by others and is a convenient way to initiate characterization of VLP formation (Brown et al. 1991, Tullis et al. 1993, Hernando et al. 2000, Yuan & Parrish 2001, Simpson et al. 2002, Toivola et al. 2004). Although others (Yuan &

Parrish 2001) have reported that there is insufficient VLP formation of CPV VP2 when insect cells were collected 24 h p.i., it is note worthy that baculovirus bind to cells up to 24 h p.i. (Dee & Shuler 1997) and the polyhedrin promoter is a late promoter with its control in expression seen at 18 h p.i. (Summers & Smith 1987). Others have also seen detection of recombinant products of rotavirus structural proteins only after 18 h p.i. when produced in BEVS (Palomares et al. 2002). This is relevant to our studies due to the fact that insect cells collected at 48 h p.i. were used to purify the parvo-VLPs.

Integrity of assembly for CPV/B19 fluorescent VLPs and non-fluorescent VLPs (I, II; IV) was confirmed with several methods. Electron microscopy confirmed parvo-VLPs morphology of the chimeric and non-chimer VLPs when compared to wt virus or similar VLPs (Agbandje et al. 1991, Brown et al. 1991, Tsao et al. 1991, Wu & Rossmann 1993, Toivola et al. 2004). FCS also showed similarly sized CPV and B19 fluorescent VLPs compared to wt viruses (I, II), which is also supported with previous data for B19 VLPs (Toivola et al. 2004). Incorporation of EGFP into the VLPs was shown by immunoelectron microscopy in that antibodies directed against GFP were able to bind to the fluorescent VLPs (I). Similarly, FCS analysis of SDS treated B19 EGFP-VP2 VLPs indicated that there were approximately 9 fluorescent units associated with the VLP. Topological imaging by AFM confirmed this previous result with the size being similar to wt virus (II). Additionally, anti-GFP was able to immunoprecipitate B19 fluorescent VLPs (II). Other immunoprecipitation experiments with acute-phase, past-immunity, and negative human serum samples showed that the IgM antibodies displayed an increased binding to the fluorescent VLPs as compared to IgG binding (II). These findings are in agreement with the fact that early antibody response consists of IgM and past-immunity only of IgG target (Söderlund et al. 1995). Collectively, these results suggest that fluorescent VLPs of CPV and B19 are structurally intact. Although VP1-specific antigenic epitopes are not present in the fluorescent VLPs described here, these fluorescent VLPs have a potential to be used as a tool in biomedicine.

Although EGFP was seen to be associated with the VLP (I, II), a degraded product migrating with a similar apparent molecular weight as VP2 was observed in insect cells expressing CPV and B19 fluorescent VLPs (I, II). It would seem reasonable that degradation of the fusion is occurring to allow assembly. The molecular weight of the fusion proteins (EGFP-VP2) is similar to that of VP1. VP1 has been shown not to self-assemble into VLPs (Tullis et al. 1992). Further, FCS data (I, II) suggests that there is heterogeneity in the number of EGFP domains on each fluorescent VLP. Every 5-fold vertex does not seem to have an EGFP domain protruding. This is evident in the AFM image (II). A schematic picture of the fluorescent VLPs of CPV and B19 from the experimental data (I, II) is presented in Fig. 6. This model portrays the claim that EGFP is outside and is likely to be connected to the internal N-terminus of VP2 via the linker, which protrudes through the pore at the 5-fold axis.

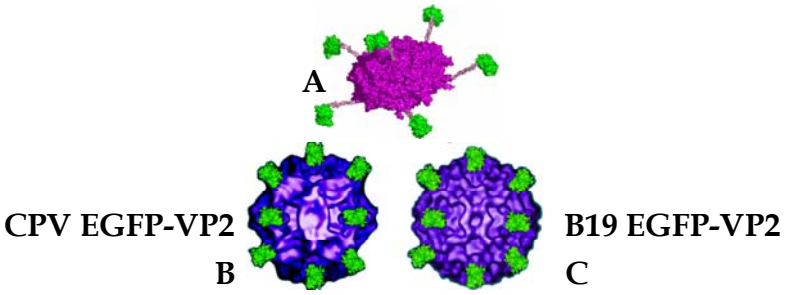

FIGURE 6 Representation of the fluorescent VLPs for CPV and B19. (A) Cartoon of a 45 subunit from the chimeric VLP. (B & C) Molecular models of the fluorescent biotechnological tools.

