



FACULTY OF VETERINARY MEDICINE
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Laboratory of Virology
Department of Virology, Parasitology and Immunology
Faculty of Veterinary Medicine
Ghent University

Entry of feline infectious peritonitis virus in blood monocytes

Evelien Van Hamme

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Promotor
Prof. Dr. Hans Nauwynck

Co-promotor
Dr. ir. Hannah Dewerchin

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LIST OF ABBREVIATIONS

aa	amino acids
ACE2	angiotensin-converting enzyme 2
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AP	adaptor protein
APN	aminopeptidase N
CALM	clathrin-assembly lymphoid-myeloid leukaemia
cav	caveolin
CCoV	canine coronavirus
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CHO (cells)	Chinese hamster ovary (cells)
CHO-DC-SIGN	CHO cells stably expressing DC-SIGN
Chol tox B	Cholera toxin B
c-lig	control ligand
CME	clathrin-mediated endocytosis
CPE	cytopathogenic effect
CrFK (cells)	Crandell feline kidney (cells)
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule (ICAM3) grabbing nonintegrin
DC-SIGNR	DC-SIGN related
dip	dynamamin inhibitory peptide
DN	dominant-negative
dyn	dynamamin
E	envelop (protein)
E-64	trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane
eFCoV	enteric feline coronavirus
ERGIC	ER tot Golgi intermediate compartment
fAPN	feline aminopeptidase N
FCoV	feline coronavirus
fDC-SIGN	feline DC-SIGN
FCS	foetal calf serum
Fcwf (cells)	Felis catus whole foetus
FIPV	feline infectious peritonitis virus

FIV	feline immunodeficiency virus
GEEC	GPI-anchored protein enriched endocytic compartments
GPI	glycosylphosphatidylinositol
hAPN	human aminopeptidase N
HCoV	human coronavirus
hDC-SIGN	human DC-SIGN
HIV1	human immunodeficiency virus 1
HR	heptad repeat
IBV	infectious bronchitis virus
IL-2R	interleukin-2 receptor
irr	irrelevant
L-SIGN	liver/lymph node specific ICAM3 nonintegrin
M	membrane (protein)
mAb	monoclonal antibody
MDCK (cells)	Madin-Darby canine kidney (cells)
MHV	mouse hepatitis virus
moi	multiplicity of infection
N	nucleocapsid (protein)
ORF	open reading frame
PAK	p21-activated kinase
pAPN	porcine aminopeptidase N
PBS	phosphate buffered saline
PBS-G	PBS supplemented with gelatine
PEDV	porcine epidemic diarrhoea virus
PI3K	phosphatidylinositol 3-kinase
PRCoV	porcine respiratory coronavirus
PRRSV	porcine reproductive and respiratory syndrome virus
RdRp	RNA-dependent RNA-polymerase
Rec X	unknown receptor 'X'
RTC	replication-transcription complex
SARS	severe acute respiratory syndrome
TBS	Tris-buffered saline
TBS-GS	TBS supplemented with sucrose and inactivated goat serum
Tfn	transferrin
TGEV	transmissible gastroenteritis virus

TNF- α	tumour necrosis factor α
TRS	transcription-regulatory sequence
S	spike (protein)
WT	wild type
γ c	common cytokine receptor γ

Chapter 1

INTRODUCTION

1.1 THE FELINE INFECTIOUS PERITONITIS VIRUS

1.1.1 Introduction - taxonomy

Nidoviruses, members of the order *Nidovirales*, are one evolutionary lineage among the positive stranded RNA viruses. Their genome organization and the relatedness of their proteins involved in RNA replication and transcription ties them together and distinguishes them from other viruses (Siddell & Snijder, 2008). The *Nidovirales* enclose three families: the *Coronaviridae*, the *Arteriviridae* and the *Roniviridae*, found to be evolutionary distinct based on sequence analysis of their RNA-dependent RNA-polymerase (RdRp) (Gorbalenya *et al.*, 2006). The *Coronaviridae* consist of two genera: *Coronavirus* and *Torovirus*. The coronavirus genus is further divided in three groups. Coronavirus species are grouped based on genetic and antigenic criteria (Gonzalez *et al.*, 2003). Table 1 lists all recently accepted species of coronaviruses and situates them within one of the three groups. Further, the strains or biotypes of species often referred to in literature, are also mentioned. These coronaviruses are associated with either respiratory or enteric diseases (Siddell & Snijder, 2008).

Feline coronaviruses are found in group 1. Based on neutralization reactivity with specific antibodies, they are divided in two serotypes: I and II (Fiscus & Teramoto, 1987). Type I grows poorly in cell culture, while type II grows easily in many different cell lines (Pedersen *et al.*, 1984). Type II has arisen from double recombination events between type I FCoV and CCoV (Herrewegh *et al.*, 1998). FCoV type I is the most prevalent serotype with a prevalence of approximately 85 % in Switzerland and Austria (Benetka *et al.*, 2004; Kummrow *et al.*, 2005).

Apart from the different serotypes, there are also two pathotypes or virulence variants. First there is the mild, mostly unapparent enteric feline coronavirus (eFCoV). Almost all Swiss cats and 17 % of the British cats are seropositive. Second, there is the systemic, highly lethal feline infectious peritonitis virus (FIPV). Approximately 5 % of the seropositive cats eventually develop FIPV (personal communication Dr Addie). This implies that 1 to 5 % of the European cat population is a potential victim of this infection.

Table 1: List of the currently known coronaviruses (adapted from Siddell & Snijder, 2008).

CoV group	species	strains or biotypes	abbreviation
Group 1	Canine coronavirus	Canine coronavirus	CCoV
	Feline coronavirus	(Enteric) feline coronavirus	(e)FCoV
		Feline infectious peritonitis virus	FIPV
		Human coronavirus 229E	Human coronavirus 229E
		Human coronavirus NL63	HCoV-NL63
	Porcine epidemic diarrhea virus	Porcine epidemic diarrhea virus	PEDV
	Transmissible gastroenteritis virus	Transmissible gastroenteritis virus	TGEV
		Porcine respiratory coronavirus	PRCoV
	Bat coronavirus	Bat coronavirus	BtCoV
Rabbit coronavirus	Rabbit coronavirus	RbCoV	
Group 2a	Canine respiratory coronavirus	Canine respiratory coronavirus 4182	CRCoV-4182
	Bovine coronavirus	Bovine coronavirus	BCoV
	Human coronavirus OC43	Human coronavirus OC43	HCoV-OC43
		Human coronavirus HKU1	HCoV-HKU1
	Human enteric coronavirus	Human enteric coronavirus	HECoV
	Murine hepatitis virus	Murine hepatitis virus JHM	MHV-JHM
		Murine hepatitis virus A59	MHV-A59
	Porcine hemagglutinating encephalomyelitis virus	Porcine hemagglutinating encephalomyelitis virus	HEV
	Puffinosis coronavirus	Puffinosis coronavirus	PCoV
	Rat coronavirus	Rat coronavirus	RtCoV
		Sialodacryoadenitis	SDAV
Group 2b	Severe acute respiratory syndrome virus	Severe acute respiratory syndrome virus	SARS-CoV
	Bat coronavirus	Bat coronavirus 133/2005	BtCoV-133/2005
Group 3	Infectious bronchitis virus	Infectious bronchitis virus	IBV
	Pheasant coronavirus	Pheasant coronavirus	PhCoV
	Turkey coronavirus	Turkey coronavirus	TCoV

1.1.2 Pathogenesis of the feline coronaviruses

Feline enteric coronavirus (eFCoV)

Feline enteric coronavirus is a ubiquitous, worldwide intestinal virus (Pedersen *et al.*, 1981; 2004). The importance of eFCoV as a primary intestinal pathogen is however minimal as the infection is usually unapparent or manifested by a transient gastroenteritis (Pedersen *et al.*, 1981; Hayashi *et al.*, 1982; Pedersen *et al.*, 2008).

The virus is spread by fecal-oral route. eFCoV has a tropism for enterocytes in the epithelium of intestinal villi (Pedersen *et al.*, 1981). During the course of a natural infection some eFCoVs may be detected in the blood, but much less than seen with FIPV (Meli *et al.*, 2004). Further, virus can also be detected in mesenteric lymph nodes, spleen, bone marrow, tonsils and thymus (Pedersen *et al.*, 1984; Herrewegh *et al.*, 1995; Gunn-Moore *et al.*, 1998). Obviously, infection leads to systemic disease. Virus shedding occurs within a week after exposure and remains at high levels for 2 to 10 months. Then, it evolves into one of three excretion patterns: some cats shed the virus persistently, some have periods of shedding interlaced with periods of non-shedding (recurrent shedders) and some just cease shedding (Pedersen *et al.*, 2008). Immunity generated upon infection is slow to develop, variable in strength and duration and lacks memory explaining why reinfection can occur easily (Addie *et al.*, 2003; Pedersen *et al.*, 2008).

The importance of eFCoV lies in its potential to mutate *in vivo* into a biotype that causes the highly fatal disease, feline infectious peritonitis (Poland *et al.*, 1996; Vennema *et al.*, 1998). Mutations that might be responsible for the FIPV biotype were identified in the 3c gene and/or to a lesser extent in the 7b gene (Vennema *et al.*, 1998). Pedersen claims that eFCoVs and FIPVs can be discriminated based on the 3c gene (Pedersen, 2009). The acquisition of an enhanced macrophage tropism is also essential for the development of FIP. Rottier *et al.* showed that the C-terminal domain of the spike protein is the determinant for efficient macrophage infection and that it distinguishes eFCoV strains from FIPV strains (2005). Taken together, these results suggest that the mutational transition between eFCoV and FIPV requires mutations in both the spike and the accessory gene(s). The higher the level of eFCoV replication, the more chance a mutation occurs and the cat develops FIP. Therefore, FIPV arises mostly during primary eFCoV infection and in kittens (Pedersen *et al.*, 2008).

Feline infectious peritonitis virus (FIPV)

The evolution from eFCoV to FIPV is accompanied by the acquisition of an enhanced macrophage tropism (Stoddart & Scott, 1989; Rottier *et al.*, 2005). Circulating blood monocytes are the predominate target cells for FIPV. These are migratory cells and after infection, they may pass through the endothelium of small vessels and initiate infection in various parts of the body (Fenner *et al.*, 1974; Weiss & Scott, 1981). Targets are the mesenteric lymph nodes, serosal surfaces of the gut and organs, and to a lesser extent the pleura, and the omentum. Some virus also appears to reach the meninges, the ependyma

surrounding the ventricles, the spinal cord, and the uveal tract and retina of the eyes (Pedersen, 2009).

Subsequent inflammatory reactions at these locations can lead to intravascular coagulation and vasculitis (Weiss *et al.*, 1980). Circulating immune complexes sediment in small vessels leading to complement activation. This triggers vasodilatation and the release of chemotactic mediators which attract monocytes. Therefore, inflammation is associated with expanding cycles of monocyte/macrophage infection, virus replication and release. Released virions in turn infect the attracted macrophages and the infection site expands dramatically. This reaction is centered on small venules and the result is a lesion called pyogranuloma (Pedersen & Boyle, 1980; Jacobse-Geels *et al.*, 1980; 1982). Further, FIPV-induced vasculitis is characterized by macrophage-dominated circular infiltrates in small veins and focal infiltrates in larger veins. Activated monocyte attachment to endothelial cells, subsequent emigration and perivenous macrophage accumulation cause the destruction of the vascular basal lamina through the secretion of matrix metalloproteinase B. This results in vasculitis and possible leakage (Kipar *et al.*, 2005).

