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Endocytic Entry and Nuclear Import

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UNIVERSITY OF JYVÄSKYLÄ

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Law of Nature

Little by little and day by day
The Universe fritters itself away
As gases mix, as starlight wanes,
till randomness alone remains.

As rivers flow, as nations meet,
All order slowly turns to heat.
You can´t escape the great decline
Disorder is the worlds´s design.

So who am I to buck the odds
And flout disorder-loving Gods?
It´s for this reason, I confess,
I always leave my desk in mess.

Michael Cowan

To seek
To find

ABSTRACT

Vihinen-Ranta, Maija
Canine parvovirus: endocytic entry and nuclear import
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Yhteenveto: Koiran parvovirus: endosyyttinen sisääntulo ja tumakuljetus
Diss.

Canine parvovirus (CPV), a nonenveloped DNA virus, emerged in 1978 as a new virus infecting dogs. The early phase of CPV life cycle involves a series of sequential events starting with the attachment of virion to receptors on the cell surface. Then, viruses are internalized by pH dependent endocytic pathway. Upon escape of the virus from endosomes into cytoplasm, the viral DNA and associated proteins are transported to the nucleus, where viral transcription and replication occurs. For assembly of new virions, viral proteins enter the nucleus. At present, relatively little is known about the nuclear targeting signals of parvoviral proteins.

The present study was designed to investigate the detection of CPV and closely related parvoviruses with antibodies to synthetic peptides, to examine the mechanism by which CPV enters canine fibroma cells and to elucidate the mechanism of the nuclear transport of CPV proteins during the CPV infection.

Our main findings were: first, it was evident that sequences derived from highly conserved VP2 and NS1 regions of CPV elicited antibodies which can be used in the detection of CPV and some related parvoviruses. Second, CPV entered a host cell via an endocytic route and microtubule-dependent delivery of CPV to late endosomes was required for productive infection. Low pH-treated CPV particles injected directly into the cytoplasm, thus avoiding the endocytic pathway, were unable to initiate progeny virus production, showing that factors of the endocytic route other than low pH were necessary for the initiation of infection by CPV. Third, the N-terminal region of the VP1 capsid protein contains a potential nuclear localization signal (NLS), which is sufficient alone to localize a carrier protein into the nucleus. A cluster of basic residues was important for localization activity.

Keywords: Canine parvovirus; endocytic pathway; nuclear import; nuclear localization signal.

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CONTENTS

List of original publications

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

I Vihinen-Ranta, M., Lindfors, E., Heiska, L., Veijalainen, P. & Vuento, M. 1996. Detection of canine parvovirus antigens with antibodies to synthetic peptides. Arch. Virol. 141: 1741-1748. https://doi.org/10.1007/BF01718296

II Vihinen-Ranta, M., Kakkola, L., Kalela, A., Vilja, P. & Vuento, M. 1997. Characterization of a nuclear localization signal of canine parvovirus capsid proteins. Eur. J. Biochem. 250: 389-394. https://doi.org/10.1111/j.1432-1033.1997.0389a.x

III Vihinen-Ranta, M., Kalela, A., Mäkinen, P., Kakkola, L., Marjomäki, V. & Vuento, M. 1998. Intracellular route of canine parvovirus entry. J. Virol. 72: 802-806. https://doi.org/10.1128/JVI.72.1.802-806.1998

Abbreviations

aa	amino acids
AAV	adeno-associated virus
B19	human parvovirus B19
BFPV	blue Fox parvovirus
BSA	bovine serum albumin
CPV	canine parvovirus
DNA	deoxyribonucleic acid
dsDNA	double-strand DNA
ECV	endosomal carrier vesicles
FPV	feline panleucopenia virus
GDP	guanosine diphosphate
GTP	guanosine triphosphate
hnRNP	heterogenous nuclear ribonucleoproteins
HIV-1	human immunodeficiency virus type1
IBB	importin-β-binding
ICTV	international Committee of Taxonomy of Viruses
IgG	immunoglobulin G
KDa	kilodalton
MBS	*m*-maleimidobenzoyl-N- hydroxysuccinimide ester
MEV	mink enteritis virus
mRNA	messenger RNAs
MVM	minute virus of mice
MuLV	Moloney murine leukemia virus
NE	nuclear envelope
NLS	nuclear localization signal
NPC	nuclear pore complex
NS	nonstructural proteins
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
Ran-GDP	GDP-bound form of Ran
Ran-GTP	GTP-bound form of Ran

RDPV	raccoon dog parvovirus
RNA	ribonucleic acid
RNP	ribonucleoproteins
RPV	raccoon parvovirus
SFV	Semliki Forest virus
snRNA	small nuclear RNA
SV40	simian virus 40
TGN	trans Golgi network
tRNA	transfer RNA
VP	viral capsid protein
WGA	wheat germ agglutinin

Responsibilities of Maija Vihinen-Ranta in the articles of this thesis

While appreciating highly the cooperation with my group I am mainly responsible for planning, producing and writing of these original publications with following exceptions:

Article I: The Immunofluorescence microscopical work was done mainly by M.Sc. Erja Lindfors in National Veterinary and Food Research Institute, Helsinki and the the article was written together with professor Matti Vuento.

Article 2: The peptides were synthesized by Dr. Pekka Vilja from Medical school, Tampere.

Article 3: Work involving the effect of reduced temperature on CPV infection was mainly done by M.Sc. Anne Kalela.

1 INTRODUCTION

CPV is a member of the feline parvovirus subgroup which includes several host range variants infecting members of many different carnivore families. There is high DNA sequence homology (›98 %) and antigenic similarity between these host range variants.

Understanding of the infectious entry pathway of viruses has increased due to extensive research efforts during the last few years. Although nonenveloped and enveloped viruses penetrate into cells by using different mechanisms, both groups can exploit the same cellular properties to facilitate their entry. For example, many enveloped and nonenveloped viruses use the acidic conditions in endosomes and lysosomes to drive the reactions that lead to penetration. Compared to enveloped viruses, less is known about the penetration and uncoating of nonenveloped viruses like parvoviruses. For most parvoviruses the early steps of viral entry into host cell are far from understood. For CPV, it is known however, that uptake of virus occurs by endocytosis and requires passage of the virus through an acidic intracellular compartment. At present, the mechanism involved in the release of the virus from endosomal vesicles is still unknown. After crossing cellular membranes some viruses like parvoviruses have to deliver their genomes to the nucleus, in a (at least partially) uncoated form, for replication to occur. Nuclear import of the viral genome and possible associated proteins plays a central role in replication. For assembly of new virions, capsid proteins enter the nucleus through nuclear pores. Although the trafficking of individual virus proteins into the nucleus has been studied for some virus systems, the mechanism of nuclear transport of parvoviral genome and proteins has only recently become a subject of research.

In this study, we have set up a method for detection of CPV antigens by using antibodies to synthetic peptides. We also wanted to raise peptide antibodies cross-reactive with other members of the feline parvovirus

subgroup. To this end, we used peptides mimicking sequences from areas of VP2 and NS1 proteins of CPV known to be highly conserved in this group of closely related parvoviruses. The aim of our study was also to verify the importance of microtubule-linked endosomal membrane traffic and the role of CPV passage through late endosomes in CPV productive infection. Finally, we wanted to elucidate the mechanism of the nuclear transport of CPV proteins by studying the specific sequence motifs that constitute their nuclear localization signals.

2 REVIEW OF THE LITERATURE

2.1 Parvoviridae

2.1.1 Taxonomy

The parvoviruses are among the smallest of the DNA animal viruses. They are nonenveloped viruses with icosahedral symmetry and a linear single-stranded DNA genome. Parvoviruses have been isolated from animals or animal tissues, both vertebrates and invertebrates. The family *Parvoviridae* contains two subfamilies: the *Parvovirinae* and *Densovirinae*. The subfamily *Parvovirinae*, which naturally infects vertebrates is divided into three genera: *Parvovirus*, *Erythrovirus* and *Dependovirus*. *Dependoviruses*, also known as adeno-associated viruses (AAV), depend generally upon adenovirus or herpesvirus coinfection for replication. In contrast, members of the autonomous parvovirus genera, *Parvovirus* and *Erythrovirus*, are capable of productive replication without the aid of a helper virus (Table 1). The subfamily *Densovirinae*, which infects insects, contains three genera: *Densovirus*, *Iteravirus* and *Contravirus* (Berns 1996, Cotmore & Tattersall 1987, Siegl et al. 1985).

2.1.2 The virion

The parvoviral particles are 18-26 nm in diameter and have a molecular weight of 5.5-6.2 x 10^6 units. Because of their high DNA/protein ratio and absence of lipids, the intact virions have a relatively high (1.39-1.42 g/cm^3) buoyant density. Empty capsids and mixed population of variant particles, containing no genome or an incomplete DNA genome, have buoyant density of 1,30 to 1,37 g/cm^3. The sedimentation coefficient of the virion in neutral sucrose

gradient is 110 to 122S for full and 70S for empty particles (Arella et al. 1990, Berns 1996).

The capsid is composed of only three structural proteins, VP1, VP2 and VP3. VP2 is the major structural protein, composing approximately 90 % of the capsid. Virions lack lipids and carbohydrates (Berns 1996). The atomic 3-D structure of CPV and FPV have been determined (see below). One of the characteristics of *parvoviridae* is their extreme resistence to physical inactivation. Virions are stable between pH 3 to 9 and at 56°C for 60 min and can be inactivated by treatment with formalin, β-propriolactone, hydroxylamine, and oxidizing agents (Arella et al. 1990, Berns 1996).

TABLE 1 Selected autonomous parvoviruses of vertebrates and their primary host species (Berns 1996, Berns et al. 1995, Chapman & Rossmann 1993).

	Virus	Abbreviation	Host
Genus *Parvovirus*			
	Aleutian mink disease virus	ADV	mink
	Blue Fox parvovirus	BFPV	blue fox
	Bovine parvovirus	BPV	cattle
	Canine parvovirus	CPV	dog
	Chicken parvovirus	ChPV	chicken
	Feline panleucopenia virus	FPV	cat
	Goose parvovirus	GPV	geese
	HB virus	HB	human?
	H-1 parvovirus	H-1	rodents, human?
	Kilham rat	RV(KRV)	rat
	Lapine parvovirus	LPV	rabbits
	LuIII	LuIII	unknown
	Mink enteritis virus	MEV	mink
	Minute virus of canines	MVC	dog
	Minute virus of mice	MVM	mice
	Porcine parvovirus	PPV	pig
	Raccoon dog parvovirus	RDPV	raccoon dog
	Raccoon parvovirus	RPV	raccoon
	Rat parvovirus	RT	rats
	Tumor virus X	TVX	human?
Genus *Erythrovirus*			
	Human parvovirus B19	B19	human

2.1.3 The genome organization

Parvoviruses contain a linear single stranded DNA genome on the order of 5000 nucleotides. Parvoviruses of all genera may pack either a negative- or a positive- stranded DNA. In general, most autonomous parvoviruses encapsidate primarily strands of one polarity, while AAV package strands of both polarities with equal frequency (Berns 1996, Berns & Adler 1972). Most

members of the genus *parvovirus* preferentially pack negative-stranded DNA (Cotmore & Tattersall 1987).

Characteristic to parvoviruses are the terminal palindromic repeat sequences at both ends of the ssDNA genome. DNA replication occurs via a double-stranded replicative form, initiated by a self-priming mechanism via the 3´terminal palindromic sequence (Astell 1990, Berns 1996, Cotmore et al. 1993, Cotmore & Tattersall 1994).

Parvoviruses have relatively small genomes, but use their single-stranded DNA with great economy. They encode multiple structural and nonstructural proteins using partially overlapping DNA sequences and alternative open reading frames. The autonomous parvovirus genome contains two large open reading frames (ORFs), that do not overlap, which together span almost the entire genome, and a number of small ORFs, the exact size and location of which vary somewhat between members. In each case the long, left-hand ORF is known to encode a major, nonstructural protein, while the right-hand ORF provides the sequences expressed in the various capsid polypeptides (Berns 1996, Cotmore 1990, Cotmore & Tattersall 1987). The proposed genome organizations of MVM and CPV are shown in Fig.1 (Cotmore & Tattersall 1987, Reed et al. 1988).

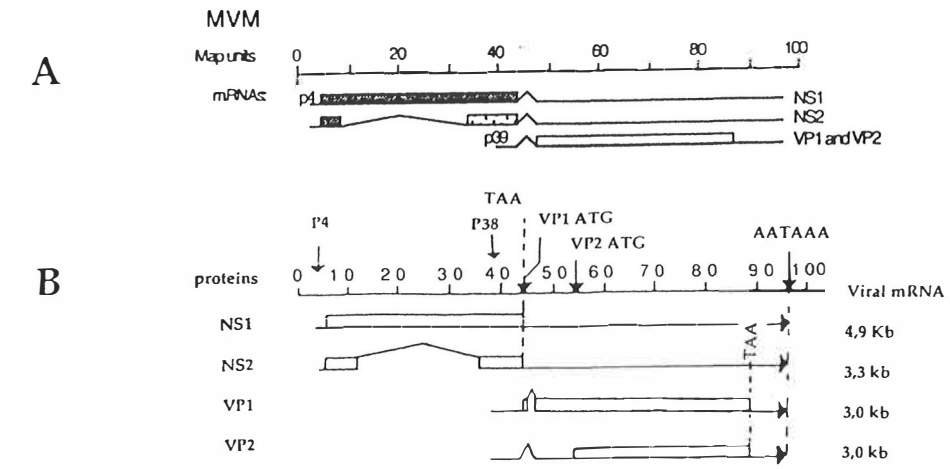

FIGURE 1 Coding strategy of the minute virus of mice (MVM, A) and the canine parvovirus aligned with the viral DNA strand (orientation 3´ to 5´); straight lines are exons and sloped lines are introns. Boxes indicate proteins. Respective promoters are also indicated. Major blocks of open reading frame are shown for MVM in each of the three reading frames; open box: ORF 1, dotted box: ORF 2, and gray box: ORF 3 (A). The CPV genome contains two large ORFs. The first covers much of the left half of the genome and encodes two nonstructural proteins (NS1 and NS2). The second large ORF occupies much of the right half of the genome and encodes the structural proteins (VP1 and VP2). Proteins VP1 and VP2 are derived from the same gene by differential splicing. The major CPV xmRNAs and sizes are indicated on the right in kilobases, while the proposed protein each encodes are presented on the left (B). (Berns 1990, Cotmore & Tattersall 1987, Reed et al. 1988).