Collectively, these recent increases in visualization of parvovirus structural components and the constant supply of said proteins mediated by rBVs have allowed accounts of individual targeting roles in the life cycle of parvoviruses.

6.2 Recombinant proteins used to investigate parvovirus life cycle

The use of baculovirus vectors in studying the synthesis and pathway of parvovirus proteins has not been reported before. A *de novo* expression system of CPV structural proteins facilitated by rBVs transduction of mammalian cells allowed individual or collective roles of the structural proteins in nuclear entry to be studied (III). This system allows the evaluation of nuclear targeting of recently synthesized proteins (III) instead of intracellular trafficking and nuclear entry of incoming viral proteins (I, II, IV). Others have exploited this theme from rBV transduction of mammalian cells (Sollerbrant et al. 2001, Ojala et al. 2004) and have seen that this method is a unique way to elucidate trafficking events of viral proteins.

Synthesized proteins of VP1, VP2, co-expression of said proteins and a C-terminal fusion of EGFP, mediated by rBVs were scrutinized for their nuclear targeting abilities. Infection of wt CPV was compared to transduction of viral proteins via rBVs (III). Inspection of recombinant VP1 in the absence of other CPV viral proteins displayed cytosolic and a nuclear distribution (III). This finding seems reasonable given that VP1 has a potential nuclear localization signal (Vihinen-Ranta et al. 1997) and is seen to target the nucleus in a wt infection (III; Yuan et al. 2001, Lombardo et al. 2000). On the other hand, it is seen that recombinant VP1 did not have a predominant nuclear accumulation as previously described (III, Lombardo et al. 2000), and may suggest that the

nuclear localization signal for VP1 is not the only requirement for this protein to have high accumulation in the nucleus.

Inspection of expressed CPV VP2 indicated that this protein when probed with anti-capsid antibodies was found to be associated with vesicular-like structures and that very little of this antigen is seen in the nucleus (III). Similarly, when CPV or B19 VP2 was studied in insect cells with confocal microscopy, these proteins were only found in the cytosol (I, II). Collectively, this suggests that nuclear transport of VP2 in the assembled form is hampered in absence of other viral components that would be present in a wt CPV infection. Unassembled VP2 on the other hand is seen both in the nucleus and cytosol (III). Others have also seen this feature of unassembled VP2 to be in the cytosol and nucleus of other parvoviruses (Hoque et al. 1999, Lombardo et al. 2000, Yuan & Parrish 2001, Reguera et al. 2004). It has been suggested that VP2 enters the nucleus as a trimeric complex with VP1 (Lombardo et al. 2000, 2002) and that the assembled VP2 remaining in the cytoplasm is consistent with the inaccessibility of a nuclear transport motif mapped in the internal capsid surface (III).

Transduction with a combination of rBVs for the expression of CPV structural proteins with or without a wt infection were performed to reach near physiological states of the viral proteins (III). These experiments allowed investigation of the cooperative roles of the recombinant VP1 and VP2 in regards to nuclear targeting. When VP1 was co-expressed with VP2, nuclear targeting increased for both viral proteins (III). Co-expression of both structural proteins in insect cells also showed nuclear targeting (III). It is significant that in our studies VP1 and VP2 did accumulate in the nucleus more efficiently when both proteins were present (III) because similar results were shown when VP2-EGFP fusion proteins were targeted to the nucleus more efficiently when the same cell had been infected with wt CPV (III). Collectively, these results suggest that interaction of VP2 with VP1 and other components of a wt infection are needed to aid in targeting of assembled VP2 into the nucleus. Cooperation of structural proteins to aid in nuclear translocation has been previously shown for other viruses (Ruffing et al. 1992, Delos et al. 1993, Cai et al. 1994).

6.3 Entry and subcellular trafficking of parvovirus biotechnological tools

Interestingly, rotavirus-like particles have been used to mimic wt virus-host interactions (Liprandi et al. 1997, Rollo et al. 1999, Charpilienne et al. 2002). In our study, VLPs were used to investigate the parvoviral life cycle in permissive and non-permissive cells (I, III, IV). More explicitly the intracellular trafficking and nuclear entry of incoming viral proteins and VLPs were followed to assess these nano-particles as biotechnological tools (I, III, IV).