There are two forms of FIPV: wet/effusive FIPV and dry/non-effusive FIPV. Wet/effusive FIPV is characterized by leakage of protein-rich fluid into the peritoneal cavity or (less frequent) the pleural or cardiac cavity. Effusion in the peritoneal cavity leads to the typical physiology of a skinny FIP cat with a swollen abdomen. Often dry FIPV eventually evolves to wet FIPV when the immune system totally collapses. When a cat develops dry FIPV, it is believed that the animal is partially protected against infection by the cellular immune system.

Virus specific antibodies fail to control or clear the infection despite the presence of high titers (Paltrinieri *et al.*, 1998). They even seem to facilitate infection. First, this may be explained by the occurrence, at least *in vitro*, of antibody-dependent enhancement (ADE) of infection of macrophages (Hohdatsu *et al.*, 1991; Olsen *et al.*, 1992). The *in vivo* relevance of ADE of infection is questioned as in natural infections of seropositive animals no enhancement of infectivity seems present. Seropositive cats showed a normal FIP progression and did not die more rapidly (Addie *et al.*, 1995). Second, antibodies mediate internalization of surface expressed viral proteins on infected cells, making the infected monocyte invisible for the immune system (Dewerchin *et al.*, 2007; Cornelissen *et al.*, 2007). Further, despite the presence of antibodies and complement, there is no antibody-dependent complement-mediated lyses of infected monocytes (Cornelissen *et al.*, 2009).

The humoral immune response is obviously not capable of successfully fighting the infection. Protection or partial protection against FIPV is therefore supposed to be cell-mediated. It is

suggested that the efficacy of early T-cell responses critically determines the outcome of infection (de Groot-Mijnes *et al.*, 2005). Nevertheless, the cell-mediated immune system is also targeted by the FIP virus. In FIPV infected cats, T-cell depletion occurs in both lymphoid tissues and in the blood (Kipar *et al.*, 2001; Paltrinieri *et al.*, 2003; de Groot-Mijnes *et al.*, 2005). This T-cell depletion is caused by a soluble factor, possibly glycoprotein 7b (see further in 1.1.3.2), present in the supernatant of infected cells, through apoptosis (Haagmans *et al.*, 1996). Much remains to be learned on cell-mediated immunity in FIPV infection. This knowledge is indispensable for a better understanding of the pathogenesis of FIPV.

1.1.3 Structure of the virion

1.1.3.1 Genomic organization

FIPV contains an extremely large positive-stranded RNA genome of 29 125 nt in length excluding the poly(A) tail, with a 5' cap (Fig. 1) (Dye & Siddell, 2005). The replicase gene encompasses two thirds of the genome and consists of 2 open reading frames (ORFs), ORF 1a and 1b. ORF 1a encodes polyprotein pp1a, while ORF 1a and 1b together encode pp1ab through a ribosomal frameshift during translation of genomic RNA (Brierley, 1995). The polyproteins are processed by viral proteinases to 16 non-structural proteins (Ziebuhr *et al.*, 2000). These replicase proteins assemble to form the membrane-bound replication-transcription complex (RTC) in the cytoplasm of the cell (Gosert *et al.*, 2002). ORFs 2, 4, 5 and 6 encode the structural proteins that are incorporated in the virion. ORFs 3 and 7 encode the accessory proteins. Expression of genes 2-7 occurs from a nested set of subgenomic mRNAs with a common leader sequence (Fig. 1). mRNA synthesis is controlled by a transcription-regulatory sequence (TRS) (Haijema *et al.*, 2007).

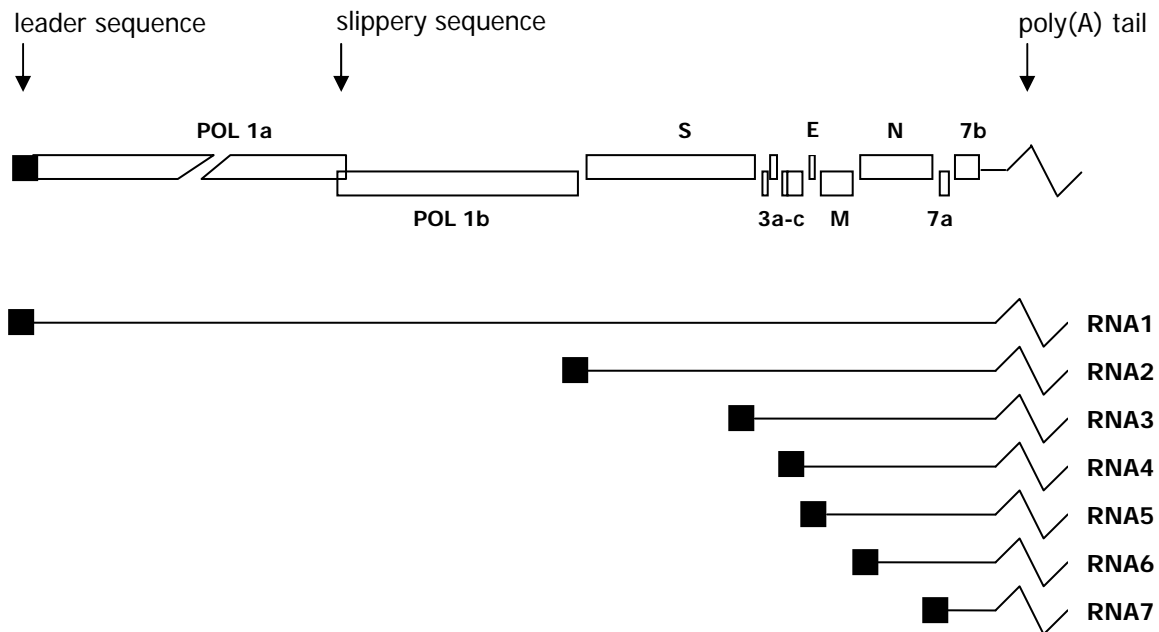


Figure 1: Genomic organization of FIPV with the leader sequence, 8 ORFs and the poly(A) tail. The nested set of 7 subgenomic mRNAs is also depicted.

1.1.3.2 Viral proteins

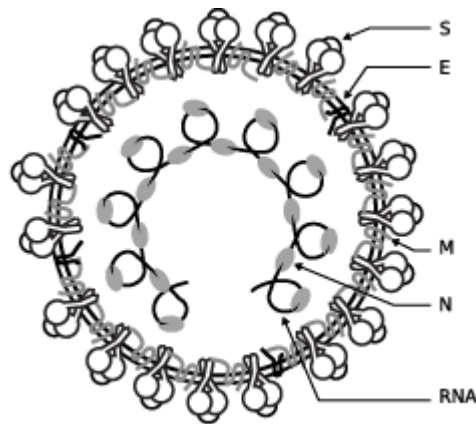


Figure 2: Schematic representation of a FIPV virion (Dewerchin, 2008).

Spike protein

The large petal-shaped spikes determine the characteristic morphology of the coronavirus virion. These protrusions of approximately 20 nm are formed by trimers of the monomeric spike (S-) protein (Fig. 2). The nascent S polypeptide is directed to the ER by a signal peptide (Masters, 2006). During maturation in the ER, S is heavily glycosylated and the ~150-200 kDa monomers form trimers. S-trimers are incorporated in virions by associating with the membrane protein during the budding process in the ER-to-Golgi intermediate compartment (ERGIC).

The spike protein is responsible for diverse interactions that are critical for establishing and maintaining infection in the host. First, the spike protein determines host range by inducing viral entry after specific interaction with its receptor (Dye *et al.*, 2007). Second, the spike protein contains epitopes important for antibody neutralization and on the other hand for various immune evasion processes, namely antibody-mediated enhancement of infection and antibody-induced internalization of viral proteins expressed in the plasma membrane (Hohdatsu *et al.*, 1991; Hohdatsu *et al.*, 1994; Dewerchin *et al.*, 2006).

Coronavirus spike proteins are class I fusion proteins and they are primed for fusion through cleavage with furin (Bosch *et al.*, 2003). However, the group 1 coronaviruses, including FIPV, are generally considered to carry uncleaved S-proteins. Regan *et al.* claim that the FCoV S-protein is cleaved by cathepsins (2008), while de Haan *et al.* state that cathepsin cleavage does not occur (2008). Instead, they showed that some specific group 1 coronavirus spikes carried a furin enzyme recognition motif that could be lost by a single mutation upon culture adaptation resulting in a heparin binding motif (de Haan *et al.*, 2008).

Envelope protein

The envelope (E-) or small membrane protein is a small protein of approximately 10 kDa that is expressed in virions and infected cells in small amounts. E-protein is an integral membrane protein whose highly hydrophobic N-terminal two thirds region is a transmembrane domain that spans the bilayer twice (Fig. 2) (Maeda *et al.*, 2001). The C-terminal region extends into the virion interior. Through its interaction with M-protein, E-protein is necessary for envelope formation and budding (Lim & Liu, 2001). To fulfil its task, E-protein is retained in the pre-Golgi membranes of the intermediate compartment, prior to virus budding in the ERGIC.

Membrane protein

The coronavirus membrane or M-protein is a protein, ranging from 20-38 kDa according to glycosylations, that spans the viral envelope three times, leaving only small parts 'hanging out' on either site of the membrane (Fig. 2) (Rottier *et al.*, 1986). M is supposed to be the determinant for the intracellular budding site. It is localized in the Golgi region which correlates with the ER to Golgi intermediate compartment (ERGIC) being the budding site (Rottier & Rose, 1987). By associating with the S-protein, the M-protein retains it at the budding site and prevents transport of S to the plasma membrane (Opstelten *et al.*, 1995). Probably the M-protein may be involved in viral entry in monocytes/macrophages as there are neutralizing antibodies against M (Kida *et al.*, 2000). Upon addition of antibodies,

surface-expressed M-protein is internalized (like described above for S-protein), so this protein is also involved in this immune evasion process (Dewerchin *et al.*, 2006).

Nucleocapsid protein

N-protein is a 42.7 kDa protein, highly phosphorylated on serine residues (Spaan *et al.*, 1988). After phosphorylation in the cytoplasm, the proteins become associated with intracellular membranes (Stohlman *et al.*, 1983). N-proteins interact with viral RNA and stabilize the helical symmetric nucleocapsid structure that is packaged into virions (Fig. 2) (Zuniga *et al.*, 2007). Antibodies against N-protein were able to block viral RNA transcription, suggesting another potential role for N-protein in infection (Compton *et al.*, 1987; Baric *et al.*, 1988). This is confirmed by the co-localization of N-protein with the replication complex in the sites of RNA synthesis at early times post-infection (Denison *et al.*, 1999). Next to its presence at the replication site, N also accumulates in the Golgi region near the budding site and co-localizes with the viral spike (Stertz *et al.*, 2007). N-M interactions might also be important for viral structure (Sturman *et al.*, 1980).