2.1.4 Proteins

Depending on the particular parvovirus, the mature virion consists of structural proteins of two to three species (Chapman & Rossmann 1993). Both autonomous parvoviruses and AAV contain three capsid proteins named VP1, VP2 and VP3 (with exceptions of human parvovirus B19 and Aleutian disease virus ADV, which contain only two coat proteins). Two of these proteins VP1 (80-86 kDa) and VP2 (64-75kDa), appear to be primary translation products, while the third, VP3 (60-62 kDa), is derived by the proteolytic cleavage of 15 to 20 amino acids from the amino terminus of VP2. The VP1 and VP2 are formed by alternative splicing of the messenger RNA from the viral DNA (Cotmore & Tattersall 1987, Reed et al. 1988). Purified empty particles (without DNA) contain only the primary translation products VP1 and VP2. Infectious virus particles contain also VP3 suggesting that in full capsids the N terminus of VP2 is exposed on the virion surface and is proteolytically cleaved to the VP3 (Berns 1996, Cotmore & Tattersall 1987, Iversen & Rhode 1990).

Parvoviruses have two different types of nonstructural proteins, NS1 and NS2. Nonstructural proteins are phosphorylated and show localization in infected cells that is mainly nuclear (NS1) (Nüesch & Tattersall 1993) or both cytoplasmic and nuclear (NS2) (Cotmore & Tattersall 1990). The large nuclear phosphoprotein NS1 is absolutely required for productive replication in all cell types (Naeger et al. 1990). NS1 of MVM (in cooperation with cellular site-specific DNA-binding proteins), mediates the nicking event and the initiation of replication at the MVM 3′ origin sequence, followed by the establishment and maintenance of a unidirectional and highly processive replication fork (Christensen et al. 1995, Christensen et al. 1997, Cotmore & Tattersall 1994, Iversen & Rhode 1990, Pujol et al. 1997). Moreover, NS proteins of MVM have proved to be cytotoxic (cell killing), especially for transformed cells (Mousset et al. 1994). The cytotoxity of the NS1 of MVM is related to changes in the synthesis and phosphorylation of cell proteins (Anouja et al. 1997). The other nonstructural protein, NS2, seems to be required in a host-range-dependent manner, both in tissue culture and in vivo, for efficient viral DNA replication and virus production (Naeger et al. 1990, Naeger et al. 1993). NS2 has been found to be important for the correct assembly of the MVM capsid proteins. MVM mutants, which do not encode full-length NS2 polypeptides, showed a defect in virion assembly, but not in capsid protein synthesis (Cotmore et al. 1997, Wang et al. 1998). In contrast to the rodent parvoviruses, the effect of the NS2 of CPV (predicted molecular weight is 19 kDa) onvirus capsid assembly or DNA replication is relatively subtle (Wang et al. 1998).

2.1.5 Viral life cycle

Parvoviruses replicate only in dividing cells and hence have a predilection for either young or unborn animals or tissues of older animals that contain proliferating cells (Tattersall 1972). Viral replication requires host cell functions of the late S phase of the cell division cycle, indicating close association between host and viral DNA synthesis, probably involving host DNA polymerase(s) (Durham & Johnson 1985, Fenner et al. 1987).

Productive parvoviral infection is initiated by adsorbtion of the virion to specific cell surface receptors. For most parvoviruses these receptors are still uncharacterized, but N-acetylneuraminic acid residues are known to play an important role in the attachment of MVM (Cotmore & Tattersall 1987). It is also proposed that a 40- to 42-kDa glycoprotein represents a specific attachment molecule for CPV in a canine fibroma cell line A72 (Basak et al. 1994). The cell surface binding of MVM is followed by internalization, probably through coated pits (Cotmore & Tattersall 1987) whereas the uptake of CPV has been suggested to be mediated mainly by small uncoated vesicles (Basak & Turner 1992). For most parvoviruses the early steps of viral entry are far from understood. For CPV, however, uptake of virus has been shown to occur by endocytosis and to require passage of the virus through an acidic intracellular compartment (Basak & Turner 1992). At present, the mechanism involved in the release of the virus from endosomal vesicles is still unknown. After crossing cellular membranes parvoviruses have to deliver their genomes to the nucleus for replication, in (at least partially) uncoated form. Since the incoming capsid is believed to be involved in the initiation of viral gene expression, the final uncoating step has been suggested to take place in the nucleus (Cotmore & Tattersall 1987). The mechanism of import of (possibly partially uncoated) virions to the nucleus remains to be determined. However, a basic, lysine-rich sequence similar to the SV 40 large T antigen nuclear localization signal has been found at the VP1 amino terminus of MVM, CPV, and FPV (Cotmore & Tattersall 1987). The role of this potential nuclear localization signal in the viral entry route remains to be investigated.

Parvoviral DNA replication, gene expression and assembly take place in the nuclei of rapidly dividing cells which are required to go through the S phase (Tattersall 1972). The first event in viral DNA replication is the conversion of the single-stranded genome into double-stranded DNA followed by amplification of these duplex DNA forms (Rhode III 1974). DNA synthesis derives from a self-priming mechanism involving palindromic terminal sequences, which can fold to hairpin structures and function as primers in DNA replication (Berns 1990, Cotmore et al. 1993, Cotmore & Tattersall 1987, Cotmore & Tattersall 1994). The parental DNA sequence is transferred to progeny genomes according to the modified rolling hairpin model (Astell 1990, Astell et al. 1985, Cotmore & Tattersall 1992, Tattersall & Ward 1976). The first transcripts represent regulatory proteins (one or two NS proteins), which affect viral gene expression. The genes for capsid proteins are transcripted later (Berns 1990). Cytoplasmically synthesized capsid proteins accumulate in the nucleus of the infected cell where packing of single-stranded progeny DNA takes place. Relatively few of the preformed viral capsids pack DNA and mature into infectious virus particles. Eventually a mixture of empty capsids and full virions is released from the cell, after the degeneration or apoptosis (Richards et al. 1977). A variety of viruses are known to be cytotoxic for their host cells and part of these viruses are able induce an apoptosis in host cells. Human parvovirus B19 NS1 protein have been shown to be able induce apoptosis in erythroid lineage cells (Moffatt et al. 1998).

2.2 Genus Parvovirus

Members of the genus parvovirus replicate selectively in the nuclei of rapidly proliferating cells. For some members of the genus, mature virions contain negative-stranded DNA of 5 kb. In other members, positive-strand DNA occurs in variable proportions (1-50%). Many members hemagglutinate red blood cells of one or more species (Berns 1996) .

The parvoviruses responsible for infections in cat (FPV), blue fox (BFPV), dog (CPV), mink (MEV), raccoon (RPV) and raccoon dog (RDPV) are closely related (Parrish et al. 1988, Parrish et al. 1985, Truyen et al. 1995, Veijalainen 1988). FPV, MEV, and CPV are classified as the feline parvovirus subgroup of the genus *parvovirus* (Siegl et al. 1985). The nucleotide sequences of the capsid protein genes of CPV and FPV are greater than 98 % homologous (Parrish 1991, Reed et al. 1988, Rhode III 1985). However, both viruses can be distinguished by a number of biological properties, such as host range *in vitro* and *in vivo*, antigenicity, and by the conditions required for hemagglutination (Chang et al. 1992, Parrish 1991, Parrish et al. 1988, Tresnan et al. 1995).

2.2.1 Canine parvovirus

Canine parvovirus, a member of the feline parvovirus subgroup, is an example of the emergence of a new viral pathogen. The first evidence of canine enteritis caused by CPV was seen in sera collected in 1976 in Belgium and it appears that the virus became globally distributed within a year of its first recognition (Parrish 1990, Parrish et al. 1988). CPV-like viruses are now endemic in most populations of domestic and wild canids that have been examined (Parrish et al. 1991). The origin of CPV is unknown. Extensive antigenic and genomic similarities exist between CPV and FPV/MEV. It is possible that CPV arose by mutation from one of these viruses or from another closely related parvovirus. The exceptionally rapid spread of CPV has caused some researchers to hypothesize that CPV arose as a mutant from FPV vaccine viruses (Pollock & Carmichael 1990). Parrish and co-workers have recently proposed that parvoviruses from wild carnivores rather than modified live virus vaccine have been involved in the emergence of canine parvovirus (Truyen et al. 1997).

2.2.1.1 Structure

The infectious CPV virion is a 26 nm-diameter particle of icosahedral symmetry, made up of a single-stranded DNA genome (5 323 nucleotides) of negative polarity and 60 protein subunits that are a combination of VP2, VP3 and, some VP1 (Cotmore & Tattersall 1987, Reed, et al. 1988). Structural proteins VP1 and VP2 have apparent sizes of 82,3K and 67,3K. Third structural protein (VP3), derived by the proteolytic cleavage of VP2 of infectious (DNA-containing) virions has a size of 63,5K (Cotmore & Tattersall 1987). Propagation of the virus *in vitro* or *in vivo* generally produces about half of empty particles,

which contain no VP3, mostly VP2, and a few VP1 subunits. Empty capsids are morphologically similar to infective virus particles (Wu & Rossmann 1993).

The three-dimensional atomic structure of canine parvovirus has been determined by X-ray crystallography (Tsao et al. 1991, Tsao et al. 1992, Wu & Rossmann 1993). The structure of the capsid-forming subunits comprises an eight-stranded antiparallel β–barrel which has four extensive loops connecting the β-strands. Five such β–barrels in each fivefold axis are arranged together forming hollow cylindrical structures. The connecting loops form most of the capsid outer surface, including a prominent protrusion, "spike", at the threefold axis of symmetry; a circular depression, "canyon", surrounding the fivefold axis of symmetry; and a depression, "dimple", spanning between spikes at the twofold axis of symmetry (Tsao et al. 1991). The DNA encapsidation is associated with a conformational change of CPV capsids, exposing the N-terminal cryptic sites of VP2 to proteases (Tattersall & Cotmore 1988). The basic amino termini of VP1 are proposed to be associated with the nucleic acid (Tsao et al. 1991).

2.2.1.2 Antigenic structure

Two antigenic variants of the original CPV have been recognized since 1987. The newer virus strains (termed CPV type 2a and CPV type 2b) have completely or partially replaced the original CPV type 2 (Parrish 1990, Parrish, et al. 1988, Parrish et al. 1991). Two major antigenic determinants of CPV defined by escape mutant analysis are in the protruding region of the capsid, the threefold spike (Strassheim et al. 1994). Ten antigenic sites in the VP2 subunit of CPV have been defined using epitope-mapping analysis with peptides. Six of the 10 sites have residues on the viral surface at highly protruding locations, and others also contained surface-exposed regions (Langeveld et al. 1993). The amino terminus of VP2 was shown to be an immunogenic domain which is exposed at the viral surface and can elicit neutralizing antibodies, which makes this region of special interest for development of vaccines (Langeveld et al. 1993, Langeveld et al. 1995).

2.2.1.3 Host range

The host range of virus defines both the kinds of tissues, cells and the animal species that it can infect and in which it can multiply. The host range of CPV is quite complex and there are differences in the ability of CPV or its subtypes to replicate *in vivo* and in *vitro*. CPV can replicate in both canine and feline cells in cultures but not in cats (Truyen et al. 1996, Truyen & Parrish 1992). The major host determinants have been located into three regions of the surface of the capsid. There are two or three residues which differ between CPV and FPV and are particularly important for the host range. These sequences control host range, antigenicity and also the pH dependence of hemagglutination differences between the two viruses (Agbandje et al. 1993, Chang et al. 1995, Horiuchi et al. 1992, Parker & Parrish 1997, Parrish 1991, Truyen et al. 1994).

2.2.1.4 Clinical manifestations

The manifestations of CPV infection in dogs range from subclinical to acute and fulminating. The pathogenesis of diseases in dogs of various ages is influenced primarily by the requirement of CPV for actively dividing cells. For example, the lymphoid and intestinal epithelial tissues, which contain rapidly dividing cell populations, are primary targets for virus replication. Especially in young and newborn pups the infection is severe and may lead to death. Many infections in dogs older than 6 weeks are mild or subclinical (Macartney et al. 1984, Meunier et al. 1985, Parrish 1995, Pollock & Carmichael 1982, Pollock & Carmichael 1990, Robinson et al. 1980). Clinical CPV disease in dogs usually takes one of two forms: enteritis or myocarditis. The enteric form of the disease occurs in dogs of all ages, but severe illness is encountered more often in pups. Symptoms like vomiting and diarrhea are believed to result from destruction of normal intestinal architecture and function. (Fenner et al. 1987, Parrish 1990). Acute myocarditis, on the other hand, is believed to occur exclusively in puppies (less than 3 months old), although older animals may develop cardiomyopathy and congestive heart failure as a sequela to neonatal infection. Clinical signs of acute myocarditis appear suddenly and progress rapidly and can cause death as a result of heart failure (Macartney et al. 1984, Meunier et al. 1985, Parrish 1995, Pollock & Carmichael 1982, Pollock & Carmichael 1990, Robinson et al. 1980).

Vaccines against CPV have been developed from attenuated live CPV or FPV strains, or from inactivated CPV or FPV (Pollock & Carmichael 1982, Saliki et al. 1992), and from synthetic peptides and recombinant proteins (Langeveld et al. 1995). A limitation of the attenuated and inactivated vaccines is that in pups (up to 18 weeks of age), have still maternally derived antibodies which disturb the development of protective immunity (Pollock & Carmichael 1982, Pollock & Carmichael 1990). For such cases, synthetic or recombinant protein vaccines might present a preferable alternative (Langeveld et al. 1993).

The first synthetic vaccine against CPV based on peptides was described in 1994. This synthetic vaccine was based on residues from antigenic sites located on the N-terminal domain of VP2 (Casal et al. 1995, Langeveld et al. 1993, Langeveld et al. 1994).

2.3 Infectious entry of animal viruses

Before viruses can replicate in the host cell they must first gain entry to the target cell. To begin a successful infection, viruses must first cross the host cell plasma membrane barrier and they must target their genome and accessory proteins to the right organelle. Finally, before the genome of the incoming virus can replicate viruses has to undergo uncoating, a process causing the release of viral genome from its condensed transport configuration.

Viruses use two general pathways to cross the host cell plasma membrane: (I) surface fusion between the viral lipid envelope and the plasma membrane and (II) receptor mediated endocytosis (Fig. 2). After crossing

plasma membrane via endocytic pathway the release of viruses from endosomes to cytoplasm is supposed to be triggered by the acidic pH existing in endosomal vesicles (Lanzrein et al. 1994, Marsh 1984, Marsh & Helenius 1989, Marsh & Pelchen-Matthews 1993). Viral uncoating consist of the steps of disassembly involving removal of the envelope and removal of the protein capsid. Uncoating events can occur at several sites in the cell, ranging from the cell surface to the nuclear matrix (Greber et al. 1993, Greber et al. 1994, Helenius 1992, Knipe 1996).