The entry route of parvovirus into the cell is initiated with binding of its receptor, transferrin (Parker et al. 2001) followed by dynamin-regulated clathrin-mediated endocytosis (Parker et al. 2001, Basak & Turner 1992). Progress of infection continues with virus entry into early endosomes, late endosomes, and then transportation into the perinuclear region (Suikkanen et al. 2002, 2003). Following this, trafficking to the lysosomal compartments is seen and sequential release into the cytosol before trafficking into the nucleus (Suikkanen et al. 2002). Movement of parvoviruses toward the nucleus is thought to be mediated by microtubules (Suikkanen et al. 2002). In the present study the entry and trafficking behavior of the fluorescent VLPs and other recombinant CPV proteins were investigated (I, III, IV).

The ability of recombinant VP1, VP2 VLPs and fluorescent VLPs to follow the endocytotic entry route of parvoviruses was tested (I, III, IV). The fluorescent VLPs of CPV (I) and B19 (III) were able to enter mammalian cells and vesicular trafficking to the perinuclear region along microtubule filaments was accomplished. The EGFP fusion proteins did not affect the endocytotic route of these parvo-VLPs and did not alter the critical surface conformation responsible for entry into the cell (I, III). Similarly, CPV fluorescent VLPs were seen to colocalize with transferrin and lysosomes (I) as in wt CPV trafficking (Suikkanen et al. 2002, 2003). After binding to the cell surface, both CPV VP2 and CPV VP1/VP2 VLPs were able to efficiently enter mammalian cells and were found in discrete cytoplasmic perinuclear spots having a vesicular appearance, presumably lysosomal compartments (IV). When time progressed, VP2 VLPs were still present in these vesicular structures, but CPV VP1/VP2 VLPs were seen in the nucleus (IV). These vesicular-like structures bear a resemblance to that observed for endocytosed empty (and full) CPV particles (Suikkanen et al. 2003). It has been outlined that wt CPV is still seen up to 10 h p.i. in perinuclear vesicles (Suikkanen et al. 2002) and that the release from endocytotic vesicles is extremely slow up to 8 h before its entry into the cytosol (Parker & Parrish 2000, Ros et al. 2002, Suikkanen et al. 2002). AAV on the other hand, has been shown to penetrate from early endosomes into the cytoplasm as early as 30 min p.i. and reach the nucleus at 2 h p.i. (Bartlett et al. 2000). Together, our results demonstrated that the fluorescent VLPs showed similar entry and trafficking routes as wt CPV and that they could be used as visualization tools in studies related to CPV entry and trafficking mechanisms.

To characterize the perinuclear vesicular compartments containing CPV VP2 and CPV VP1/VP2 VLPs, several organelle makers were used (IV). Fed recombinant VP1 and VP2 VLPs individually were seen to enter cells, found to be trafficked identically into the lysosomal compartment, the early endosomal compartment and seen to colocalize with microtubules (IV). What is note worthy is that VP1 is effectively targeted to the nucleus in contrast to VP2 VLPs where it is extensively seen in the subcellular compartments, specifically in the lysosomes. The slight colocalization of VP1 with Lamp-2 (marker for lysosomes) and EEA1 (marker of early endosomes) could indicate that this protein is delivered quickly to the cytosol as compared to VP2 where it is retained in the lysosomes. The VP1 staining is in agreement with studies with

wt CPV infected cells, where colocalization with lysosomal compartments is seen 0.5 h to 3 h p.i. with little colocalization at 8 h p.i. (Suikkanen 2003).

When VP1/VP2 VLPs and VP2 VLPs with recombinant VP1 present were used in feeding studies similar endocytic trafficking events were seen (IV). In both cases the VLPs were found to traffick identically to early endosomes, reach the late endosomal and lysosomal compartments. This entry behavior of the VLPs when associated with VP1 in the capsid, or just as recombinant VP1, were similar to the entry route of empty capsids of CPV (Suikkanen 2002). There is a mark difference in the entry of recombinant VLPs, including combinations with VP1, when compared to wt parvoviruses in that recombinant VLPs are not sequestering in the nucleus at 5.5 h p.f.