Accessory proteins

All coronaviruses encode a number of accessory proteins in group-specific genes, that are thought to be dispensable for replication in cell culture, but apparently provide a selective advantage *in vivo* (de Haan *et al.*, 2002; Ortego *et al.*, 2003). For FIPV 79-1146, two regions of the genome have been identified that encode putative accessory proteins. They are known as ORFs 3abc and 7ab (Fig. 1). The gene products and function of ORF 3abc remain to be identified. The predicted size of the protein encoded by ORF 7a is 11 kDa. ORF 7b is known to encode a non-structural, secretory viral glycoprotein 'gp7b' of 26.5 kDa (Vennema *et al.*, 1992). This secretory factor might be responsible for the observed induction of T-cell depletion during FIPV pathogenesis (Haagmans *et al.*, 1996). The importance of the accessory proteins in FIPV pathogenesis was illustrated by the development of a live, attenuated vaccine by deletion of the group-specific genes from FIPV 79-1146 (Haijema *et al.*, 2004).

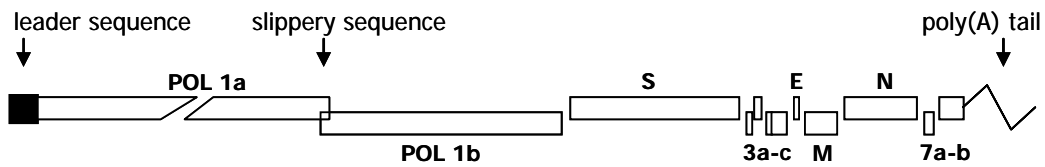
1.1.4 Replication cycle

1.1.4.1 Virus entry

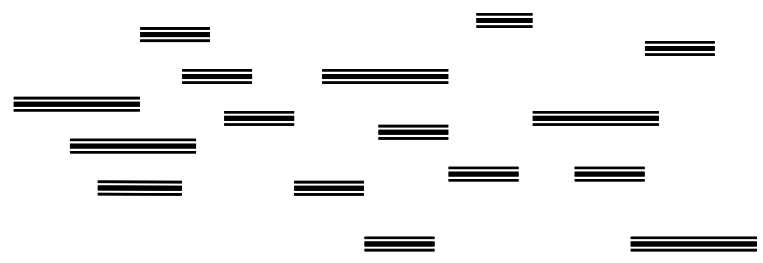
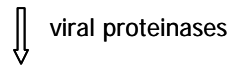
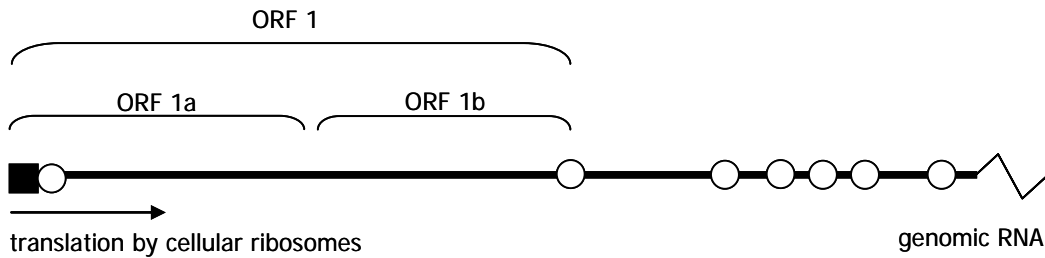
The process of virus entry, including binding to virus receptor(s), internalization into the cell and eventually uncoating or release of the genome in the cytosol will be discussed in detail in part 1.2 (Fig. 5, step 1-3). After release of the genome, replication and transcription can start in the cytoplasm of the infected cell. Replication is the process whereby genome-sized RNA, which also functions as mRNA, is produced. Transcription is defined as the process whereby subgenome-sized mRNAs are produced (Sawicki *et al.*, 2007).

1.1.4.2 Replication

The 16 non-structural proteins encoded in ORF 1 are directly translated upon 'arrival' in the cytoplasm (Fig. 3 & Fig. 5, step 4). Together with viral N-protein and, possibly, cellular proteins, they assemble into the membrane-bound replication-transcription complex (RTC). In these complexes the production or copying of the genome- or subgenomic length RNA occurs. RTCs accumulate at perinuclear regions and are associated with double-membrane vesicles (Brockway *et al.*, 2003; Snijder *et al.*, 2006) (Fig. 5, step 5). The non-structural proteins include proteinase, polymerase and helicase (Snijder *et al.*, 2003). The RTC copies the genome either continuously into genome-length template or discontinuously into various subgenome-length minus-strand templates. The minus-strand genomic template is used for genome synthesis (Fig. 4) (Sawicki *et al.*, 2007).



Translation of non-structural proteins



16 non-structural proteins
including proteinases, polymerase and helicase



assembly into membrane-bound replication-transcription complex

Figure 3: Translation of the non-structural proteins encoded by open reading frame (ORF) 1. **—** represents positive-strand RNA. **≡** represents protein. pp stands for polyprotein.

1.1.4.3 Transcription

Coronavirus transcription is characterized by the generation of a nested set of mRNAs, with each mRNA having the identical “leader” sequence (Fig. 1). During subgenome-length minus-strand mRNA synthesis, the nested set of mRNAs is generated through discontinuous extension (Sawicki & Sawicki, 1995). Each subgenome-length mRNA contains a 5' leader sequence corresponding to the 5' end of the genome. This 5' leader is joined to a mRNA “body”, which represents sequences from the poly(A) stretch to a position that is upstream of each genomic ORF encoding a structural or accessory protein (cluster). The junction between leader and the mRNA body in each mRNA is called the transcription regulatory sequence (TRS) of about 10 nucleotides (Fig. 4) (Sawicki *et al.*, 2007). The process of discontinuous transcription during minus-strand synthesis starts by the recruitment of the components of a functional RTC and the initiation of synthesis at the 3' end of the genomic RNA until the first TRS is encountered. Then, some RTCs will just continue the synthesis, while a fraction will stop strand synthesis and relocate to the 5' end of the genome to the leader. As there are 7 TRSs, there are 7 minus-strand mRNAs synthesized including the genomic mRNA template (Fig. 5, step 6). When the nested set of minus-strand subgenomic mRNAs is generated, positive-strand genomic and subgenomic mRNAs are produced as templates for translation (Fig. 4).

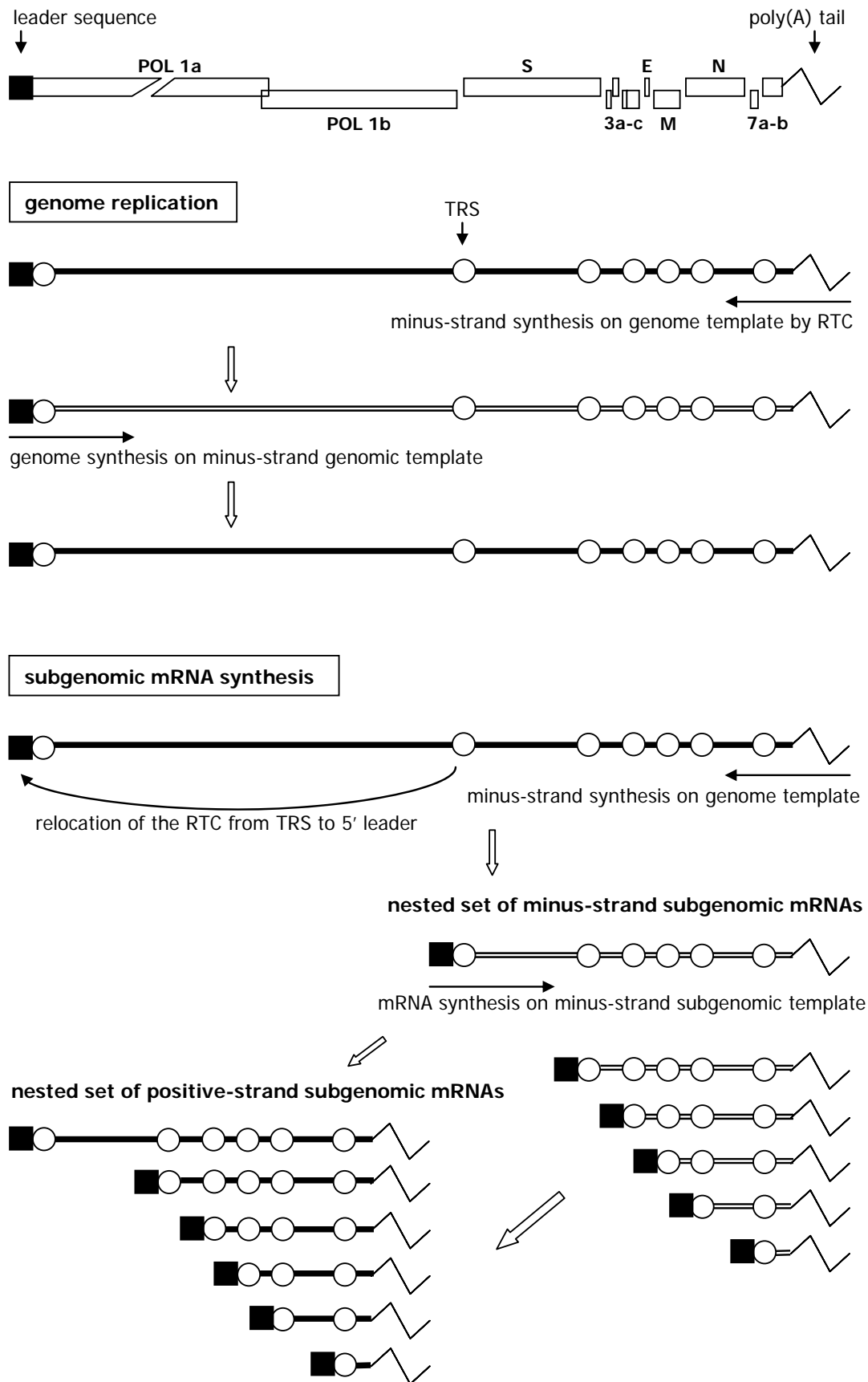


Figure 4: Genome replication and transcription. == represents minus-strand RNA.
 — represents positive-strand RNA.

1.1.4.4 Translation

Viral (sub)genomic mRNAs resemble host mRNAs and are recognized by the host translation machinery (Fig. 5, step 7). The initiation of translation occurs through ribosomal scanning starting at the 5' cap structure provided by the 5' leader to an AUG codon (Kozak, 1989). Protein synthesis then continues until a termination codon is encountered and the ribosome complex detaches from the RNA. Coronavirus mRNAs are structural polycistronic but can be both functional monocistronic as polycistronic, the latter when a coding sequence contains more than one translational active ORF.

Little is known about the regulation of translation. For functionally monocistronic RNAs, the level of protein expression depends on the amount of mRNA and therefore transcription. However, there are some indications that the leader sequence may influence translation levels (Luytjes *et al.*, 1995). For the polycistronic mRNAs the mechanisms are more evident. The best known example is the ribosomal -1 frameshift in ORF 1 made by one third of the ribosomes on the slippery sequence AAUUAUC (Brierley *et al.*, 1987; Bredenbeek *et al.*, 1990).