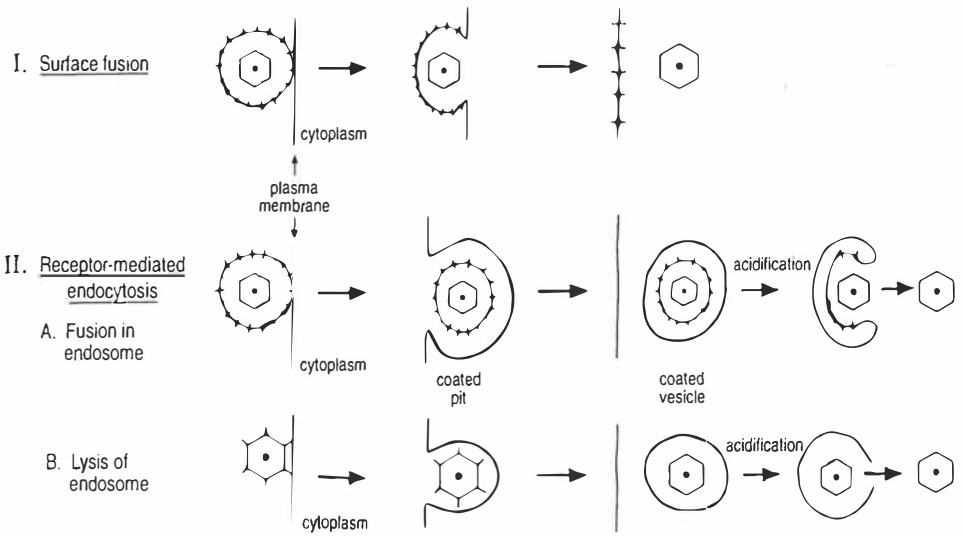

FIGURE 2 Pathways for viral entry of the host cell (adapted from Knipe, 1995).

2.3.1 Endocytosis

Endocytosis is an important internalization process by which cells obtain extracellular molecules. Endocytosis is involved in uptake of extracellular nutrients, regulation of cell-surface receptor expression, and many other physiologic processes. Mammalian cells have evolved a variety of mechanisms to internalize molecules and particles, including phagocytosis ("cell eating"), pinocytosis ("cell drinking"), clathrin-dependent receptor-mediated endocytosis (Fig. 3), and clathrin-independent endocytosis (Mukherjee et al. 1997, Robinson et al. 1996). Furthermore, many viruses, microorganism, and toxins gain their entry into the cell by endocytosis.

Receptor-mediated endocytosis enables selective uptake of macromolecules by the cell (Fig. 3). After internalization via receptor-mediated endocytic pathway, the most common fates of molecules are degradation in the lysosomal pathway or recycling back to the cell surface. The process begins with receptors and their ligands selectively concentrating in clathrin-coated pits on the plasma membrane, after which they are internalized and delivered

to endosomes, a group of functionally diverse vesicles. In parallel with clathrin-mediated endocytosis the nonclathrin-mediated pathway also occurs; it is supposed that caveolae may be responsible for at least some of the latter pathway (De Tulleo & Kirchhausen 1998). Clathrin-coated vesicles lose their clathrin prior to fusion with the sorting endosomes (a subgroup of the early endosomes). Sorting and targeting of endocytosed solute and receptor-bound ligands to other intracellular destinations, including the recycling pathway and the degradative lysosomal compartment, takes place in sorting endosomes (Gruenberg et al. 1989, Gruenberg & Howell 1989). Acidification (pH 6,2) of the sorting endosomes (pH 6,5) is achieved by the activity of the vacuolar (V-type) proton ATPase (Al-Awqati 1986, Nelson & Taiz 1989). Many receptors and ligands dissociate from each other upon exposure to the acidic pH. Receptors which enter the recycling pathway continually recycle from the cell surface to the endosomes and back (Kishimoto et al. 1987, Miettinen et al. 1989, Stoorvogel et al. 1987). In contrast, most of the contents of the sorting endosomes (including released ligands and some receptor-ligand pairs, are destined for degradation in late endosomes or lysosomes.

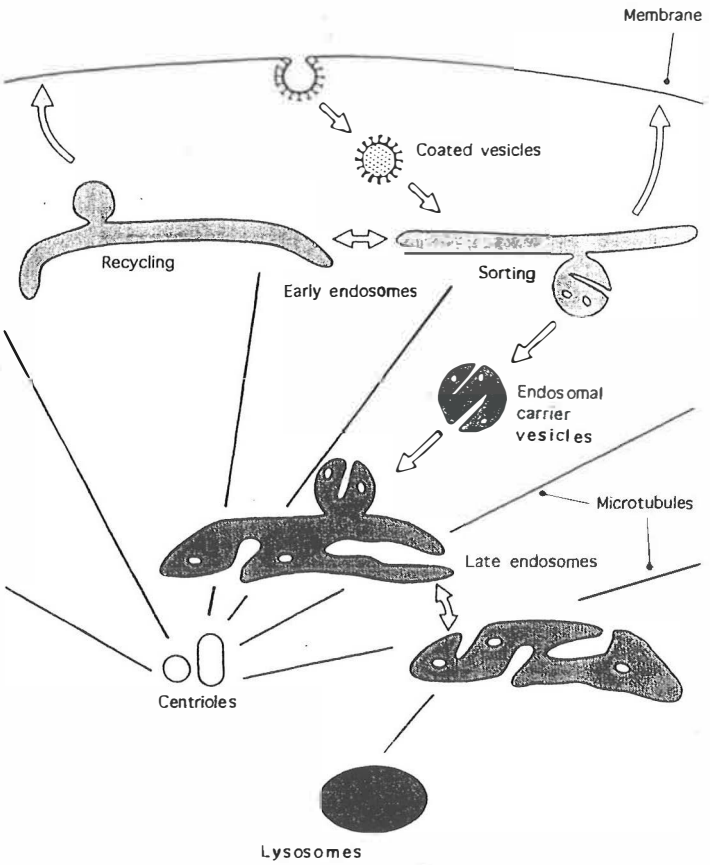

FIGURE 3 The major steps of clathrin-mediated endosomal membrane transport. Routes of membrane transport between endosomes and the trans-Golgi network, as well as other possible recycling routes, have not been included (modified from Gruenberg, 1995).

From early endosomes the internalized molecules can proceed to further stations along the endocytic pathway via transport intermediates. These structures, termed endosomal carrier vesicles (ECVs) (pH ≈5.5), can fuse with late endosomes in a microtubule-dependent fashion (Aniento et al. 1993, Aniento & Gruenberg 1995, Gruenberg et al. 1989). Microtubules play an important role in membrane traffic, acting as a track along which carrier vesicles can move from peripherally localized early endosomes to perinuclear late endosomes (pH ≈5.5) (Bomsel et al. 1990, Pierre et al. 1992). Altogether, transport processes between the early and late endosomes via endosomal carrier vesicles depends on an intact microtubule network, microtubule-associated proteins, and on cytoplasmic dynein (Aniento et al. 1993).

The rab proteins, a family of low-molecular weight GTPases, have been implicated in nearly all types of membrane trafficking like vesicle budding, docking, and fusion (Pfeffer 1994). Because mutations of rab proteins can have drastic consequences in terms of blocking protein transport along a given route or actually changing the sizes of entire organelles, it appears that rab proteins play a key regulatory role in membrane trafficking. The precise mechanism is unknown, but it is thought that these proteins ensure the specificity or directionality of vesicle budding or fusion events by coupling the completion of these reactions to the hydrolysis of bound GTP (Mukherjee et al. 1997, Pfeffer 1994). Different members of the rab family are associated with particular types of organelles. In the endocytic system, rab4 and rab5 are found primarily on early endosomes (Bucci et al. 1992, Gorvel et al. 1991, van der Sluijs et al. 1992). Rab7 and rab9 are primarily on late endosomes, with some rab9 being on the trans Golgi network (TGN) (Feng et al. 1995, Lombardi et al. 1993, Riederer et al. 1994).

Molecules destined to be degraded are routed from the late endosomes to lysosomes, which are acidic (pH ≈4.5) and rich in hydrolytic enzymes. Although the precise mode of delivery of material from the endosomes to the lysosomes is not well understood, it is supposed that endosomes either mature into lysosomes or fuse with pre-existing lysosomes (Griffiths & Gruenberg 1991, Gruenberg & Howell 1989, Mellman 1996). Microtubules have been shown to be involved in the meeting and fusion between late endocytic structures and lysosomes (Deng et al. 1991). In addition, evidence for bidirectional traffic of soluble material between lysosomes and late endosomes has been reported (Jahraus et al. 1994). Transport from late endosomes to lysosomes depends on the vacuolar proton pump. Selective inhibition of the vacuolar H^+ ATPase (bafilomycin A_1 and 3-methyladenine) prevents this transport (Punnonen et al. 1994, van Deurs et al. 1996).

2.3.2 Enveloped viruses

Enveloped viruses enter the host cell either by receptor-mediated endocytosis or by direct fusion with the host cell membrane (Kartenbeck et al. 1989, Marsh 1984, Marsh & Helenius 1989). These two different forms of virus-host cell interaction can be distinguished by their pH requirements (Table 2).

In the endocytic pathway viruses encounter an endosomal acidic pH (Gruenberg & Maxfield 1995, Marsh & Helenius 1989). The role of conformational changes which in the viral capsid proteins are induced either by interaction with the receptor and/or by the acidic endosomal enviroment is very important in the delivery of viral particles or genome from endosomes (Guinea & Carrasco 1995, Lanzrein et al. 1994, Marsh & Helenius 1989, Marsh & Pelchen-Matthews 1993). Viruses with dependence on low pH such as influenza (orthmyxoviruses) (Daniels et al. 1985, Guinea & Carrasco 1995) and Semliki Forest virus (SFV, alphaviruses) (Kielian & Helenius 1985, Marsh & Bron 1997, Wahlberg et al. 1992) show sharp pH profiles regarding fusion with endosomal membranes and their entry is inhibited by acidotropic weak bases and carboxylic ionophores (Marsh 1984). For these viruses the acidic pH appears to induce a conformational change in viral membrane glycoproteins, assumed to increase the interaction of the proteins with membrane. Thus, as a result of pH-induced alterations in the virus structure, the viral genome and other components inside the viral membrane are proposed to be released into the cytoplasm (Greber et al. 1994, Marsh 1984, Marsh & Pelchen-Matthews 1993). The group of enveloped viruses following an acid-dependent pathway includes the following families: toga, rhabdo, orthomyxo, baculo, and retro (Table 2) (Marsh 1984).

However, low pH is not always needed for the initiation of infection (Hagelstein et al. 1997, Kooi et al. 1991, Köck et al. 1996, Marsh & Pelchen-Matthews 1993, Pérez & Carrasco 1994, Rigg & H. Schaller 1992, Wittels & Spear 1990) and the role of endocytosis in the entry of pH independent viruses is less clear and somewhat controversial. Some pH-independent viruses like the herpes simplex virus (HSV) (Mellman 1996, Wittels & Spear 1990), human cytomegalovirus (Compton et al. 1992) and paramyxoviruses (Lamb 1993) appear to penetrate directly through the plasma membrane, while Epstein-Barr virus (EBV) (Miller & Hutt-Fletcher 1992) and human immunodeficiency virus type 1 (HIV-1) (Grewe et al. 1990, Pauza & Price 1988) appear to fuse with the plasma membrane in certain cells and to use the endocytic pathway in others. Finally, there is also evidence that some enveloped viruses, which are usually internalized by endocytosis, like influenza virus can fuse directly with the plasma membrane in an acidic culture medium (Guinea & Carrasco 1995, White 1990).

2.3.3 Nonenveloped viruses

Although the process of entry for several enveloped animal viruses is fairly well-known, much less is known about the mechanism by which nonenveloped viruses enter a host cell. Nonenveloped viruses cannot use membrane fusion since they lack the lipids and associated proteins of enveloped viruses. Rather, interactions of nonenveloped viruses with membranes may be expected to involve the readily exposed proteins of the outer capsid and the cellular lipids, membrane proteins, or both. Nonenveloped viruses are known to enter the cell through either a pH-dependent (picornaviruses, adenoviruses, reoviruses, parvoviruses) or independent (papovaviruses) endocytic pathway (Table 2) (Hagelstein et al. 1997, Neubauer et al. 1987, Prchla et al. 1994) and some use

direct penetration through the plasma membrane (rotaviruses) (Kaljot et al. 1988).

Adenoviruses, picornaviruses, reoviruses, and parvoviruses enter cells by receptor mediated endocytosis and encounter in endosomes an acidic pH, which is assumed to somehow trigger their penetration into the cytosol. Adenoviruses and parvoviruses deliver their genomes into the nucleus for replication, whereas picornaviruses and reoviruses replicate and assemble in the cytoplasm. Before replication, viruses or their genomes must penetrate membranes. This may be achieved in endocytic pathway either by disruption of the endosome (Greber et al. 1993) or through a pore formed by viral proteins (Marsh & Helenius 1989, Marsh & Pelchen-Matthews 1993, Prchla et al. 1995). Adenoviruses go through a stepwise uncoating process during the entry into cells and the penetration of destabilized viruses causes acid-triggered rupture of the early endosomes. The stepwise dismantling starts with the dissociation of adenovirus fibers during endocytic uptake and culminates in the release of the viral DNA into the nucleus through the nuclear pore complexes and dissociation of the capsid (Greber et al. 1993, Greber et al. 1996, Varga et al. 1991). In contrast to the membrane-disrupting mechanism of adenovirus, human rhinoviruses (picornavirus) are released from late endosomes through a specific pore forming mechanism. Uncoated rhinovirus RNA is passed through the endosomal membrane via a pore of limited size (Giranda et al. 1992, Neubauer et al. 1987, Prchla et al. 1994, Prchla et al. 1995). The mechanism of the penetration of the endosomal (or lysosomal) membrane barrier by reoviral or parvoviral particles or viral genome is poorly defined (Berns & Adler 1972, Cotmore & Tattersall 1987, Nibert et al. 1996).