It has been noted that endosomal entry triggers membrane penetration that could include structural changes in the viral proteins induced changes by endosome's low pH (Skehel et al. 1995), or cleavage of viral proteins by the activities of low-pH-dependent endosomal proteases (Ebert et al. 2002), and for parvoviruses some of these changes have been seen (Maroto et al. 2000). During the endosomal transport of parvoviruses, partial decapsidation can occur through acidification-dependent proteolytic cleavage of VP2, which consequently causes conformational alterations in the capsid (Ros et al. 2002, Ros & Kempf 2004). In addition, the unique VP1 N-terminal domain, which has a number of potential nuclear localization signals, becomes exposed outside of the spherical capsid and is essential for infectivity given that deletions of this area leads to non-infectious viruses (Tullis et al. 1993, Cotmore et al. 1999, Vihinen-Ranta et al. 1997). In addition, exposure of this unique region is also crucial for productive infection, since it has a novel viral phospholipase A_2 (PLA2) activity (Zadori et al. 2001, Dorsch et al. 2002, Bleker et al. 2005) that plays a role in capsid release from vesicles (Zadori et al. 2001, Suikkanen et al. 2003). In our study VP2 VLPs assembled with VP1 or co-fed with VP1 could be released from the lysosomal compartments. This supports the claim that capsids are most likely released into the cytoplasm from a lysosomal vesicle at the perinuclear location (Suikkanen et al. 2003). The mechanism of escape from endocytic vesicles into the cytosol is still unknown; however, PLA2 activity and other factors have been seen to aid in this step (Parker & Parrish 2000, Suikkanen et al. 2003).

There seems to be a relative increase in nuclear targeting of VP2 when VP1 is present. VP1 could facilitate movement of VP2 VLPs from the lysosomes (IV). Indeed, recombinant VP1 was shown to have PLA2 activity (IV). There seems to be further processing in the cytoplasm before transportation to the nucleus due to the fact that fed VP2 VLPs could not be seen in the nucleus where as VP1/VP2 VLPS could, but even in the presence of VP1, VLPs still show no nuclear sequestering (IV). This could be explained if the N-terminus of VP1 in the VP1/VP2 VLPs were exposed on the surface as seen with human parvovirus B19 chimer VLPs with fusion proteins of VP1 when produced in insect cells (Miyamura 1994). In contrast, when truncated versions of VP1 were fused to poly-his-tags the N-terminal portion of the VP1 protein remained interior to the capsid (Wong et al. 1994). Nevertheless it has been recognized

that CPV and minute virus of mouse interacts with proteasomes after escape from vesicles by 9 h post-infection (p.i.) and most of the viral particles have interacted with cellular proteasomes (Ros et al. 2002, Ros & Kempf 2004). It seems that for some parvoviruses the ubiquitin-proteasome pathway plays an essential role in its life cycle, probably assisting at the stages of capsid disassembly and/or nuclear translocation. We have suggested that even though the presence of VP1 allows lysosomal escape, nuclear sequestering could only be accomplished if other capsid processing occurs or other viral components of a wt infection are present. Together these results indicated that VP1 allowed release of recombinant CPV proteins from the lysosomes. Due to the phospholipase A_2 activity of VP1, there is potential in using these proteins along with other proteins in the release from lysosomes into the cytosol for further trafficking within the cell.

7 CONCLUSIONS

The main conclusions of this thesis are:

1. The fluorescent partner, EGFP, was successfully displayed on the surface of CPV and B19 VLPs. (I, II)

2. The recombinant VP2, VP1/VP2 and EGFP-VP2 VLPs of CPV and EGFP-VP2 VLPs B19 were useful biotechnological tools to study binding, entry and trafficking in parvovirus permissive and non-permissive cell lines. (I, II, IV)

3. Recombinant baculoviruses hosting the structural proteins of CPV with or without their fluorescent partner allow investigation of trafficking events and nuclear targeting of the structural proteins in transduced mammalian cells. (III)

4. Recombinant CPV VP1 PLA_2-activity was observed. (IV)

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