N-proteins are expressed in the cytosol, while S, E and M are co-translationally embedded in the membranes of the rough ER.

1.1.4.5 Budding

Positive-strand genomic RNA interacts with N-protein in the cytosol to form the nucleocapsid (Fig. 5, step 7b). The viral structural proteins S, E and M migrate from the membranes of the ER to the ER-to-Golgi intermediate compartment (ERGIC). The presence of the E-protein is essential for efficient assembly (Lim & Liu, 2001). In the ERGIC, the nucleocapsid is surrounded by an envelope containing viral proteins through the interaction between M- and N-protein as the budding process proceeds (Fig. 5, step 8).

It has not been established how the virus spreads to other cells. Egress via exocytosis could occur at lower levels of virus production and leaves the infected cell intact. Replication could also result in lysis and destruction of the host cells, releasing all produced virions (Baker, 2008).

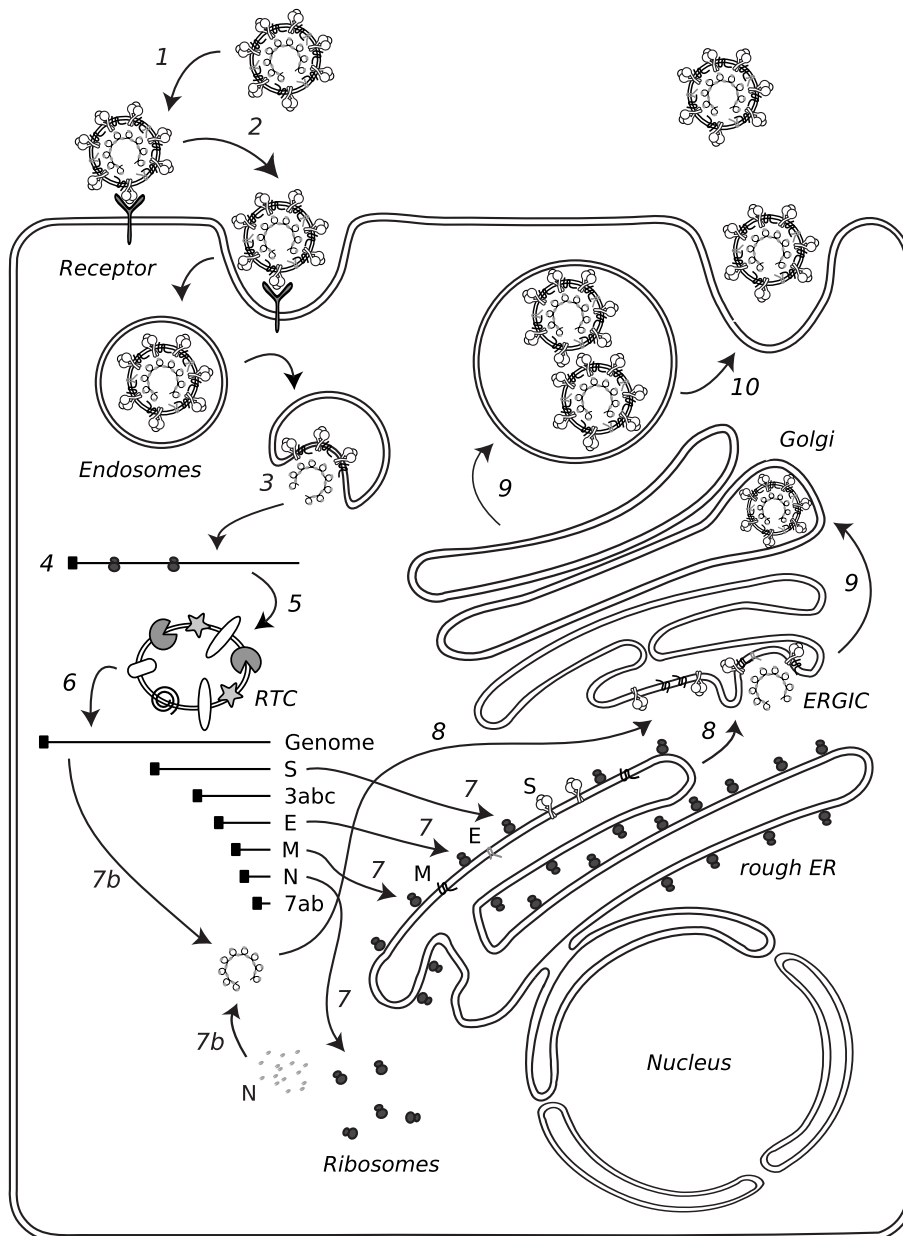


Figure 5: Replication cycle of FIPV. 1-2 Binding and internalization. 3 Release of the genome into the cytosol. 4 Translation of the 16 non-structural proteins. 5 Assembly of the RTC. 6 Genome synthesis and transcription to mRNAs. 7 Translation mRNAs to proteins. 7b Association of N proteins and genome to form nucleocapsid. 8 Accumulation of viral proteins in the ERGIC for budding into new virions. 9 Transport through the secretory pathway. 10 Release of progeny virus (Dewerchin, 2008).

1.1.5 Prevention and treatment

1.1.5.1 Prevention

Many attempts were made in the past to induce protective immunity against the development of FIP in cats through vaccination. Most of these attempts failed and even led to accelerated disease. The enhancement of disease was probably due to the induction of a humoral immune response rather than a protective, cellular immune response. The first and only commercially marketed vaccine is Primucell FIP[®], a temperature-sensitive mutant of FIPV that is applied as a modified live vaccine. It induces a strong mucosal immune response (IgA) and a cell-mediated immune response measured by lymphocyte proliferation (Gerber *et al.*, 1990). The vaccine does not offer full protection and needs to be administered frequently. The usefulness of this vaccine is being questioned as it only works when administered to seronegative cats. Further promising results - also with a modified live FIP vaccine - were obtained by Haijema *et al.* through vaccination with deletion mutants of a serotype II FIPV strain lacking the group specific genes 3a-c or 7ab (2004). The mutants replicate well in cell culture and show an attenuated phenotype in cats. Vaccinated cats are protected against a lethal homologous challenge. An extension of these data with challenges with serotype I strains are being expected.

Another strategy to prevent or at least minimize FIPV infection is reducing the FCoV infection pressure in households. This can be achieved by separating seronegative from seropositive animals and applying high hygiene standards.

1.1.5.2 Treatment

As FIP is an immune-mediated disease, treatment is mostly aimed at controlling or modulating the immune response. Glucocorticoids are often used in an attempt to slow disease progression but do not provide a cure. The antiviral drug ribavirin, a nucleoside analogue, is active against FCoV *in vitro* but cannot be used to treat cats as it causes severe side effects when administered *in vivo* (Weiss *et al.*, 1993). In cats treated with a combination of human interferon- α and *Propionibacterium acnes*, the mean survival time was prolonged for a couple of days (Weiss *et al.*, 1990). The latest strategy in the search for an effective treatment is combining feline interferon- ω with glucocorticoids. While some results indicate prolonged survival, others did not (Ishida *et al.*, 2004; Ritz *et al.*, 2007). Clearly, there is no effective treatment available for FIPV. When a cat is diagnosed with FIP, it will most likely die.

The development of an effective vaccine or treatment for FIP remains a huge challenge in CoV research.

1.2 VIRUS ENTRY

1.2.1 Introduction

Essential for a virus to cause infection is gaining entry into a cell. For a number of steps in the viral replication cycle, the virus relies on cellular factors and mechanisms to guide the way. Virus entry is a multistep process during which each step is pre-programmed and tightly regulated in space and time (Smith & Helenius, 2004). Virus entry starts with recognition of the target cell through cell surface receptors or attachment factors. These can vary from being abundant and ubiquitous to rare and cell specific. Receptors often determine the cell type that can be infected. They can be just binding receptors that capture the virus or they can be 'true' receptors that also lead the virus across the plasma membrane (Smith & Helenius, 2004). The internalization of the virus into the cell is the second step in the entry process. The viral membrane can fuse directly with the host cell plasma membrane or the virus particle can be endocytosed in a vesicle pinched off the plasma membrane. Upon direct fusion, the viral genome is delivered directly into the cytosol. Upon endocytosis, a further uncoating of the virion is necessary (Lanzrein *et al.*, 1994). Uncoating can be triggered by a number of factors, eg interaction with an additional receptor, exposure to low pH or reimmersion into a reducing environment (Smith & Helenius, 2004). Uncoating results in release of the genome from the vesicle into the cytosol where transcription, and translation by the host cell ribosomes can start. For most RNA viruses, no transport to the nucleus is required.

1.2.2 Receptors important for group 1 coronaviruses

1.2.2.1 Aminopeptidase N

Aminopeptidase N (APN) or CD13 is a type II metalloprotease that belongs to the M1 family of the MA clan of peptidases (Rawlings & Barret, 1999). The 'MA' clan of peptidases is the first ('A') clan of metallopeptidases ('M'). Human APN is an integral membrane protein of approximately 967 amino acids (aa) and 110 kDa (Sjöström *et al.*, 2000). Structural studies show that it consists of 7 domains (Fig. 6). Domain I is a short cytoplasmic N-terminal domain of only 8 to 10 aa. No signalling sequences have been found in this domain, therefore it has been suggested that APN signals over the membrane via an auxiliary

membrane protein (Sjöström *et al.*, 2000). Domain II is a single transmembrane part. The large cellular ectodomain contains a stalk region (domain III) that 'carries' the four globular-like domains (domain IV to VII). The Zn-dependent active catalytic site for the enzyme preferentially releases N-terminal neutral or basic residues of oligopeptides and is located between domain V and VI. In most species aminopeptidase N forms homodimers (Sjöström & Norén, 1982). The interaction between monomers is non-covalent and localized within domains V to VII. The charged C-terminal helix at domain VII might be involved in dimerisation. Dimerisation occurs before Golgi-associated processing, suggesting that this might be crucial for the transport of the intracellular high-mannose form of APN out of the ER (Danielsen, 1990a,b). In the Golgi, APN is N- and O-glycosylated (Fig. 6) (Sjöström *et al.*, 2000).

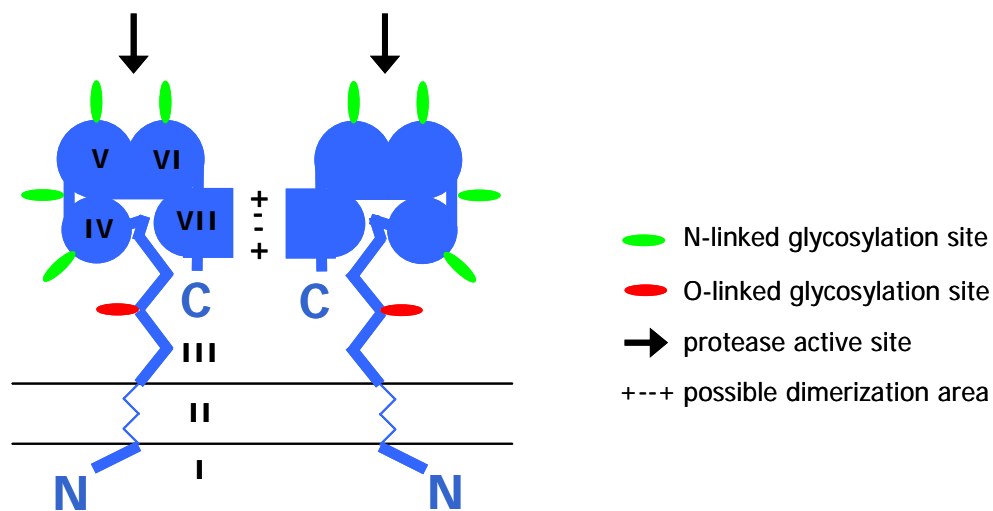


Figure 6: Model of aminopeptidase N. The monomer consists of 7 domains and is highly glycosylated. APN forms non-covalently linked homodimers.