The role of endocytosis in the entry of polyoma virus, a member of the papovavirus family, is unusual in several respects. In the early phase of viral entry these viruses, which belong to the group of acid-independent viruses, have been seen to be endocytosed into small tight-fitting uncoated vesicles (monopinocytotic vesicles) derived from the plasma membrane (Griffith et al. 1988, Kartenbeck et al. 1989), instead of clathrin-coated vesicles used by most viruses with endocytic entry pathway (Marsh & Helenius 1989). To infect a host cell, polyoma virus has to deliver its genome to the nucleus. It has been proposed that virions enter the nuclei via direct fusion of monopinocytotic (virus-containing) vesicles with the host cell nuclear membrane (Griffith et al. 1988).

The entry of SV40, another member of the papovavirus family, has been shown to differ in many ways from the entry of polyoma virus and other viruses. Firstly, the majority of SV40 particles enter the cell via endocytosis in uncoated vesicles while a minor portion uses coated vesicles. Secondly, viral particles inside the vesicles seem to be targeted to the endoplasmic reticulum (ER), which is an unusual target for endocytic traffic. In the ER they induce formation of specialized tubular smooth membrane structures interconnected with each other. The mechanism by which the viral genome is translocated from the ER to the nucleus, the site of its replication, is not yet known (Anderson et al. 1996, Kartenbeck, et al. 1989).

The mechanism of the rotavirus infection is not fully understood, but it is probably a multistep event, including binding, protease cleavage, and entry. Two modes of rotavirus entry have been suggested, direct membrane

penetration (main pathway) and receptor-mediated endocytosis which may occur independently and simultaneously (Gilbert & Greenberg 1997, Kaljot et al. 1988). Proteolytic cleavage of virus particles with trypsin strongly enhances rotavirus infectivity in cell culture and is believed to occur in the lumen of the intestine prior to infection of the enterocytes (Dryden et al. 1993, Estes et al. 1981). It has been proposed that trypsinized viruses induce an increase of cell membrane permeability preceding viral entry (Kaljot et al. 1988). The biochemical studies showed the existence of direct interactions between virus particles and membrane vesicles (isolated from a variety of cells) (Ruiz et al. 1994), and the increase of cell membrane permeability induced by infectious particles (Kaljot et al. 1988, Liprandi et al. 1997). Nontrypsinized particles entered the cell via endocytosis and appeared to be less infectious (Kaljot et al. 1988).

TABLE 2 pH-dependence of virus entry[a]. Modified from Marsh and Pelchen-Matthews (1993).

Virus group	Virus	Envelope
Acid-dependent viruses		
Alphavirus	Semliki Forest virus, sindbis	yes
Orthomyxovirus	Influenza A, B, C	yes
Flavivirus	West Nile virus	yes
Rhabdovirus	Vesicular stomatitis virus, rabies	yes
Bunyavirus	La Grosse virus	yes
Retrovirus	Mouse mammary tumor virus	yes
Iridovirus	African swine fever	yes
Picornavirus	Rhino, foot and mout disease virus	no
Adenovirus	Adenovirus type 2	no
Reovirus	Reovirus type 3	no
parvovirus	Canine parvovirus	no
Possible acid-dependent viruses		
Coronavirus	Mouse hapatitis virus 3	yes
Baculovirus	Nuclear polyhedrosis virus	yes
Retrovirus	Murine ecotropic leukaemia virus	yes
Poxvirus	Vaccinia	yes
Arenavirus	Junin virus	yes
Acid-independent viruses		
Paramycovirus	Sendai, Newcastle disease virus	yes
Retrovirus (avian)	Rous sarcoma virus	yes
Retrovirus (primate)	Mason-Prizer monkey virus, HTLV-1	yes
Retrovirus (lenti)	HIV-1, HIV-2,	yes
	Simian immunodeficiency virus	yes
Herpesvirus	Herpes simplex virus 1	yes
Papovavirus	SV40, polyomavirus	no
Rotavirus	Porcine rotavirus	no
Hepadnavirus	Hepatitis A, Duck hepatitis B	yes

[a] References can be found in Marsh and Helenius 1989 or in Marsh and Pelchen-Matthews 1993.
HTLV, human T-cell leukaemia virus; HIV, human immunodeficiency virus

2.3.4 Inhibitors of viral entry and endosomal pathway

Weak bases like NH_4Cl, amantadine, chloroquine, dansylcadaverine, methylamine and tributylamine, which accumulate in endosomes and raise the pH, can inhibit virus infection in culture and in some cases in vivo (Helenius et al. 1982, Marsh 1984, Schlegel et al. 1982). The inhibition of infection is consistent with the depression of endosomal and lysosomal pH which is required to trigger the fusion between viral envelope and endosomal membrane (Pless & Wellner 1996).

Carboxylic ionophores, such as monensin and nigericin, induce endosome neutralization by transmembrane exchange of sodium or potassium ions for protons, thus leading to inhibition of the low pH-dependent infectious entry of animal viruses into the cell (Guinea & Carrasco 1995, Irurzun et al. 1997). Monensin has been shown to have no effect on virus binding to cell surface but it blocks the endocytic pathway. For example with SFV, virus is clearly endocytosed in the presence of monensin, and in electron micrographs viruses can be seen in coated pits and endosomes, but penetration of nucleocapsid into the cytoplasm was blocked. Unlike the weak bases, monensin completely inhibited degradation of the viral proteins (Guinea & Carrasco 1995).

Antibiotics, such as bafilomycin A1 and concanamycin A are potential inhibitors of vacuolar proton ATPase, responsible for acidification of endosomes without accumulation of cations (Bowman et al. 1988, Dröse et al. 1993, Guinea & Carrasco 1994, Muroi et al. 1993). Studies on the actions of bafilomycin A1 indicated that micromolar concentrations of this antibiotic inhibited the entry of Semliki Forest virus, influenza virus, and vesicular stomatitis virus, whereas polio virus entry is not affected (Guinea & Carrasco 1994, Pérez & Carrasco 1993). Concanamycin A has also been shown to be a powerful inhibitor of infection by enveloped animal viruses, like influenza virus (Guinea & Carrasco 1994, Muroi et al. 1993). Bafilomycin is also supposed to disturb the traffic of internalized molecules from endosomes to lysosomes (van Deurs et al. 1996).

Some chemicals interfere with the endosomal traffic between peripheral early and perinuclear late endosomes, a transport process which depends on an intact microtubule network. Drugs like colchicine, vinblastine, nocodazole and podophyllotoxin block the endocytic pathway by causing depolymerization of microtubules (Avitable et al. 1995, Gruenberg et al. 1989, Hammonds et al. 1996, Satake & Luftig 1982). Inhibition of virus entry and proliferation in the presence of these agents would support a role for the endocytic pathway in the viral entry (Satake & Luftig 1982). A reduction in the temperature to 16-22°C also interrupts the endocytic membrane traffic between peripheral early and perinuclear late endosomes (Griffiths et al. 1988, Punnonen et al. 1998, Wolkoff et al. 1984) causing interference with viral entry.

2.4. Nuclear import

The asymmetric distribution of macromolecules between the nucleus and the cytoplasm is an essential component of cellular regulation and function. There is continuous exchange of macromolecules between the nucleoplasm and the cytoplasm. Although small molecules (smaller than 40-60 kDa) can diffuse through the nuclear pore complex (NPC), most macromolecules are imported in an energy-dependent, regulated, and highly specific manner (Nigg 1997).

The import of molecules through NPC is mediated by a number of soluble cytosolic factors (Görlich & Mattaj 1996, Panté & Aebi 1996). Nuclear import of proteins is a highly selective process which can be divided into several distinct phases (Newmeyer & Forbes 1988, Richardson et al. 1988). First, the nuclear localization signal (NLS) within the import substrate is specifically recognized by an import receptor. The receptor-substrate then binds to the NPC. The subsequent step is energy-dependent translocation of this complex across the NPC (Dingwall 1991, Görlich & Mattaj 1996, Nigg 1997, Silver 1991, Verner & Beers 1995) (Fig 4.).

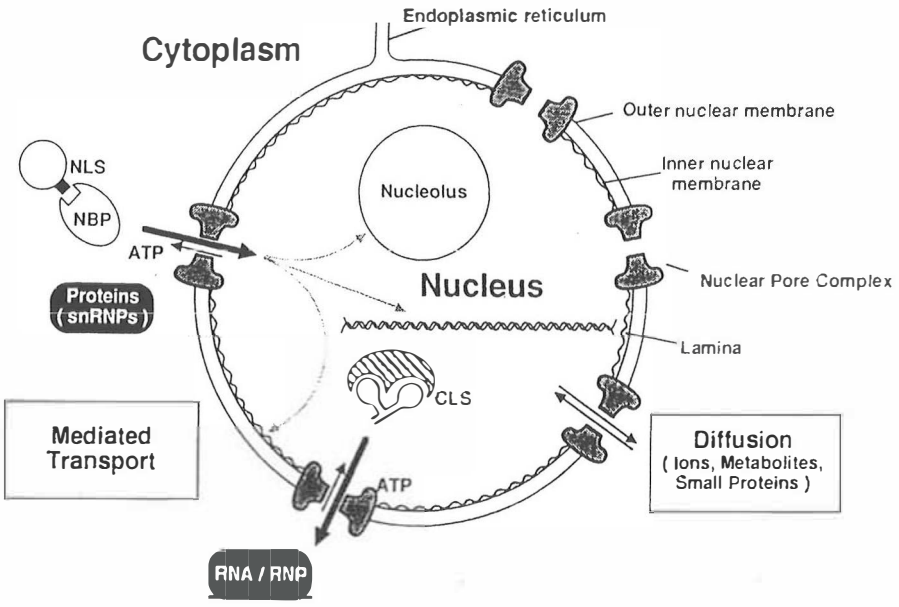

FIGURE 4 Nuclear-import export. NLS, nuclear localization signal; NBP, NLS-binding protein (importins); CLC, cytoplasmic localization signal (adapted from Nigg et al. 1991).

2.4.1 Nuclear pore complex

The nucleus is enclosed by the nuclear envelope (NE), a double membrane continuous with the endoplastic reticulum, which separates the nucleoplasm from the cytoplasm. NPCs provide aqueous channels, about 9 nm in diameter

as observed by electron microscopy, which allow the exchange of macromolecules between the two compartments (Davis 1995). Transport across the pore occurs in both directions. For example, all newly-synthesized nuclear proteins must be transported from the cytoplasm, whereas transfer RNAs (tRNAs) and messenger RNAs (mRNAs), are exported from the nucleus to the cytoplasm, their site of function (Izaurralde & Mattaj 1995). Some RNAs like small nuclear ribonucleoprotein (snRNA) are transported in both directions as part of their assembly into small nuclear RNP (snRNP) complexes (Izaurralde & Mattaj 1995). NPC can accommodate the active transport of particles as large as several million dalton in weight or 26-28 nm in diameter (Davis 1995, Dworetzky et al. 1988). In exponentially proliferating cells, hundreds of proteins and ribonucleoprotein particles are translocated through each NPC every minute.

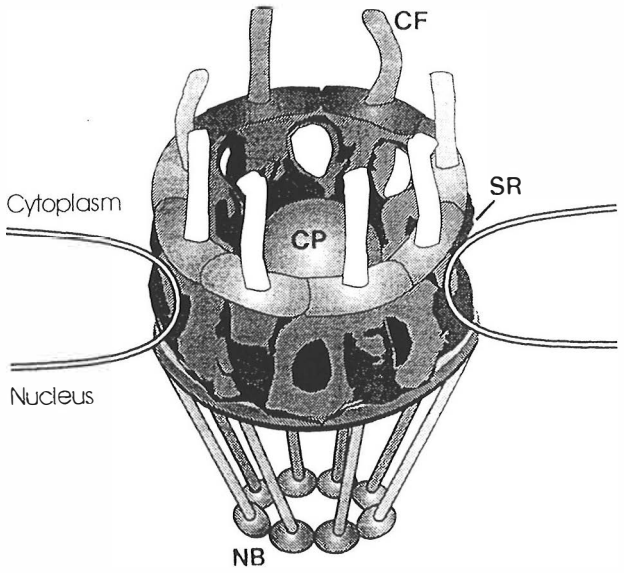

FIGURE 5 Structural elements of the NPC, including the spoke-ring complex (SR), central plug (CP), cytoplasmic filaments (CF), and nuclear basket (NB) (adapted from Davis 1995).

The NPCs have a mass of about 125 megadaltons in higher eukaryotes, span the double lipid bilayer of the NE and are estimated to contain roughly 100 different polypeptides, termed nucleoporins, which are present in multiple copies (Davis 1995). By electron microscopy, NPCs appear as roughly cylindrical structures, with eight-fold rotational symmetry in the plane of the NE. The NPC creates a large central channel, through which active nucleocytoplasmic transport is known to occur, and eight smaller peripheral channels that are probable routes for passive diffusion of ions and small molecules (Akey & Radermacher 1993, Goldberg & Allen 1995, Hinshaw et al. 1992). NPC appears to be composed of four basic elements. The waist of the NPC consists of a spoke-ring assembly anchored within a specialized region of

NE, where the lipid bilayers of the inner and outer NE are fused. A central plug lies within the aqueous channel formed by the spoke-ring complex. Extending from the ring are cytoplasmic filaments on one side and a nuclear basket on the other. This basket structure appears to consist of eight filaments, extending from the nucleocytoplasmic ring to a smaller ring (Fig. 5) (Akey & Radermacher 1993, Goldberg & Allen 1995, Hinshaw et al. 1992).

2.4.2 Nuclear localization signals

Small macromolecules, ions and metabolites freely diffuse through the pore, while the facilitated import of proteins larger than ~40 kDa requires ATP, a set of cytosolic factors, and is mediated by nuclear localization signals (NLSs) on the import substrates. The nuclear import pathway employed by proteins containing a "classical" NLS has been the subject of intense study (Görlich & Mattaj 1996). Classical NLSs are characterized by stretches of basic amino acids (Dingwall & Laskey 1991) and can be divided into two related types. The prototypic NLSs, consisting of short stretches of basic amino acids resembling the single basic domain of the simian virus 40 large T antigen (Kalderon et al. 1984, Kalderon et al. 1984, Landford & Butel 1984), while the *Xenopus laevis* nucleoplasmin-resembling, bipartite-basic types (Robbins et al. 1991) are composed of two clusters of basic residues separated by a spacer region (Table 3). A novel import pathway is mediated by the M9 domain of heterogenous nuclear ribonucleoprotein A1 (hnRNPA1), a NLS without any classical basic stretches (Michael et al. 1995) (Table 3). Classical NLS- and M9-containing proteins do not compete with each other for import and are recognized by distinct receptors (Goldfarb 1997, Pollard et al. 1996). The import of U snRNPs (U-rich small nuclear ribonucleoproteins) defines a pathway, also without any basic clusters, into the nucleus (Fischer et al. 1991, Michaud & Goldfarb 1991) (Table 3).