Aminopeptidase N is both ubiquitous and multifunctional (Luan & Xu, 2007). It is present in a number of organs, tissues and cell types including endothelial and epithelial cells, and fibroblasts and leukocytes. It is, among other processes, related with tumorigenesis, trimming of antigen and the process of antigen presentation, and serves as a receptor for several pathogens. The function that APN plays, depends on the location and tissue of origin (Nocek *et al.*, 2007). In monocytes, APN is linked with signal transduction pathways. Crosslinking of APN leads to an increase in intracellular Ca^{2+} ions and phosphorylation of mitogen-activated (MAP) kinases. Phosphatidylinositol 3 kinases (PI3K) are linked with this signalling cascade as well (Navarrete Santos *et al.*, 2000). APN can also functionally associate with receptors for immunoglobulin G ($\text{Fc}\gamma\text{Rs}$) and may act as a signal regulator of $\text{Fc}\gamma\text{R}$ function (Mina-Osorio & Ortega, 2005).

Human aminopeptidase N also mediates human cytomegalovirus infection in lung fibroblasts by binding, and possibly internalizing, virus to these susceptible cells (Söderberg *et al.*, 1993).

Most group 1 coronaviruses can use aminopeptidase N of their natural host as receptors (see further in 1.2.3.1).

1.2.2.2 DC-SIGN and L-SIGN

The dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) or CD209 and its close relative DC-SIGNR (DC-SIGN related) or L-SIGN (liver/lymph node-specific) or CD209L are type II transmembrane proteins and members of the C-type lectin family. DC-SIGN and L-SIGN consist of four domains: a cytoplasmic domain necessary for signalling with a di-leucine motif for internalization and a tri-acidic cluster for targeting to proteolytic vacuoles, a single-spanning transmembrane region, a region with a series of seven and a half repeats of 23 aa and a carbohydrate-recognition domain (CRD) (Fig. 7) (Snyder *et al.*, 2005). The extracellular domain of each receptor is a tetramer of approximately 145 kDa stabilized by an α -helical stalk or neck. DC-SIGN and L-SIGN recognize specifically high-mannose carbohydrates through the CRDs. The binding depends on Ca^{2+} and can be inhibited by mannan, mannose and EGTA. When the CRDs are clustered in the tetrameric extracellular domain, their arrangement provides a means of amplifying specificity for multiple glycans on host molecules targeted by DC-SIGN and L-SIGN (Mitchell *et al.*, 2001).

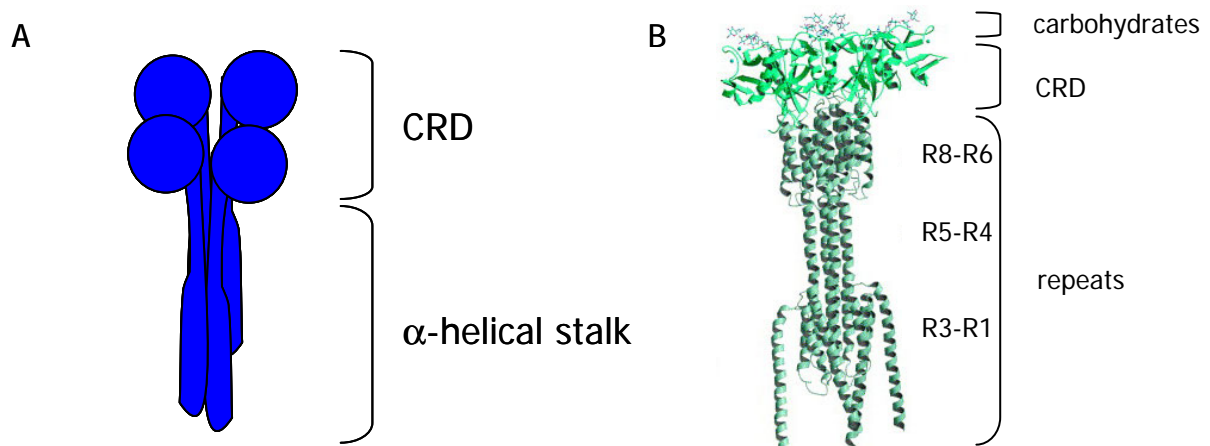


Figure 7: Structure of the extracellular domains of DC-SIGN. A) Model of tetrameric DC-SIGN with the carbohydrate-recognition domain (CRD) and the α -helical stalk. B) Predicted model based on homology modelling and sequence-based prediction of secondary structure elements showing the repeats in the α -helical stalk (adapted from Snyder *et al.*, 2005).

DC-SIGN is highly expressed in monocyte- and CD34⁺-derived dendritic cells (DCs) and in subsets of mature and immature DCs at various sites. L-SIGN is not expressed by these cells but by endothelial cells in lymph nodes, liver and lungs. Both DC-SIGN and L-SIGN act as cell adhesion and pathogen-recognition receptors (Khoo *et al.*, 2008). As pathogen recognition receptors, both lectins recognize a wide range of micro-organisms. DC-SIGN captures viruses like human immunodeficiency virus 1 (HIV1), Ebola virus, hepatitis C virus, Dengue virus, cytomegalovirus and severe acute respiratory syndrome coronavirus (Alvarez *et al.*, 2002; Geijtenbeek *et al.*, 2000; Halary *et al.*, 2002; Lozach *et al.*, 2003; Pöhlmann *et al.*, 2003; Tassaneeritthep *et al.*, 2003; Marzi *et al.*, 2004; Jeffers *et al.*, 2004; Yang *et al.*, 2004). Unlike L-SIGN that seems to function solely as an adhesion receptor, DC-SIGN is thought to mediate endocytosis next to adhesion, more specifically as a recycling receptor that releases its ligand at endosomal pH (Khoo *et al.*, 2008). Both DC- and L-SIGN might also function as antigen-capturing receptors that bind antigens at the cell surface and internalize them into a low pH endosomal compartment for release and degradation for loading onto major histocompatibility complex molecules (Khoo *et al.*, 2008).

The interaction between DC-SIGN and some viruses is well studied. DC-SIGN captures HIV1 from the periphery to transport it via unsusceptible DCs into the secondary lymphoid organs where it is transmitted to susceptible T-cells to enhance infection *in trans* (Geijtenbeek *et al.*, 2000). For hepatitis C virus, both DC-SIGN and L-SIGN function as attachment factors possibly to target virus to the site of replication. DC-SIGN might facilitate entry into DCs *in cis*. While binding to L-SIGN on endothelial cells in the liver, might concentrate the virus in this target organ and enhance infection *in trans* (Lozach *et al.*, 2003; Pöhlmann *et al.*, 2003).

Some group 1 coronaviruses can interact with DC-SIGN or L-SIGN (see further in 1.2.3.1).

1.2.3 Receptor use by different coronaviruses

1.2.3.1 Group 1 coronaviruses

Feline coronaviruses (FCoV)

Feline aminopeptidase N (fAPN) can serve as a receptor for feline coronaviruses as well as canine, porcine and human coronaviruses. This was concluded after the observation that transfection with cDNA coding for fAPN isolated from a feline cell line, led to infection of non-susceptible cells (Tresnan *et al.*, 1996). However, the efficiency of inducing infection was different between FCoV serotype I and II strains. Later, Hohdatsu *et al.* explained this

difference by showing that fAPN was only a receptor for FCoV serotype II, but not serotype I (1998). This study was based on the capability of a monoclonal antibody (R-G-4) to block infection of various cell lines with type II FCoV strains, but not type I strains. Dye *et al.* confirmed the inability of type I feline coronavirus spike proteins to recognize aminopeptidase N as a receptor on cell lines (2007).

Different regions of fAPN are involved in the interaction with different viruses. According to Hegyi & Kolb, aa 670 to 840 are necessary for the FCoV and transmissible gastroenteritis virus (TGEV) receptor function whereas aa 135 to 297 are essential for the human coronavirus 229E (HCoV-229E) receptor function (1998). Tusell and coworkers also performed a mutational analysis to refine the knowledge on aa regions of APN that are determining viral host range (2007). Three small, discontinuous regions in fAPN determine the host ranges of FCoV, TGEV, CCoV and HCoV-229E. They are located from aa 288-290, aa 732-746 and aa 764-788. The first region from aa 288-290, is essential for HCoV-229E. TGEV requires the second region from aa 732-746, while CCoV and FCoV require both the second and the third region from aa 732-746 and from aa 764-788. The monoclonal antibody (mAb) R-G-4 produced by Dr Hohdatsu can block infection with all these different viruses (Hohdatsu *et al.*, 1998). The antibody binds to an epitope within the region from aa 251-582 which correlates with domain V of APN (Tusell *et al.*, 2007). This would imply that the three discontinuous regions determining the host range lie closely together on the folded protein. These studies were all performed using APN sequences obtained from a feline cell line. Rottier *et al.* studied the blocking effect of mAb R-G-4 on bone marrow-derived macrophage infection and saw a reduction from approximately 33 up to 3 % (2005). On the *in vivo* target cells, monocytes, the entry of FCoV has not been studied. Recently, Regan & Whittaker claimed that mAb R-G-4 also significantly reduced infection of monocytes (2008). Further, the expression level of fAPN is increased in macrophages of FIP cats. *In vitro*, replication of FIPV in macrophages leads to tumour necrosis factor α (TNF- α) production, which subsequently up regulates the expression of fAPN. Possibly this induces an enhanced susceptibility to infection. When there is ADE of FIPV infection, the expression of TNF- α and the upregulation of APN are even stronger (Takano *et al.*, 2007).

For the FCoVs, expression of human DC-SIGN in Crandell feline kidney cells induced an increase of infection that was blocked by mannan, a competitor of DC-SIGN binding. Further infection of feline monocytes, the *in vivo* target cell, was strongly reduced by mannan according to Regan & Whittaker (2008).

Canine coronavirus (CCoV)

The entry of canine coronavirus is not extensively studied but it is known that CCoV binds to canine APN (Benbacer *et al.*, 1997).