All these different nuclear import pathways for proteins are mediated by soluble import factors. Using an in vitro assay to reconstitute nuclear import (Adam et al. 1990), four soluble proteins have been identified which are essential for the import reaction. These are the two subunits of the NLS-binding receptors (importin-α and β), the small Ras-related GTPase Ran (which provides a potential source of energy), and its cofactors (Adam & Adam 1994, Görlich et al. 1995, Görlich et al. 1996, Imamoto et al. 1995, Moore & Blobel 1993), it is known that importin-α binds to proteins containing NLSs consisting of one or two clusters of basic amino acid residues and also binds to importin-β via an N-terminal domain. Importins carry their substrates through the NPC into nucleus, where the gargo is released from the receptor (Dingwall 1991, Dingwall & Laskey 1991, Görlich & Mattaj 1996, Nigg 1997) (Fig. 6.). The understanding of the nuclear import process is rapidly emerging and also the family of import receptors is growing. The importin α/β complex recognizes NLSs of the classical type, i.e., those typified by SV 40 T-antigen and nucleoplasmin (Table 3). An alternative pathway for nuclear import, the import of hnRNPs containing a nonclassical NLS, is mediated by an importin β-related

molecule (Pollard et al. 1996). Pathways used to import ribosomal subunits and mRNA-binding proteins are also mediated by related, but distinct, importin β-like molecules. NLSs of these subunits are so far unknown (Pemberton et al. 1997, Rosenblum et al. 1997, Rout et al. 1997, Schlenstedt et al. 1997). In contrast to conventional NLSs, the nuclear import of the human immunodeficiency virus type 1 (HIV-1) Tat protein (activator of viral gene expression and replication) is not mediated by cytosolic NLS-binding importin subunits (Efthymiadis et al. 1998).

TABLE 3 Examples of signals involved in protein import into the nucleus[a]. Modified from Görlich and Mattaj (1996).

Signal (length)	Sequence	Function
SV40 large T antigen NLS (7)	P**KKKRK**V	Nuclear import
Nucleoplasmin bipartite NLS (16)	**KR**PAAI**KK**AGQA**KKKK**	Nuclear import
IBB domain from importin-α(41)	RMR**K**F**KN**K**GK**DTAEL**R**- **RRR**VEVSVEL**RKAKK**D- EQIL**KRR**NV	Targets the nuclear import receptor to the nucleus
HIV-1 Tat NLS (12)	G**RKKRRQRRR**AP	Nuclear and nucleolar import[b]
hnRNPA1 M9 (38)	NQSSNFGPM**K**GGNFGG- **R**SSGPYGGGGQYFAKP **R**NQGGY	Confers rapid shuttling between cytoplasm and nucleus

Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; **K, Lys**; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; **R, Arg**; S, Ser; T, Thr; V, Val and Y, Tyr (basic residues highlighted in bold type)

[a]Unless indicated references can be found in Görlich and Mattaj, 1996.

[b]Efthymiadis et al., 1998. Nucleolar import; targeting from cytoplasm to nucleus.

2.4.3 Identification of nuclear localization signal

In the identification of NLSs two questions must be answered. First, is the proposed sequence of the parent protein necessary for the nuclear targeting, and second, is the necessary sequence sufficient to direct a protein into the nucleus. Two approaches have been used. In the "subtractive" approach (Kalderon et al. 1984, Landford & Butel 1984), a putative NLS is either deleted or mutated to demonstrate that it is necessary for the nuclear localization of a given protein. A second strategy, the "additive" approach, involves the transposition of a putative nuclear signal onto a non-nuclear protein or macromolecule to demonstrate that it is sufficient for nuclear localization (Goldfarb et al. 1986, Landford et al. 1986). Neither of these approaches is sufficient itself to prove that a given amino acid sequence is a "real" NLS. Additive and subtractive approaches have to be used in concert to identify NLSs.

Synthetic peptides have been used to induce nuclear import (Goldfarb et al. 1986, Kalderon et al. 1984). Several investigations have demonstrated the ability of synthetic peptide homologous to the NLS to induce the nuclear import of nonnuclear proteins. Peptides have been cross-linked to proteins like bovine serum albumin (BSA) or immunoglobulin G (IgG) (Goldfarb et al. 1986, Landford et al. 1986). The peptide-conjugates have been introduced into suitable cell lines using microinjection (Goldfarb et al. 1986, Landford et al. 1986) or cell permeabilization techniques (Adam et al. 1990). Synthetic peptides have been utilized to examine the effect on import of basic or nonbasic amino acid substitutions (Landford et al. 1988). Nuclear import of peptide-protein conjugates has been evaluated by immunofluorescence microscopy, or by electron microscopy using peptides cross-linked to gold particles (Feldherr & Akin 1990).

The use of recombinant DNA technology provides a rapid method for defining sequences involved in nuclear import. To test the ability of a potential NLS sequence to induce nuclear import, plasmids containing chimaeric genes have been constructed such that a small sequence encoding the putative NLS is fused to genes encoding large nonnuclear proteins like *E. coli* β-galactosidase or chicken muscle pyruvate kinase. A variety of point and deletion mutants have been constructed and examined after microinjection of plasmids (Clever & Kasamatsu 1991, Kalderon et al. 1984, Landford & Butel 1984).

2.4.4 Mechanism of nuclear import

Proteins above the size limit for passive diffusion can enter the nucleus only in an energy-dependent manner. However, even some small nuclear proteins, such as histones, generally enter the nucleus actively rather than by diffusion (Breeuwer & Goldfarb 1990). The import process is characterized by energy-dependence (Zasloff 1983), signal dependence (Dingwall & Laskey 1991) and is mediated by saturable import receptors (Goldfarb et al. 1986, Zasloff 1983). Import of proteins into the nucleus is an active process consisting of at least two steps, both of which require the presence of NLS. First, rapid energy-independent docking of the substrate to the nuclear envelope, and second, a slower, energy-dependent translocation through the nuclear pore complex (Newmeyer & Forbes 1988, Richardson et al. 1988) (Fig. 6).

The first step of nuclear translocation, the protein binding to the NLS-receptor protein, has been suggested to occur at the cytoplasmic periphery of the NPC, possibly in association with fibrils that emanate from the cytoplasmic NPC surface (Imamoto et al. 1995, Newmeyer & Forbes 1988). During this step NLS-containing protein bind to the importin α–β heterodimer (importin consist of a 60 and a 90 kD subunit) which constitutes a cytosolic receptor for NLSs that enables import substrates to bind to the NE (Görlich et al. 1995). Subunit α of importin contains both the NLS-binding site and the importin-β-binding (IBB) domain which mediates the α–β heterodimerization. The β subunit mediates docking of the complex at the NPC (Adam & Gerace 1991, Görlich et al. 1994, Imamoto et al. 1995, Weis et al. 1995). During the second step of

translocation, the trimeric NLS protein-receptor complex is translocated through the pore by an energy-dependent mechanism (Richardson et al. 1988). The energy required by translocation is probably directly provided from GTP hydrolysis by Ran, a member of the Ras superfamily of small GTP-binding proteins (Moore & Blobel 1993, Moore & Blobel 1994). It is likely that Ran shuttles between the nucleoplasm and cytoplasm, and that the state of the bound nucleotide determines compartment-specific interactions with components of the transport machinery (Görlich & Mattaj 1996, Koepp et al. 1996). The translocation process of protein-receptor complex ends approximately at the terminal ring (inside the nucleus) of the nuclear baskets in NPC (Fig. 5, 6) where the termination reaction disassembles the importin heterodimer and releases the import protein into the nucleoplasm. The import substrate and importin-α reach the nucleoplasm, whereas importin-β remains at the nuclear envelope (Görlich et al. 1996). Importin-β does not accumulate in the nucleoplasm, presumably because its recycling to the cytoplam is too rapid. In the nucleus the dissociation of the importin-α from NLS containing import substrate is likely to be caused by the conversion of importin-α to a form with a low affinity for the NLS (Görlich et al. 1996). Finally, the subunits of importin are proposed to return to the cytoplasm separately, possibly by different routes (Görlich et al. 1996, Weis et al. 1996) (Fig. 6)

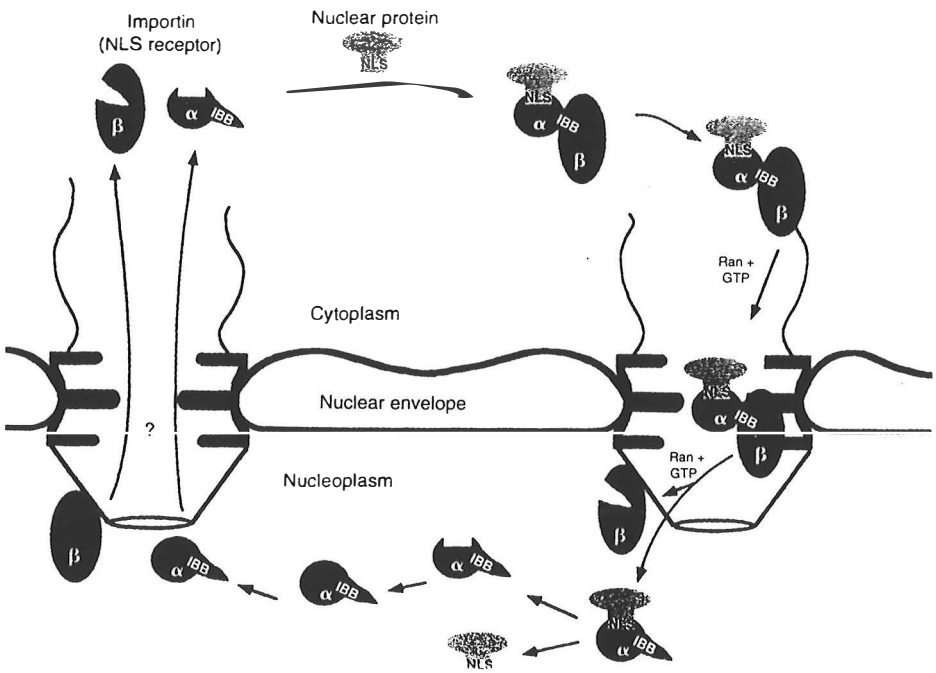

FIGURE 6 Steps of the nuclear protein import cycle. NLS, Nuclear localization signal; IBB, importin-β-binding; Ran-GTP, GTP-bound form of Ran. For additional description of the proteins, see text (adapted from Görlich 1996).

2.4.5 Methods for study of nuclear transport

A handful of techniques to introduce macromolecules, like putative NLS or inhibitors of nuclear import, into eukaryotic cells have been developed. These include (i) microinjection, (ii) cell permeabilization techniques and (iii) techniques using cell-free systems.

(i) Capillary microinjection has proved to be one of the most efficient methods for introducing a known number of test molecules either into cytoplasm or into the nuclei of recipient living cells (Ansorge 1982, Celis 1984, Graessman et al. 1980). The degree of damage caused by microinjection can be minimized by several factors including cell type, capillary tip size and shape, injection site, and nature and volume of the material injected (Agutter & Taylor 1996, Miller et al. 1984).

(ii) Cell permeabilization techniques have been developed to study in the import of macromolecules across the nuclear envelope. The permeabilized cells efficiently mimic nuclear protein import in intact cells in terms of energy requirement and the effects of import inhibitors. The in vitro nuclear import assay involving digitonin-permeabilized cells was developed by Adam et al (1990). While treatment of cells with digitonin permeabilizes the plasma membranes to macromolecules, the nuclear envelopes remain structurally intact and nuclei retain the ability to import and accumulate proteins containing NLSs. The nuclear protein import requires addition of exogenous cytosol to permeabilized cells, indicating that the soluble cytoplasmic factors required for nuclear import are released during digitonin treatment (Adam et al. 1990, Moore & Blobel 1992, Moore & Blobel 1993, Moore & Blobel 1994).

(iii) The cell-free import systems that have been descriped thus far have the limitation of utilizing nuclei that have been dissociated from other cellular structures. One solution for the problem is provided by Xenopus egg extracts, which can assemble nucleus-like structures around naked DNA and reseal the nuclear envelopes of isolated rat liver nuclei. These nuclei exclude nonnuclear proteins and selectively accumulate proteins containing NLSs (Lohka & Masui 1984, Newmeyer et al. 1986, Newmeyer & Forbes 1988, Newmeyer & Forbes 1990, Newmeyer et al. 1986). Ultrastructural techniques demonstrated that this in vitro import occurs through NPCs (Wolf et al. 1988). While the association of proteins with nuclei reflects some of the characteristics of in vivo protein import, it has not yet been demonstrated that the nuclei are structurally intact.

2.4.6 Inhibitors of nuclear protein import

Import of most nuclear proteins through the nuclear pore complex has been shown to require energy and to be temperature-dependent (Richardson, et al. 1988).

ATP dependence of nuclear accumulation of nucleoplasmin and SV40 large T antigen has been shown by using reversible inhibition of ATP production (Richardson et al. 1988), in vitro and, in vivo (Newmeyer & Forbes

1988, Richardson et al. 1988). Although import inhibited by ATP depletion and lowered temperature, NLS-containing proteins accumulated at the NE (Newmeyer & Forbes 1988, Richardson et al. 1988), showing that the first step of nuclear import, docking, is independent of temperature or energy. The second step, translocation across the NE, is an active process which is both ATP- and temperature-dependent.

Wheat germ agglutinin (WGA) lectin, which binds to a family of NPC proteins, is able to inhibit import, while still allowing signal sequence recognition and binding to the nuclear NE (Finlay et al. 1987, Newmeyer & Forbes 1988). WGA interacts with the O-glycosylated nucleoporins within the cytoplasmic ring of the pore (Akey & Goldfarb 1989). The fact that WGA binding affects import and not binding suggest that the binding site for an imported protein may be at some distance from the translocation site (Akey & Goldfarb 1989).

2.4.7 Nuclear import of viral proteins and genomes

Nuclear entry of viral components is a significant part the life cycle of many viruses as a part of viruses use the nucleus as a place of viral transcription, replication and assembly of mature virions from cytoplasmically synthesized viral proteins (Table 4). It is evident that most viruses or viral proteins entering the nucleus take advantage of the cell´s nuclear import machinery: nuclear pore complexes, receptors and import factors (Marsh & Helenius 1989, Whittaker & Helenius 1998).

Although little is known about how incoming viruses deliver their genomes and possible associated proteins into the nucleus, it is clear that the mechanism differ considerably among virus families. There are at least four different strategies for nuclear entry of viral nucleocapsid or genome during the infection. (i) The viral nucleocapsid is small enought to pass through the nuclear pores. (ii) The incoming virus undergoes uncoating in the endosomes, and core particles are imported into the nucleus. (iii) Some viruses bud across the nuclear membrane and others (iv) rely on dissociation of the nuclear membrane during cell division (Marsh & Helenius 1989, Whittaker & Helenius 1998).

For example, influenza viruses deliver into the nucleus for replication their (multiple single-stranded RNA) genomes following pH-dependent uncoating. The nuclear import of the viral genomic segments is mediated by NLS in viral RNA-associated nucleoprotein, not in the RNA itself (Bui et al. 1996, Martin & Helenius 1991, O´Neil et al. 1995). Retroviral murine leukemia virus releases its core into cytoplasm, where additional uncoating events take place, and reverse transcription is initiated. The produced proviral DNA must integrate into the host chromosomal DNA in order to complete the infection of the cell (Coffin 1996). The integration-competent complexes containing viral DNA, capsid proteins and integrase, have been suggested to enter the nucleus (Risco et al. 1995). It remains to be seen whether nuclear entry of the viral genomes via specifically or nonspecifically associated proteins and an NLS-mediated pathway is common to other RNA viruses.

Table 4. lists the families of viruses known to replicate in the nucleus. The majority of such viruses are DNA viruses. The obvious reason for the rather low number of RNA viruses is that the RNA viruses use quite different replication strategies. The nucleus cannot supply enzymes for RNA replication as it lacks RNA-dependent RNA polymerases. Furthermore, the gene-regulation machinery present in the nucleus is essentially useless for a typical RNA virus. However, some RNA viruses can replicate in nucleus by using mRNA transcription mechanism

TABLE 4 Virus families which replicate in the nucleus. Modified from Whittaker and Helenius (1998).

Virus family	Selected example(s)	host	Genome
Retroviridae		animal	RNA
a) Oncovirus	Simian retrovirus type 1		
b) Lentivirus	Human immunodeficiency virus type 1		
Orthomyxoviridae	Influenza virus	animal	ss RNA
Bornaviridae	Borna disease virus	animal	ss RNA
Rhabdoviridae	Lettuce necrotic yellows virus	Plant	ss RNA
Hepadnaviridae	Hepatitis B virus	Animal	ds DNA
Caulimoviridae	Cauliflower mosaic virus	Plant	ds DNA
Geminiviridae	Bean dwarf mosaic virus Squash leaf curl virus	Plant	ss DNA
Parvoviridae	Minute virus of mice Parvovirus B19	Animal	ss DNA
Papovaviridae	Papovavirus Simian virus 40	Animal	ds DNA
Adenoviridae	Adenovirus 2	Animal	ds DNA
Herpesviridae	Herpes simplex virus Cytomegalovirus	Animal	ds DNA
Polydnaviridae	Ichnovirus	Animal	ds DNA
Iridoviridae	Frog virus 3	Animal	ds DNA
Baculoviridae	Autographa californica Multiple nuclear polyhedrosis virus	Animal	ds DNA

DNA viruses employ a variety of nuclear import strategies. Herpesvirus and adenovirus have been reported to dock at NPC and are thought to inject their DNA across the NPC into the nucleus (Batterson et al. 1983, Dales & Chardonnet 1973, Tognon et al. 1981). It is proposed that during early events in polyomavirus infection virions enter the nuclei via direct fusion of

monopinocytotic (virus-containing) vesicles with the host cell nuclear membrane (Griffith et al. 1988). Studies with the autonomous parvovirus minute virus of mice (MVM) have shown the virus to be capable of productive infection only when two capsid proteins, VP1 and VP2 are present in the nucleus. It is also suggested that VP1 is required for the import of MVM to the nucleus. It is not known whether MVM enters the nucleus as an intact virion or partially disassembled DNA-protein complex (Tullis et al. 1993).

During the late phase of infection, viruses using the nucleus as a place of assembly, import cytoplasmically synthesized structural and nonstructural viral proteins to the nucleus, where they assemble into virions. At present the mechanisms of the nuclear targeting and nuclear entry of capsid proteins before virion assembly are poorly understood. The protein sequence PPKKKRKV appears to mediate translocation of SV 40 large T antigen to the nucleus (Kalderon et al. 1984) and many other viruses have a similar basic, proline-rich sequences. Whether this sequence is essential either in passage of input virions to the nucleus, or in the intranuclear accumulation of de novo synthesized capsids later in the infection, remains to be investigated.

3 AIM OF THE STUDY

The specific aims of this study were:

1. To raise antibodies to CPV by using synthetic peptides.

2. To study the importance of vesicular traffic for CPV productive infection.

3. To characterize the mechanism of the nuclear transport of CPV proteins.

4 SUMMARY OF MATERIALS AND METHODS

4.1 Cells and viruses

The canine parvovirus strain used was serotype CPV-2 (Parrish & Carmichael 1983, Parrish et al. 1982), a wild-type strain isolated in 1980 from a clinically ill dog. CPV was propagated in canine fibroma cell line A-72 (Binn et al. 1980), grown (72 hr, 37°C, 5% CO2) in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco, Paisley/UK) (I, II, III). CPV was purified according a modification of the procedure of Paradiso (Paradiso 1981). The ratio of infectious to empty viral particles was estimated to be 40:60 from sedimentation profiles of hemagglutinating CPV in CsCl gradients (Agbandje, et al. 1993). The hemagglutination titer of the stock was 60, 000 to 80,000 (Carmichael et al. 1980) (I, II, III).

BPV was of commercial Haden strain (ATCC VR-767). BPV was propagated in primary calf turbinate cells, grown in MEM supplemented with 10 % horse serum. The cells were grown at 37 °C in an atmosphere containing 5 % CO_2 (I). Isolates of Blue Fox parvovirus (BFPV), mink enteritis virus (MEV) and feline parvovirus (FPV) were a kind gift of Dr Pirjo Veijalainen, National Veterinary and Food Research Institute, Helsinki (I). Samples of raccoon dog parvovirus (RPV) were specimens of stool from clinically ill animals (I).

4.2 Peptide synthesis and peptide-protein conjugation

The peptides were synthesized by Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a simultaneous multiple peptide synthesizer (SMPS 350, Zinsser Analytic, Frankfurt, Germany) (II) or with an Applied Biosystems peptide synthesizer (I). A C-terminal cysteine residue was added to the peptides for

coupling. Additional terminal glycines were used as spacers in the shortest sequences. Purity of the peptides was analyzed by reversed-phase HPLC (System Gold, Beckman Instruments Inc, Fullerton, CA, USA (I, II).

Peptides used in peptide-mediated nuclear transport experiments (II) were conjugated to bovine serum albumin (BSA) (Sigma, St. Louis, USA) with a heterobifunctional crosslinking reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Sigma, St. Louis, USA) by a procedure similar to that described previously (Clever & Kasamatsu 1991, Landford et al. 1986). Peptide conjugates were labelled with fluorescent rhodamine B isothiocyanate (Sigma, St. Louis, USA). The conjugates were analysed by SDS-PAGE as described (Landford et al. 1986) (II).

For immunization, peptides were coupled to keyhole limpet hemocyanin (KLH, Sigma St. Louis, MO. U.S.A.) via their C-terminal cysteine residue by MBS (Sigma St. Louis, MO. U.S.A.) (Liu et al. 1979) (I).

4.3 Antibodies and immunization

Rabbit antiserums to CPV, RPV and BPV (bovine sera) were a kind gift of Dr. Pirjo Veijalainen, National Veterinary and Food Research Institute, Helsinki (I, II). Mouse monoclonal antibody to α-tubulin was purchased from the Radiochemical Center (Amersham, UK) (III). Goat anti-mouse IgG conjugated with fluorescein, and goat anti-rabbit IgG conjugated with rhodamine were purchased from Organon Teknika Corporation (Durham, NC) (III). Goat anti-rabbit IgG-FITC and goat anti-bovine IgG-FITC were purchased from Dako (Glostrup, Denmark) (I).

Rabbits were immunized with subcutaneous injections of peptide-KLH-conjugates in phosphate buffered saline, pH 7.4 (PBS) emulsified with Freund's complete adjuvant (Difco, U.S.A), 500 µg of peptide per immunization, on days 0, 30 and 60. Sera were collected on day 68 (I).

4.4 Enzyme linked immunosorbent assay

Polystyrene microtiter wells were coated with synthetic peptides or with CPV at a concentration of 10 µg/ml in PBS overnight at 4 °C. Excess binding sites were blocked (2 h at 22 °C) with 1 % nonfat milk powder in PBS. After thorough washing, antisera diluted 1:100 were incubated in the wells for 2 h at 22 °C. Bound immunoglobulins were detected, after washing, with alkaline phosphatase-labelled antibodies against rabbit immunoglobulins; p-nitrophenyl phosphate was used as substrate (I).

4.5 Western blotting

Samples (10 µl) containing one of CPV, FPV, BPV, RPV or BFPV were dissociated by heating in buffer containing SDS, and polypeptides were separated with polyacrylamide gel electrophoresis as described (Towbin et al. 1979). Polypeptides were transferred to nitrocellulose sheets and tested for binding of antibodies (Towbin, et al. 1979) to synthetic peptides. Nonimmune rabbit serum was used as a control (I).

4.6 Immunofluorescence microscopy

For immunofluorescence microscopy, A 72 cell cultures were inoculated with 1,5-2 x 10^4 (II,III) or 1.5-3 x 10^4 cells/cm^2 (I), and primary calf turbinate cells were seeded at a density of 2-3 x 10^4/cm^2 (I) on glass coverslips (I), or on 8-mm-diameter wells of eight-well teflon-coated coverslips (CML, France) (III). Cells were synchronized by following the procedure of Cotmore and Tattersall (Cotmore & Tattersall 1987) (III). Cells were infected with CPV (MOI of 4 to 5) and mock-infections were carried out for controls with PBS (I, III). The cover slips were dipped after an appropriate cultivation time in phosphate- buffered saline (PBS) pH 7.4, and fixed with methanol for 6 min at -20°C (III) or at room temperature (I). After being rinsed with PBS, the cells were incubated for 45 min with the primary antibodies diluted in 3% BSA in PBS and rinsed with PBS. The cells were incubated with FITC- or rhodamine-conjugated secondary antibodies (strain specific from Dako, Glostrup, Denmark or Jackson Immuno Research Laboratories, West Grove, USA) for 45 min and rinsed several times with PBS. The immunolabeling was carried out at room temperature. The monolayers were mounted in glycerol containing 10% PBS and 1 mg/ml para-phenylene diamine. The cells were viewed under a Leitz DMR fluorescence microscope (Leica, Wetzlar, Germany) (I,III).

4.7 Microinjection

For microinjection, the cell cultures were seeded with 1 x 10^4 to 3 x 10^4 cells/cm^2, and cells were grown for one to two days on round microgrid coverslips (diameter 12 mm, 175 µm grid size, Eppendorf, Hamburg, Germany) (II, III). Microinjections were performed with an Eppendorf 5246 microinjector and an Eppendorf 5171 micromanipulator (Eppendorf, Hamburg, Germany), the latter being mounted on an IMT-2 inverted microscope (Olympus Optical Co., Tokyo, Japan). Capillaries for injection were prepared from glass tubing (GC 120 F-15, Clark Electromedical Instruments, Pangbourne, UK) using a model P 97 capillary puller from Sutter Instruments (Novato, CA, USA) (II, III). The synthetic peptide conjugates were microinjected into the cytoplasm at a concentration of 1-1.4 mg/ml in 50 mm sodium phosphate buffer (pH 7.0) (II).

The volume of liquid released from injection capillaries into cells was determined (typical values were 0.1-0.5 pl) by bubble pressure measurement (Schnorf et al. 1994). For injection, CPV particles were purified as described earlier. The cells were pretreated with chloroquine (200 μM) for 1 hr and microinjection was carried out in the presence of this reagent. For some experiments an acidic pretreatment of CPV was done before microinjection. The viral particles were treated with citrate buffer, pH 5.0 or 5.5 (100mM citric acid, 200 mM Na_2HPO_3) for 30 min and then neutralized with 0,5 M Na_2HPO_4 (III).

4.8 Inhibitors of nuclear transport

To assess the effect of wheat germ agglutinin (WGA) on binding to the envelope of CPV or peptide-protein conjugates, cells were microinjected with peptide-linked rhodamine-BSA together with WGA (Sigma, St. Louis, USA, 0.4 mg/ml). The samples were incubated for 60 min at 37°C and processed for microscopy (II).

To test for the ATP requirements of nuclear transport, the cells were preincubated for 30 min in the presence of Hank´s balanced salt solution with 1 μM carbonyl cyanide p-trifluoromethyloxyphenylhydrazone (FCCP, uncoupler of oxidative phosphorylation) or 6 mM 2-deoxyglucose (inhibitor of glycolysis) at 37°C (Breeuwer & Goldfarb 1990) prior to microinjection. Following microinjection the cells were further incubated in the presence of FCCP and 2-deoxyglucose for 30 min at 37°C before processing for microscopy. To test if the effect of ATP depletion was reversible, the microinjected cells were washed and incubated with normal medium for 30 min prior to fixation and mounting (III).

For some experiments the cells were kept on ice for 30 min before and 30 min following injection (Breeuwer & Goldfarb 1990, Richardson, et al. 1988). To determine whether the inhibition of nuclear accumulation caused by chilling was reversible, the temperature of the cells was raised to 37°C, by adding warm medium, for 30 min prior to processing for microscopy (III).

4.9 Inhibitors of endocytic pathway

For the endocytic transport-block experiments, cell cultures were seeded with 1,5-2 x 10^4 cells/cm^2. Cells were treated with nocodazole (methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl) carbamate, 20 μg/ml, Sigma, St. Louis, Mo.) for various periods of time, and the viral infection was carried out either in the presence or absence of it at 37 °C. In some experiments the cells were preincubated with nocodazole 60 min before viral infection (III). Temperature block experiments were performed at 18°C (III).