Human coronaviruses (HCoV)

Human coronavirus 229E (HCoV-229E) binds to human APN (hAPN) on human fibroblasts (Yeager *et al.*, 1992). In human fibroblasts, APN is located in the raft fraction of the membrane (Nomura *et al.*, 2004). After binding, receptor/virus complexes relocate to the site of internalization. Binding of HCoV-229E to hAPN may trigger conformational changes in the viral spike necessary to mediate internalization (Breslin *et al.*, 2003). The region between amino acids 288-295 of hAPN is crucial for the HCoV-229E receptor function. Substitution of this region with a region originating from porcine APN (pAPN) and introducing an N-linked glycosylation site abolished recognition of HCoV. It is suggested that differences in glycosylation between coronavirus receptors from different species are critical for species specificity (Wentworth & Holmes, 2001). On the spike protein, the domain between aa 417-547 is probably required for binding of HCoV-229E to the hAPN receptor (Bonavia *et al.*, 2003).

HCoV-229E can bind and probably enter cells expressing L-SIGN via this receptor but there is no uniform cytoplasmic expression of viral proteins nor release of infectious viral particles from these cells (Jeffers *et al.*, 2006).

Human coronavirus NL63 (HCoV-NL63) is the only group 1 coronavirus that has not been linked with APN. It uses the severe acute respiratory syndrome (SARS) coronavirus receptor angiotensin-converting enzyme (ACE) 2 and possibly also DC-SIGN (Hofmann *et al.*, 2005; 2006). NL63 S-protein has a weaker interaction with the ACE2 receptor than SARS-CoV. The ACE2 binding site is located between residues 190 and 739 in the C-terminal part of the S1 subunit of the spike protein (Hofmann *et al.*, 2006; Mathewson *et al.*, 2008). Lin *et al.* (2008) specified this information by mapping a minimal receptor-binding domain of 141 residues between aa 476-616 in the spike protein. In this region 15 critical residues were identified: C497, Y498, V499, C500, K501, R518, R530, V531, G534, G537, D538, S540, E582, W585 and T591. These critical residues are clustered in three separate regions and may represent three receptor-binding sites. In ACE2, residue 354 is crucial for NL63 binding (Li *et al.*, 2007).

HCoV-NL63 depends on ACE2 for infectious entry, but expression of DC-SIGN or L-SIGN slightly augments infection of susceptible cells. Expression of DC-SIGN in non-susceptible cells is however not sufficient to enable entry in these cells (Hofmann *et al.*, 2006).

Porcine coronaviruses

Porcine aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. TGEV replicates selectively in differentiated enterocytes covering the villi of the small intestine (Delmas *et al.*, 1992). Recombinant expression of APN confers infectivity to non-susceptible cells. Transfected cells are not susceptible for FCoV, nor CCoV, but porcine respiratory coronavirus (PRCoV) does use pAPN for entry into the host cells (Delmas *et al.*, 1993). For TGEV, an additional receptor of approximately 200kDa has been described (Weingartl & Derbyshire, 1994). This receptor was not further identified, so its role in the entry process of other coronaviruses could not be examined.

pAPN also functions as a receptor for porcine epidemic diarrhoea virus (PEDV) because transfection of non-susceptible cells with pAPN, led to infection and neutralizing antibodies against pAPN blocked infection (Oh *et al.*, 2003; Li *et al.*, 2007).

For both bat coronavirus and rabbit coronavirus, receptor use has not been studied.

1.2.3.2 Other important coronaviruses

Mouse hepatitis virus (MHV)

Mouse hepatitis virus (MHV) is one of the best studied viruses in coronavirus group 2. The receptor for MHV-A59, a strain of MHV, belongs to the carcinoembryonic antigen family of glycoproteins in the immunoglobulin superfamily. More specifically, the virus binds to the carcinoembryonic antigen-related cell adhesion molecule 1a (CEACAM1a) receptor on murine cells via the MHV spike protein. It is a glycoprotein with four immunoglobulinlike domains, a transmembrane domain and a short cytoplasmic tail. Transfection into non-susceptible cells, confers infectivity (Dveksler *et al.*, 1991). Isoform CEACAM1b can also function as an MHV receptor but with 10 to 100 times less affinity (Ohtsuka *et al.*, 1996). The MHV receptor is not associated with lipid rafts and virus binding does not require cholesterol (Thorp & Gallagher, 2004). The N-terminal part of CEACAM1a is essential for binding and can induce a conformational change in the spike protein upon binding (Matsuyama & Taguchi, 2002; Zelus *et al.*, 2003; Miura *et al.*, 2004). The induced conformational change transforms the non-fusogenic S-protein into a fusogenic form.

Infectious bronchitis virus (IBV)

Avian infectious bronchitis virus (IBV) is the prototype for group 3 coronaviruses. IBV not only attaches to α 2,3-linked sialic acids on erythrocytes, but the presence of these sialic acids on target cells determines susceptibility to infection by IBV (Schultze *et al.*, 1992; Winter *et al.*, 2006). Probably, sialic acids mediate primary attachment and a second, unknown receptor mediates tighter binding and internalization. Infection of ciliated and goblet cells in tracheal organ cultures also depends on the presence of sialic acids, confirming the *in vivo* relevance of sialic acid receptors (Winter *et al.*, 2008; Rahman *et al.*, 2009). For a certain IBV strain, Beaudette, that has an extended host range in cell culture, heparan sulphate works together with sialic acids (Madu *et al.*, 2007). This could be due to cell culture adaptations that are not relevant *in vivo*. Previously feline aminopeptidase N was also claimed to be capable of mediating IBV entry (Miguel *et al.*, 2002). This was however countered by Chu *et al.*, who showed that fAPN could not rescue infection by IBV strain Mass41 (2007). The alleged IBV receptor function of fAPN was explained by a low IBV susceptibility of feline cells that was however negligible compared to susceptibility of primary chick kidney cells.

Severe acute respiratory syndrome virus (SARS-CoV)

Angiotensin-converting enzyme 2 (ACE2) is a functional receptor for SARS virus (Li *et al.*, 2003). It is a type I transmembrane protein of 805 amino acids with carboxy-metalloprotease activity. The proteolytic activity of ACE2 is however not important for SARS-CoV receptor function. This receptor is present at the primary site for SARS-CoV replication, the lungs (Kuiken *et al.*, 2003; Li *et al.*, 2003). The ACE2 binding site is localized in a small region in the S1 domain of the spike protein, more specifically in a region of 193 aa between residues 318 and 510 (Babcock *et al.*, 2004; Wong *et al.*, 2004). The presence of ACE2 in lipid rafts would be important for efficient virus receptor interactions (Glende *et al.*, 2008; Lu *et al.*, 2008). SARS-CoV can also interact with L-SIGN and DC-SIGN. This interaction would lead to an enhancement of infection in susceptible cells by transmission of virus to susceptible cells (Marzi *et al.*, 2004; Yang *et al.*, 2004). Han *et al.* showed that DC/L-SIGN expression could rescue SARS-CoV infection in Hela cells (2007). The interaction between SARS-CoV and DC/L-SIGN occurs through glycans on the spike protein, more specifically asparagine residues at aa positions 109, 118, 119, 158, 227, 589 and 699 (Han *et al.*, 2007). However, it is still unknown if DC/L-SIGN mediates infectious entry *in vivo*.

1.2.4 Virus internalization

1.2.4.1 Direct fusion versus endocytosis

The interaction with cellular receptors is indispensable for viruses to cross the plasma membrane. There are two major routes for enveloped viruses to cross the plasma membrane and deliver viral genomes in the cytoplasm: fusion with the plasma membrane (referred to as direct fusion) or receptor-mediated endocytosis followed by fusion with an intracellular vesicle membrane (referred to as endocytosis). The virus-receptor interaction can induce a conformational change in the viral fusion protein that exposes a fusion peptide. Fusion peptides originally reside in the ectodomain of the fusion protein. Upon binding to the right receptor, they are exposed to promote hydrophobic interactions between the viral envelope and the plasma membrane of the host cell (Voyles, 2002). The viral envelop is left behind in the plasma membrane and the viral nucleocapsid is released in the cytosol (Fig. 8). The virus-receptor interaction can however also induce invagination of the plasma membrane and the formation of an intracellular vesicle that contains the virus-receptor complex. This process of receptor-mediated endocytosis can occur through a number of pathways that are shown in Figure 9. Eventually, uptake via endocytosis also leads to a fusion event as described above, this time between the viral envelope and the membrane of the intracellular vesicle.

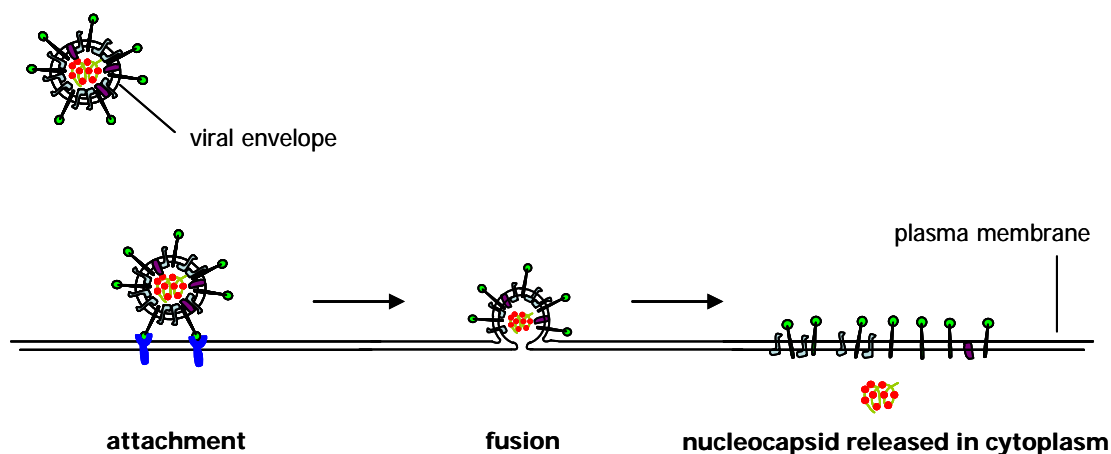


Figure 8: Virus entry via direct fusion with the plasma membrane.

1.2.4.2 Endocytosis pathways

Endocytosis pathways can be divided in two major categories: phagocytosis (the uptake of large particles) and pinocytosis (the uptake of fluid and solutes) (Conner & Schmid, 2003). Pinocytosis can be further subdivided into macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis or clathrin- and caveolae-independent endocytosis (Fig. 9).