4.10 Quantation of viral DNA

Viral DNA synthesis was quantitated in cell monolayers at 90 min or 9 hours postinfection by measuring specific [3H]thymidine incorporation. Briefly, cells were seeded at 2,5 x $10^4/cm^2$ into 3.2-cm-diameter culture plates, allowed to attach at 37°C for 2 hours, and then inoculated with 0.4 ml of virus (titer 60, 000) with 3H-thymidine (Amersham, U.K., 20 μCi/ml, specific activity 22.0 Ci/mmol). DNA was extracted from cell cultures inoculated with virus according to the method of Hirt (Hirt 1967). CPV DNA was separated from host DNA on agarose gels (Basak & Turner 1992, Parrish & Carmichael 1986), and the gel slices were counted for radioactivity associated with CPV DNA (III).

5 REVIEW OF THE RESULTS

5.1 Characterization of antibodies to VP2 and NS peptides

In the present study VP2 residues 292-309 (peptide 1) (NSLPQSEGATNFGDIGVP) (Table 5) were selected as the immunogen. In addition to VP2 one sequence residues 391-409 (peptide 2) (GKRNTVLFHGPASTGKS-C) was chosen from a narrow conserved segment of the NS1 protein (Table 6). Antisera were produced against these peptides in rabbits. The antisera were characterized by enzyme immunoassay, western blotting and immunofluorescence.

Antiserum produced against peptide 1 and 2 gave in enzyme immunoassay analysis a recognizeable signal with the peptide hapten at dilutions of 1:14 000 and 1:8 000 (I, Table 1). Antibodies to peptide 1 also showed a weak but consistently observed binding to purified CPV in ELISA (I, Table 1).

Western blot analysis showed that peptide antibody against VP2 sequence 292 to 309 was able to identify antigens with molecular weights of 60, 66 and 86 kDa representing CPV capsid proteins VP1, VP2 and VP3 (I, Fig. 1A). Moreover, the ability of the CPV VP2 peptide antibody to cross-react with VP2 and VP3 proteins of closely related parvoviruses was verified with antigen bands of 86 kDa and an antigen band close to 66 kDa in BFPV, FPV and MEV in samples obtained from cell culture (I, Fig. 1A). The antibodies to NS1 peptide 391 to 409 identified an antigen of 90 kDa antigen representing the NS1 protein of CPV (I, Fig. 1C). In addition, cross-reaction was detected with similar-sized proteins of BFPV and MEV (I, Fig. 1C). Thus, a synthetic peptide from the NS1 can elicit an antibodies which can be used in detection of NS1 proteins from several parvoviruses.

The ability of the peptide antibodies to react with newly synthesized viral proteins was tested by indirect immunofluorescence. Antibodies against peptide 1 and 2 identified, viral antigens in canine A 72 cells infected with CPV. With antisera to peptide 1 a strong nuclear fluorescence was detected beginning 20 hours after infection, together with weaker puctuate cytoplasmic

TABLE 5 Amino acid sequence of synthetic VP2 peptide (peptide 1) of CPV and corresponding peptides from other closely related parvoviruses. Differences between CPV and other parvoviruses are underlined.

Virus	Sequence	GenBank accession no.
CPV	NSLPQS EGATNFGDIGVQ	M38245
BFPV	NSLPQSEG<u>V</u>TNFGDIGVQ	U22185
FPV	NSLPQSEGATNFGDIGVQ	M38246
MEV	NSLPQSEGATNFGDIGVQ	M23999
RDPV	NSLPQSEGATNFGDIGVQ	U22192

Table 6 Amino acid sequence of synthetic NS1 peptide (peptide 2) of CPV and corresponding peptides from other parvoviruses. Differences between CPV and other parvoviruses are underlined.

Virus	sequence	Gen Bank accession no.
CPV	GKRNTVLFHGPASTGKS	M38245
BPV	GKRNSTLF<u>Y</u>GPASTGK<u>T</u>	P07296
FPV	GKRNTVLFHGPASTGKS	M38246
MEV	GKRNTVLFHGPASTGKS	M23999
B19	GK<u>K</u>NTLWF<u>Y</u>GP<u>P</u>STGK<u>T</u>	B24299

fluorescence (I, Fig. 2A). Anti-peptide 2 serum showed a nuclear fluorescence similar to that seen with anti-CPV polyclonal (I, Fig. 2B,C). Antibodies to NS1 peptide identified NS-antigens of BPV inside the nucleus of BPV-infected bovine serum turbinate cells 24 h after infection. Antigens were localized to a few small round shaped structures (I, Fig. 3A), different from those observed with antiserum against the whole BPV (I, Fig. 3B). The results show that synthetic peptides elicit antibodies capable of reacting with viral proteins synthesized inside the infected cells.

In conclusion, antisera against peptides 1 and 2 were able to recognize CPV proteins in ELISA, Western blotting and immunofluorescence assays.

5.2 Effect of nocodazole and reduced temperature on CPV infection

Published results suggest that CPV needs endosomal low pH as a trigger in the penetration into cell. The present study was designed to test for the idea that the microtubule-mediated transport between early and late endosomes is essential for the productive infection of CPV. Experiments were performed in

which two endocytosis blocking factors were used, reduced temperature (Griffiths et al. 1988, Punnonen et al. 1998, Wolkoff et al. 1984) or with nocodazole (a microtubule-depolymerizing agent) (Avitable et al. 1995, Gruenberg et al. 1989). Cells were infected in the presence of nocodazole or at 18 °C, and then incubated for 90 min and 24 hr in the same conditions. Immunofluorescence staining for CPV antigens showed fluorescence which appeared to be restricted to small cytoplasmic vacuolar-like structures scattered around the cytoplasm (III, Fig. 1C,D,E,F). In untreated cells 90 min postinfection, the viral antigens were concentrated in a rim around the nucleus (III, Fig. 1A). Untreated cells 24 hr postinfection showed nuclear fluorescence mostly due to newly synthesized CPV proteins, was observed (III, Fig. 1B). Viral DNA synthesis, as measured at 9 hours postinfection, was almost totally inhibited by nocodazole or low temperature (III). When the temperature block (18°C) was released, the viral antigens were found after 15 and 30 min mostly in small cytoplasmic and perinuclear vacuolar structures (III, Fig. 2B,C). With increasing time after block more and more cells had viral antigens around the nucleus. Until at 90 min when most of the viral antigens had reached this perinuclear distribution (III, Fig. 2E).

5.3 Intracellular distribution of microinjected CPV

We used the microinjection approach to study if the endocytic pathway can be bypassed by injecting CPV into the cytoplasm. CPV particles microinjected directly into the cytoplasm were unable to initiate progeny virus production. The viral antigens were first found throughout the cytoplasm and after 20 hours displayed an additional circular staining pattern around the nucleus (III, Fig. 3A,B).

A small part of virions can leak into the medium during the microinjection process and the productive CPV infection caused by virions internalized from the medium via the endocytic route (III Fig. 3D) was prevented with the presence of chloroquine in the medium. Some weak bases like chloroquine block endocytic pathway by causing the raise of pH in endosomes (Helenius et al. 1982, Marsh 1984).

Finally, to determine the role of acidic conditions in endosomes for infection, we monitored the proceeding of CPV infection after microinjection of the low-pH-treated virions. Virions treated at pH 5.0 were unable to initiate virus production and viral antigens were observed by immunofluorescence microscopy to remain in the cytoplasm for 60 min and 20 h (III, Fig. 3C).

5.4 Nuclear import facilitated by CPV capsid protein sequences

We investigated the abilities of synthetic peptides mimicking the potential nuclear localization signal of CPV capsid proteins, to translocate a carrier protein to the nucleus following microinjection into the cytoplasm of A72 cells.

Potential nuclear localization sequences were chosen for synthesis from CPV capsid protein VP1, VP2 sequences on the basis of the presence of clustered basic residues (Fig.1), which is a common theme in most of the previously identified targeting peptides. The abilities of synthetic peptides mimicking the potential NLS of CPV capsid proteins to translocate a carrier protein to the nucleus following microinjection into the cytoplasm of A72 cells was investigated. Nuclear targeting activity was found within the amino-terminal residues 4-13 (PAKRARRGYK) (p1) of the VP1 capsid protein (II, Fig. 1B). Five additional capsid proteins sequences were tested and residues 81 to 86 (FRAKKA) (p2), 102 to 112 (RPTKPTKRSKP) (p3), 121 to 126 (AKKKKA) (p4), 447 to 453 (FKAKLRA) (p5) and 673-679 (p6) were found to be ineffective in targeting BSA, which was tagged with a rhodamine label, to the nucleus (II, Fig. 1B).

The effect of substituting glysine for basic aminoacid on p1 peptide-induced nuclear transport was tested. Eight peptides harboring mutated sequences of p1 were synthesized (II, Fig. 2A) and the subcellular localization of the peptide conjugates was examined. Result showed that substitution of arginine 10 with glycine did not affect the nuclear targeting activity but replacement of lys 6, arg 7 or arg 9 with glycine abolished it. The targeting activity was thereby found to reside in the cluster of basic residues lys 6, arg 7 and arg 9 (II, Fig. 2B). The saturability of nuclear import was studied by microinjecting SV40-rhodamine conjugates together with 10- to 100 fold molar excess of the unlabelled p1-peptide conjugates. The SV40 peptide, based on an SV40 large-T antigen NLS sequence was used as a positive control (Kalderon et al. 1984). In conclusion, the nuclear transport of SV40-conjugate was dimished in lower concentration p1-BSA and was abolished in the presence of higher concentration of the competitor (II).