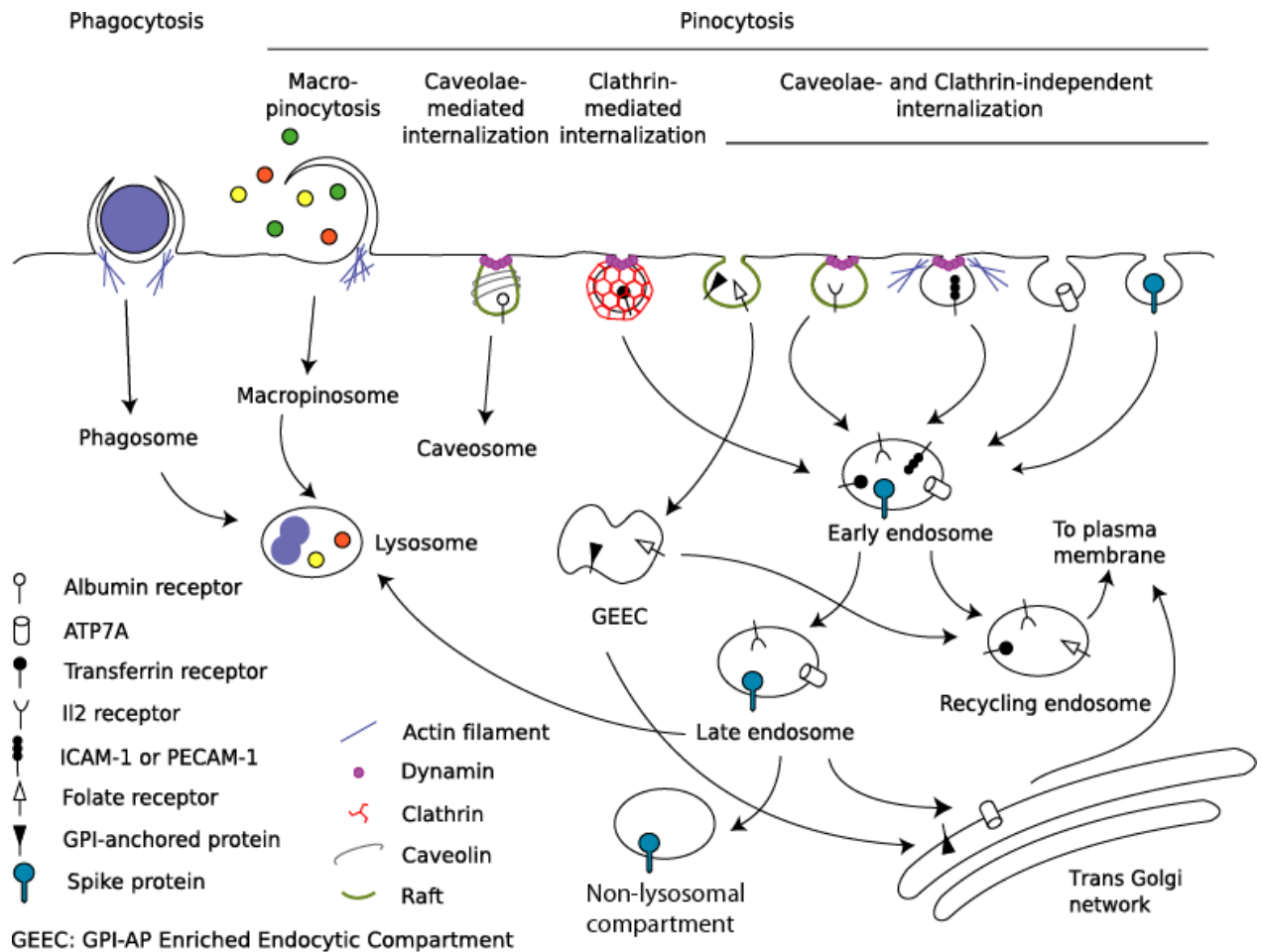


Figure 9: Overview of all possible endocytosis pathways with the main structural compounds involved and the intracellular destination (Dewerchin, 2008).

Phagocytosis

Phagocytosis is an active and highly regulated process for the uptake of 'large' (generally $> 0.5 \mu\text{m}$) particles. It participates in several processes such as development, tissue remodelling, the immune response and inflammation (Aderem & Underhill, 1999). Phagocytosis in mammals is conducted primarily by specialized cells, such as macrophages, monocytes and neutrophils (Conner & Schmid, 2003).

Formation of phagosomes

The process is initiated by binding of the ligand to specific cell-surface receptors. This triggers a signalling pathway involving activation of the Rho GTPases Cdc42 and Rac that induces actin assembly for the formation of lamellipodia. Lamellipodia are membrane extensions that zipper up around the ligand and engulf the particle into a phagosome. Another GTPase involved in the phagocytic process is dynamin. It is enriched in early phagosomes. The exact role of dynamin is unknown but blocking dynamin function arrests internalization at the stage of membrane extension around the particle (Gold *et al.*, 1999). Phosphatidylinositol 3-kinase (PI3K) mediates the closing of the lamellipodia and the capture in the phagosome. After internalization, actin is depolymerised from the phagosome and the vacuole membrane becomes accessible to early endosomes. Through a series of fusion and fission events, the vacuolar membrane and its contents mature, followed by fusion with late endosomes and ultimately lysosomes to form a phagolysosome (Aderem & Underhill, 1999).

Inhibition of phagocytosis

Latrunculin B that disrupts actin polymerization can be used to block phagocytosis as it will prevent the formation of lamellipodia (Spector *et al.*, 1989). Wortmannin is a PI3K inhibitor that can be used as an inhibitor for phagocytosis (Araki *et al.*, 1996).

Macropinocytosis

Macropinocytosis is a highly controlled and regulated process for the uptake of particles generally $> 0.2 \mu\text{m}$. Macropinocytosis fulfils diverse functions and can be induced in most cell types. For example, GTPase Rac and p21-activated kinase (PAK) are activated through platelet-derived growth factor-induced macropinocytosis and control directed cell migration (Ridley, 2001). Further, dendritic cells sample the extracellular milieu through macropinocytosis to fulfil their role in immune surveillance (Mellman & Steinman, 2001).

Formation of macropinosomes

Like for phagocytosis, Rho GTPases induce actin-rearrangements that form membrane protrusions. These protrusions are called membrane ruffles. More specifically, Rac regulates the assembly of an actin-nucleating complex at the plasma membrane where the ruffles originate from. Besides oriented actin polymerization, ruffling may also require contributions from actin-based mechanochemical enzymes, such as myosin. Cytoplasmic microtubules also appear to modulate the ruffling (Swanson & Watts, 1995). Unlike for phagocytosis, the protrusions do not zipper up along the particle but collapse onto and fuse with the plasma

membrane. In this way, large endocytic vesicles are formed, the macropinosomes, that sample large volumes of the extra-cellular milieu (Conner & Schmid, 2003). They are heterogeneous in size (generally from 0.2 up to 5 μm). During their life span of approximately 15 minutes, macropinosomes change from an early endosome-like organelle to a late endosome-like organelle, then merge completely into the lysosomal compartment (Racoosin & Swanson, 1993).

Inhibition of macropinocytosis

Latrunculin B, an inhibitor for phagocytosis also blocks macropinocytosis, as they both rely on actin polymerization (Spector *et al.*, 1989). Further, as for phagocytosis, PI3K is involved in closure of the macropinosomes. In the presence of the inhibitor wortmannin, the formed ruffles recede into the cytoplasm (Araki *et al.*, 1996).

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is probably the best studied internalization pathway. CME constitutively takes place in all mammalian cells. It is important for the uptake of essential nutrients such as cholesterol-laden low-density lipoprotein particles and iron-laden transferrin (Schmid, 1997; Brodsky *et al.*, 2001). CME is also crucial for intercellular communication during tissue and organ development (Di Fiore & De Camilli, 2001; Seto *et al.*, 2002). Further, it is also involved in serum homeostasis and neurotransmission (De Camilli & Takei, 1996; Beattie *et al.*, 2000).

Formation of the clathrin-coated vesicle

CME is initiated by the concentration of high-affinity transmembrane receptors and their bound ligands. The 'coated pits' of approximately 120 nm are subsequently formed on the plasma membrane by the assembly of cytosolic coat proteins (Fig. 11, step 1 & 2). The coat proteins consist of clathrin and the assembly proteins. Clathrin is the main assembly unit. It is a tree-legged structure formed by three clathrin heavy chains, each with a tightly associated light chain (Brodsky *et al.*, 2001). This structure is called a 'triskelion' (Fig. 10). There are two structurally and functionally different classes of assembly proteins: the monomeric assembly protein AP180 (in neuronal cells) or its isoform CALM (clathrin-assembly lymphoid-myeloid leukaemia) and heterotetrameric adaptor protein (AP) complexes, AP1 to AP4. However, only AP2, composed of α -, β 2-, μ 2- and σ 2-subunits, is involved in endocytic clathrin-coated vesicle formation (Fig. 10). Clathrin triskelions spontaneously self-assemble into lattices. AP2s direct clathrin assembly into curved lattices

to the site where cargo is recruited and where ligated receptors are concentrated through a direct interaction of AP2 with the receptors (Fig. 11, step 2) (Brodsky *et al.*, 2001). The exact role for AP180/CALM is not known but in the absence of this protein fewer vesicles are formed and they are less uniform in size (Tebar *et al.*, 1999). Eps15 binds directly to AP2 and indirectly via epsin to clathrin. Thus, the coat proteins clathrin, AP2, AP180/CALM and Eps15 encode all the functions necessary to select cargo and form a vesicle. After the formation of the 'coated pit', the vesicle is pinched off the membrane and the receptor-ligand complexes are transported into the cell (Fig. 11, step 3).

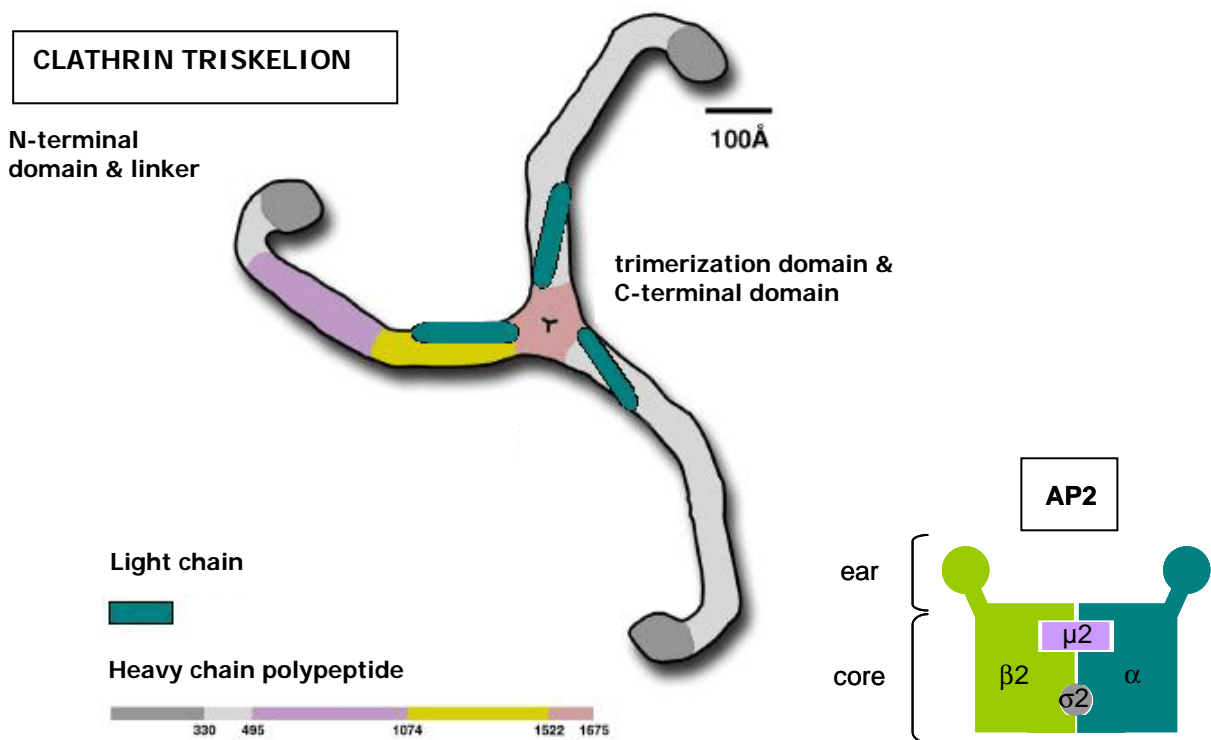


Figure 10: Structure of the main components of clathrin-coated pits: clathrin triskelions and adaptor complex 2 (AP2) (clathrin triskelion adapted from <http://www.bio.indiana.edu/~ybelab/research.html>).