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1    MAPPAKRARRGYKYLGPGKSLDQGEPTNPSDAAAKEHDEAYAAYLRSGKN
51   PYLYFSPADQRFIDQTKDAKDWGGKIGHYFFRAKKAIAPVLTDTPDHPST
101  SRPTKPTKRSKHPPHIFINLAKKKKAGAGQVKRDNLAPMSDGAVQPDGGQ
151  PAVRNERATGSGNGSGGGGGGGSGGVGISTGTFNNQTEFKFLENGWVEIT
201  ANSSRLVHLNMPESEKDRRVVVNNMDKTAVNGNMALDDIHAQIVTPWSLV
251  DANAWDVWFNPGDWQLIVNTMSELHLVSFEQEIFNVVLKTVSESATQPPT
301  KVYNNDLTASLMVALDSNNTMPFTPAAMRSETLGFYPWKPTIPTPWRYYF
351  QWDRTLIPSHTGTSGTPTNIYHGTDPDDVQFYTIENSVPVHLLRTGDEFA
401  TGTFFFDCKPCRLTHTWQTNRALGLPPFLNSLPQSEGATNFGDIGVPQDK
451  KRGVTQMGNTNYITEATIMRPAEVGYSAPYYSFEASTQGPFKTLPIAAGR
501  GGAQTDENQAADGNRYAFGRQHGKKTTTTGETPERFTYIAHQDTGRYPEG
551  DWIQNINFNLPVTNDNVLLPIDPIGGKTGINYTNIFNTYGPLTALNNVPP
601  VYPNGQIWDKEFDTDLKPRLHVNAPFVCQNNCPGQLFVKLAPNLTNEYDP
651  DASANMSRITYSDFWWKGKLVFKAKLRASHTWNPIQQMSINVDNQFNYV
701  PSNIGGMKIVYEKSQLAGRKLY
```

Figure 7 Amino acid sequence of CPV capsid proteins. The boxed amino acids represent the potential NLSs.

5.5 ATP and temperature dependence of nuclear translocation and the effect of wheat germ agglutinin on nuclear import

Transport of most nuclear proteins through the NPC has been shown to require energy and to be temperature dependent. The cellular distribution of microinjected p1 and SV40 BSA conjugates was studied in the presence of inhibitors of ATP production. In cells depleted of ATP some of the p1 conjugates concentrated in a thin rim around the periphery of the nucleus while the rest of conjugates were spread diffusely in the cytoplasm at 60 and 90 min after microinjection (II, Fig. 3A). The narrow perinuclear band was seen most clearly with low concentrations of p1-BSA conjugates. Similar patterns were obtained with SV40-BSA conjugates. When the injected, energy-depleted cells were allowed to recover for 30 min in medium lacking ATP production-blocking agents the conjugates (p1, SV40) accumulated in the nuclei, indicating the reversibility of the inhibition (II, Fig. 3B).

The nuclear transport of p1 was inhibited by low temperature. p1-BSA microinjected into cells chilled at 0 °C did not reach the nucleus but instead formed a narrow band around the nucleus or remained in the cytoplasm (II, Fig3C). When microinjected, chilled cells were warmed and allowed to recover for 30 min the peptide conjugates accumulated in the nucleus, thereby indicating the reversible nature of the temperature (II, Fig. 3D).

Wheat germ agglutinin (WGA) is a lectin that has been shown to inhibit the accumulation of nuclear proteins by blocking the translocation step of the transport, probably by interacting with O-glycosylated nucleoporins(Adam & Adam 1994, Finlay, et al. 1987). WGA has been reported not to affect binding of the transported protein to nuclear pores (Finlay et al. 1987, Moore & Blobel 1992). WGA blocked the import of the p1-conjugates into the nucleus. In most of the injected cells, the cytoplasmic staining was quite noticeable, and the narrow rim of staining was obtained in only a small proportion of the injected cells (II, Fig. 3E).

6 DISCUSSION

6.1 Detection of CPV and closely related parvoviruses with peptide antibodies

Antibodies for viral proteins, were first prepared by immunizing with peptides corresponding to the COOH terminus of the envelope polyprotein of moloney murine leukemia virus (MuLV) and the NH_2- and COOH-termini of simian virus 40 (SV40) transforming protein (Sutcliffe et al. 1980). Later it has been shown that relatively short linear peptides, not restricted to NH_2- or COOH-terminus of proteins, often elicit antibodies reacting with protein molecules with a complex tertiary and quaternary structure (Cheetham et al. 1991, Geysen et al. 1985, Lerner 1982, Sutcliffe et al. 1983). Such antibodies have proved to be valuable tools for detecting or purifying proteins.

CPV is a member of the feline parvovirus subgroup which includes several host range variants infecting carnivores of many different families. These host range variants have a high sequence homology (›98 %) and antigenic similarity (Parrish 1990, Truyen et al. 1996, Truyen et al. 1995, Truyen et al. 1997). Antigenic properties of the CPV capsid proteins VP1 and VP2 have been mapped by using a complete set of overlapping nonapeptides. Ten antigenic sites found: six are located on the virion surface (Langeveld, et al. 1993). One (VP2/residues 297-310), located in the most protruding region on the capsid surface, the so-called spike around the threefold-symmetry axis (Tsao, et al. 1991), overlaps with a sequence which is highly conserved in closely related parvoviruses (VP2/residues 292-309) (Truyen, et al. 1995). Moreover, one of two dominant antigenic sites, found by comparison of mutants and naturally variant viruses, was located into residues 299, 300, and 302 around the shoulder of the CPV threefold spike (Strassheim, et al. 1994). In this study, we used antibodies to synthetic peptides for detection of CPV antigens. We studied the cross-reactivity of the peptide antibodies with other members of the feline parvovirus subgroup. Peptides mimicking highly conserved VP2 and NS1 sequences of CPV were used and they elicited

antibodies which can be used in detection of CPV and some other related parvoviruses.

In general the non-structural proteins involved in the replication of the virus appear before the structural proteins. The amount of NS proteins is probably small in the early stage of infection. Result from immunofluorescence staining of BPV-infected cells with anti-peptide 2 serum of suggests that the spotted distribution of NS1 peptide antibody may reflect some kind of NS1 assembly centers inside the nucleus of infected cells. The fact that NSl proteins of CPV and BPV both can cross react with antibodies raised against peptide 2 can be explained in terms of the high degree of similarity in the NS1 protein in these viruses.

Taken together, these results suggest that sufficient structural information for recognition of proteins of CPV and related strains is contained in peptides mimicking sequences of VP2 and NS1 conserved areas. The ability to make antibodies to defined regions of CPV proteins will open the way to a number of cell biological experiments on virus-cell relationship as well as help in the immunological detection of CPV and some other related parvoviruses. It will also be of interest to study whether the peptide antibodies studied are neutralizing and whether the peptides could be used as vaccines.

6.2 Endocytic entry of CPV

Although the process of penetration of CPV into cells is still incompletely understood (this is true for most nonenveloped viruses), previous studies have dissected CPV entry into several steps:

(i) The productive infection of CPV has been shown to be initiated by the adsorbtion of virions to specific cell surface receptors identified as 40- to 42-kDa glycoproteins (Basak et al. 1994);

(ii) The entry of CPV particles 15 min after binding to its receptor has been suggested to occur mainly via small noncoated vesicles (Basak & Turner 1992).

(iii) After entry the virions have been proposed to been taken up by intracellular structures characterized as small endosome-like vesicles. Ultrastructural studies have shown that these small vesicles fuse with larger vacuoles at 1-1.5 hr postinfection (Basak & Turner 1992).

(iv) The infection of A72 cells by CPV can be prevented by lysosomotropic bases (NH4Cl and chloroquine) raising the intracellular pH, which indicates that the infectious entry pathway of CPV requires passage through an acidic intracellular compartment.

Materials (viruses) taken up by the endocytic pathway generally pass through discrete compartments characterized as early and late endosomes (Gruenberg & Howell 1989). Movement from early to late endosomes requires intact

microtubules and is mediated by vesicular intermediates known as endosomal carrier vesicles (Aniento et al. 1993, Clague et al. 1994, Gruenberg et al. 1989). Disruption of the microtubule network allows the formation of endosomal carrier vesicles from peripheral early endosomes but not their delivery to perinuclear late endosomes (Gruenberg et al. 1989). Endocytic transport between early and late endosomes has been shown to be blocked at reduced temperatures (Griffiths et al. 1988, Punnonen et al. 1998, Wolkoff et al. 1984) or with nocodazole (a microtubule-depolymerizing agent) (Avitable, et al. 1995, Gruenberg et al. 1989). In the presence of blocking factors productive infection was prevented and CPV was arrested in cytoplasmic localized vacuoles. Nocodazole added 2 hr postinfection was not inhibitory, suggesting that by that time the virions had already passed the microtubule-dependent step. These results demonstrate the involvement of microtubule-linked membrane traffic in CPV entry and suggest that CPV passage through late endosomes is essential for productive infection.

An attempt to circumvent the endocytic and membrane fusion steps in the entry process was made by microinjection of CPV particles into the cytoplasm. Although injected viruses could reach the nuclear envelope, they were unable to enter the nucleus. Injected CPV was not able to initiate progeny virus production, even if it was pretreated at pH 5.0; however it was able to concentrate around the nuclear membrane. Obviously, factors on the endocytic pathway other than low pH are required for productive infection. Thus, we conclude that endocytic entry, involving the exposure of virions to low pH, is a necessary but not a sufficient step for CPV to initiate a succesful infection. Besides the putative low pH-induced changes of virions, there may be conformational changes in the viral capsid proteins caused by other factors such as interaction with the cell surface receptor.

Interest in early virus-cell interactions is rapidly growing. Some well-characterized paradigms of early viral entry have now been established. The main challenges for the future are analysis of the early cytoplasmic and nuclear events, and elucidation of the viral uncoating mechanisms. The membrane penetration mechanisms of nonenveloped viruses like CPV remain particularly enigmatic. Proteolytic events that accompany CPV entry and uncoating are also unknown. For full understanding of the uncoating and DNA delivery mechanisms used by CPV these and other processes in the entry pathway have to be studied further.

6.3 Nuclear localization signals of CPV capsid proteins

Viruses replicating in the nucleus provide interesting systems for studying nuclear transport. Not only are in infected cells many newly synthesized structural and nonstructural proteins transported into the nucleus, but in many cases the incoming viral genome and accessory proteins must also gain access to the nucleoplasm. It is evident that most viruses that enter or exit the nucleus take advantage of the cell´s nuclear import and export machinery. With a few exceptions, viruses seem to cross the nuclear envelope through the nuclear

pore complexes, making use of cellular nuclear import and export signals, receptors, and transport factors. However, the large size of viral capsids makes the processes unique and complicated. Some kind of capsid disassembly is thought to be required before entry of the viral genome and possible accessory proteins can occur through the nuclear pores. In some viruses it is believed that the low pH in endosomes may trigger the capsid disassembly events necessary for nuclear transport (Whittaker & Helenius 1998). In theory, there are at least three different possibilities for nuclear entry of the viral genomes. (i) The viral genome is released and deproteinized from the nucleocapsid or virion outside the nucleus. (ii) The incoming virus loses the surface proteins during entry, and core particles are transported into the nucleus. (iii) The nuclear import of the viral genome begins with disassembly of the core particles outside of the nucleus. The subsequent genome transport could be mediated either by the covalently linked polymerase or by nonassembled core protein subunits attached to the viral genome. The viral NLS might be exposed and activated by limited proteolysis within the cytoplasm. In some viruses the incoming proteins are suggested to be involved in the initiation of viral gene replication (Görlich 1997, Kann et al. 1997, Whittaker & Helenius 1998).

Virtually nothing is known about the mechanism by which newly synthesized parvoviral proteins are transported into the nucleus before virus assembly, and how incoming viruses deliver their genomes and associated proteins into the nucleus. In this work, we studied the mechanism by which CPV capsid proteins are transported into the nucleus. According to crystallographic studies, CPV has a disordered amino-terminal portion of VP1 not required for coat assembly (Tsao et al., 1991). It may be assumed that the N terminus of VP1 is accessible also in the virion and hence be a good candidate for an active NLS. It is also suggested that VP1 is required for the transport of MVM to the nucleus. It is not known whether MVM enters the nucleus as an intact virion or partially disassembled DNA-protein complex (Tullis et al., 1993). The key finding of the present study is that the peptide PAKRARRGYK, corresponding to the amino terminal residues 4-13 of the capsid protein VP1, was able to target a carrier protein to the nucleus. The cluster of residues lys 6, arg 7, and arg 9 was sufficient to direct a carrier protein into the nucleus. The glycine substitution technique (Li et al. 1998) used here abolished the positive charge of the amino acid residue with a simultaneous reduction of the size of the side chain. However, a hydrophobic side chain was not introduced in contrast to alanine substitution which is widely used in similar experiments. It can be concluded that the positive side chain charge of residues 6-9 possibly in combination with side chain size, was the relevant feature of the activity of the peptides.

In competition experiments the nuclear localization was challenged by microinjecting SV40-conjugates (contains an active NLS) together with 10- to 100 fold molar excess of the potential NLS-containing VP1-conjugates. The nuclear transport of SV40-conjugate was dimished in lower concentration and was totally abolished in the presence higher concentration of the competitor. The presence of multiple signals affects the rate of the nuclear accumulation and the saturability of nuclear import has been demonstrated for canonical NLSs (Goldfarb et al. 1986, Landford et al. 1986). However, the diversity of peptides that can direct proteins into the nucleus suggests that multiple types

of NLS-binding protein might recognize varying classes of NLSs and deliver NLS-containing proteins to the NPC (Silver 1991). In this case results suggest that both conjugates were recognized by similar carrier proteins .

The present study indicated that nuclear transport through the NPC of conjugates containing potential NLS of CPV, requires energy and is temperature dependent. Furthermore, nuclear accumulation of the NLS-peptide conjugates could be inhibited by the lectin WGA which binds to pore complexes. Arrest of nuclear transport under these conditions is characteristic of large NLS-containing proteins and is distinct from the diffusion-driven transport of smaller macromolecules (Richardson et al. 1988). Also characteristic of NLS-containing proteins is their localization to a thin perinuclear band, a feature that others have shown to involve, at least in part, specific binding to the cytoplasmic face of the NPC (Newmeyer & Forbes 1988). Taken together with the observation of the reversibility of transport arrest in chilled or energy-depleted cells, the above results suggest that nuclear import of NLS-peptide conjugates is a facilitated, signal sequence-dependent process.

Several investigations have demonstrated the ability of synthetic peptides homologous to NLSs to induce the nuclear import of nonnuclear carrier proteins (Goldfarb et al. 1986, Kalderon et al. 1984). The influence of size in transport as well as the enhancement of transport by multiple identical signals per transport moiety has been also demonstrated using synthetic peptides. The use of multiple signal peptides per carrier protein permits detection of weak transport activities that are not apparent in polypeptides which contain only one NLS per molecule (Landford et al. 1990, Landford et al. 1988). However, one cannot ignore the possibility that synthetic peptides may not completely mimick possible conformational aspects of true NLSs. Future investigations, done by using recombinant DNA technology to generate NLS-containing fusion proteins, should definine further the character and role of sequences involved in nuclear transport of CPV capsid proteins.

7 CONCLUSIONS

The main conclusions are:

1. Sequences derived from highly conserved VP2 and NS1 regions of CPV elicited antibodies which can be used in detection of CPV and some other parvoviruses.

2. CPV entered the host cell via an endocytic route. The temperature- and microtubule-dependent delivery of CPV to late endosomes is required for productive infection.

3. CPV particles treated at pH 5.0 prior to microinjection were unable to initiate progeny virus production, showing that factors of the endocytic route other than low pH are necessary for the initiation of infection by CPV.

4. The N-terminal region of the VP1 capsid protein contains a potential NLS, which alone is sufficient to direct a carrier protein into the nucleus. A cluster of basic residues is essential for localization activity.

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YHTEENVETO (Résumé in Finnish)

Koiran parvovirus: tumakuljetus ja endosyyttinen sisääntulo

Parvo-suvun virukset ovat pieniä, vaipattomia DNA-viruksia. Koiran parvovirus havaittiin ensimmäisen kerran vuonna 1978, jonka jälkeen se levisi nopeasti ympäri maailmaa. Koiran parvovirus aiheuttaa sydän- ja suolistotulehduksia koirille. Infektio on letaali erityisesti pennuille.

Parvovirus kykenee lisääntymään vain aktiivisesti jakaantuvissa soluissa. Voidakseen infektoida solun, parvoviruksen täytyy tunkeutua isäntäsoluun ja kuljettaa genominsa tumaan monistumaan. Useiden virusten on osoitettu pääsevän solun sisälle reseptorivälitteisen endosytoosin avulla. Koiran parvovirusten sisääntulon on myös võitu osoittaa tapahtuvan endosytoottisen kalvoliikenteen avulla. Sisääntulon mekanismin yksityiskohdat ja se, kuinka viruspartikkeli vapautuu isäntäsolun solulimaan, ovat toistaiseksi tuntematomia. Olemme selvittäneet tutkimuksessamme endosytoottisen kalvoliikenteen eri vaiheiden merkitystä viruksen sisääntulossa. Havaitsimme mikrotubulusvälitteisen endosomaalisen liikenteen varhaisten ja myöhäisten endosomien välillä olevan olennainen osa viruksen sisääntuloa.

Solulimasta viruspartikkelit tai sen osat siirtyvät vielä epäselvien mekanismien avulla tumahuokosten läpi isäntäsolun tumaan, jossa virusgenomi monistuu. Uusien virionien kokoamisessa tarvittavat virusproteiinit syntetisoidaan solulimassa ja kuljetetaan tumahuokosten läpi tumaan, jossa viruspartikkelit kootaan proteiiniosasistaan ja virusgenomista. Parvovirusproteiinien tumakuljetuksen yksityiskohdat ovat toistaiseksi lähes täysin tuntemattomia ja niiden tutkimus on vasta alkuvaiheessa. Olemme tutkimuksessamme paikallistanneet ja karakterisoineet koiran parvoviruksen kapsidiproteiinin aminohappojärjestykseen sisältyvän tumakuljetussignaalin, joka on välttämätön edellytys proteiinien tumakuljetukselle.

Koiran parvovirus on osa parvovirusten alaryhmää, joka sisältää joukon toisilleen ja kissan panleukopenia virukselle läheisesti sukua olevia viruksia. Ryhmän jäsenet ovat geneettisesti (homologia > 98 %) ja antigeenisiltä ominaisuuksiltaan hyvin samankaltaisia. Olemme kehittäneet menetelmän, jonka avulla koiran parvovirus voidaan havaita käyttämällä vasta-aineita, jotka on tuotettu synteettisiä peptidejä vastaan. Peptidien aminohapposekvenssit on valittu sellaisilta alueilta koiran parvoviruksen kapsidiproteiinia, jotka ovat samankaltaisia kaikilla alaryhmän jäsenillä. Saatujen peptidivasta-aineiden avulla on mahdollista paikallistaa immunologisesti koiran parvoviruksen lisäksi myös läheisten lajien viruspartikkeleita tai pintaproteiineja.

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ORIGINAL PAPERS

I

Detection of canine parvovirus antigens with antibodies to synthetic peptides.

by

Vihinen-Ranta, M., Lindfors, E., Heiska, L., Veijalainen, P. & Vuento, M.

Archives of Virology 141: 1741-1748, 1996

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II

Characterization of a nuclear localization signal of canine parvovirus capsid proteins.

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Vihinen-Ranta, M., Kakkola, L., Kalela, A., Vilja, P. & Vuento, M.

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III

Intracellular route of canine parvovirus entry

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