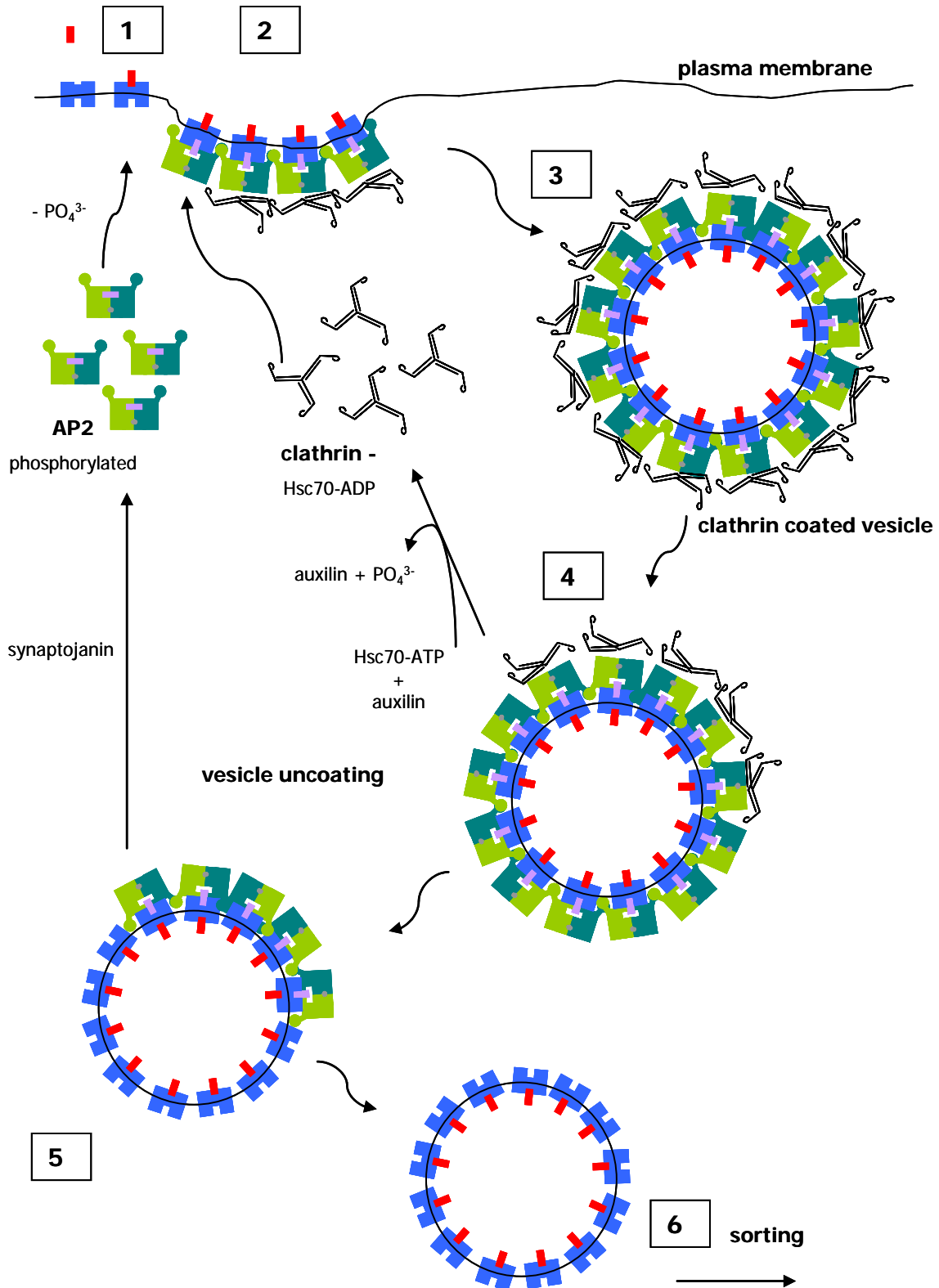


Figure 11: Clathrin-mediated internalization. 1 Binding of ligands to receptors. 2 AP2 associates with the receptors through the $\mu 2$ subunit. The β subunit interacts with clathrin. 3 Internalized clathrin-coated vesicle. 4-5 Uncoating of the vesicle by dissociation of clathrin and AP2. 6 Sorting of the uncoated vesicle.

'Pinching off' the vesicle

The 'pinching off' is attributed to the GTPase dynamin. Dynamin forms rings at the neck of invaginated clathrin-coated pits and a conformational change in the ring that correlates with GTP hydrolysis, is essential for vesicle fission (De Camilli *et al.*, 1995). The dynamin-2 isomer is expressed ubiquitously. It interacts with itself to form oligomers, predominantly tetramers, to form rings (Hinshaw & Schmid, 1995). Next to the GTPase domain, dynamin also contains domains to interact with several binding partners that stimulate the GTPase activity or target dynamin to the plasma membrane (Hinshaw, 2000). The GTPase activity stimulating domain is the 'GTPase effector domain'. Targeting to the plasma membrane occurs through interaction with the 'pleckstrin homology (PH) domain' of dynamin with phosphoinositides (Salim *et al.*, 1996). Further, the 'proline-rich domain' at the C-terminus of dynamin interacts with SH3 domains of profilin and cortactin that interact with the actin cytoskeleton (Simpson *et al.*, 1999). Another important binding partner for dynamin are amphiphysines. They not only bind dynamin but often also clathrin and AP2 to stabilize the clathrin-coated vesicle (Slepnev *et al.*, 2000). Together with amphiphysine, dynamin binds endophilin and actin tails through pacsin/syndapin, to pinch-off the vesicle from the membrane.

Intracellular fate of the internalized vesicle

The uncoating of the internalized vesicle is initiated through auxilin that binds Hsc70-ATP (Lemmon, 2001). Hydrolyzation to Hsc70-ADP induces a conformational change in clathrin that is released from the vesicle and binds to Hsc70-ADP (Fig. 11, step 4). Dissociation of AP2 is induced by synaptojanin and after release from the vesicle AP2 is phosphorylated to prevent it from binding again to clathrin (Fig. 11, step 5) (Wilde & Brodsky, 1996; Cremona *et al.*, 1999). The uncoated vesicle can subsequently fuse with other intracellular vesicle and/or continue its intracellular journey (Fig. 11, step 6).

Inhibition of clathrin-mediated endocytosis (CME)

Amantadine stabilizes the structure of clathrin-coated vesicles in purified pig brain (Phonphok & Rosenthal, 1991). Another, perhaps more specific inhibitor of CME, is chlorpromazine. It causes clathrin lattices to assemble on endosomal membranes and at the same time prevents coated pit assembly at the plasma membrane (Wang *et al.*, 1993). It was also shown that the extraction of cholesterol with methyl- β -cyclodextrin perturbs formation of clathrin-coated endocytic vesicles (Rodal *et al.*, 1999). Each protein involved in CME can also be targeted. Dynamin is often used as a target for blocking several endocytosis pathways, including CME.

Caveolae-mediated endocytosis

Caveolae are approximately 60 nm, flask-shaped invaginations of the plasma membrane that occur on many cells and demarcate cholesterol and sphingolipid-rich microdomains of the plasma membrane. Most cell types contain some caveolae, while they are really abundantly present in other cells, like eg adipocytes. Some cells completely lack caveolae, such as lymphocytes and neuronal cells from the central nervous system. Caveolae-mediated endocytosis is important for intracellular cholesterol trafficking and intracellular cholesterol homeostasis. Further, it plays an organismal role in lipid homeostasis in adipocytes and it mediates transcellular transport in endothelial cells (Conner & Schmid, 2003).

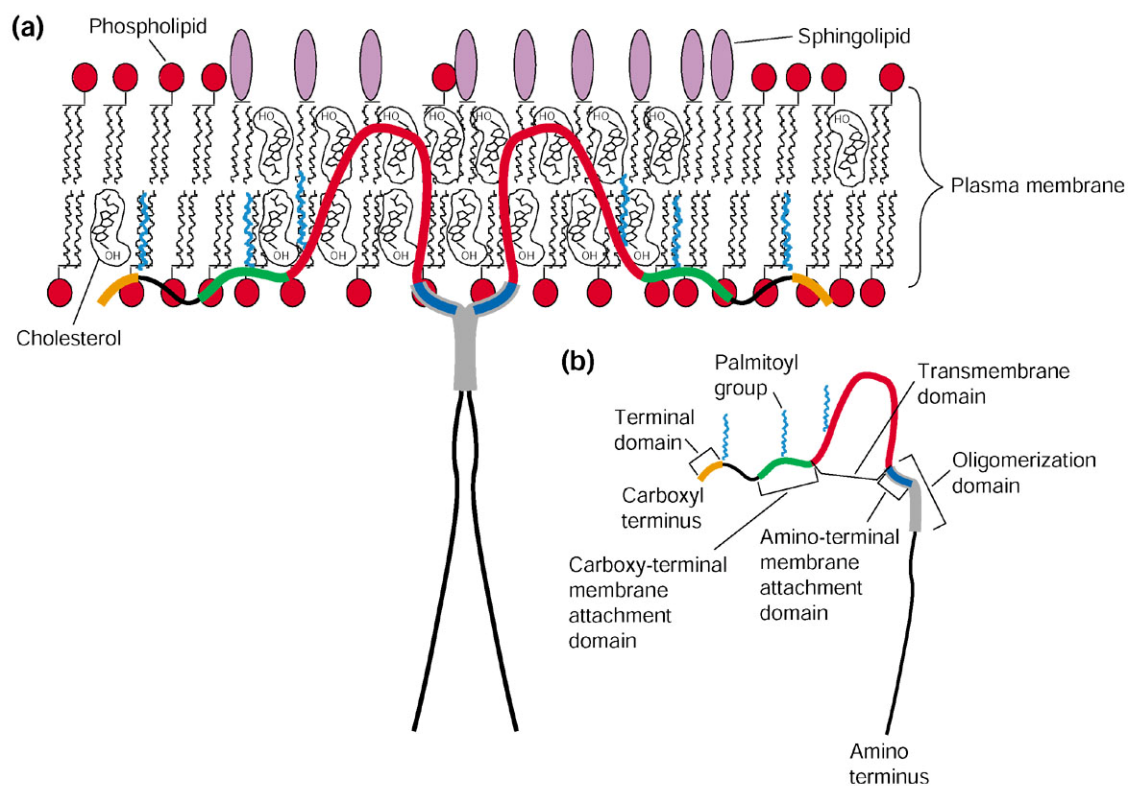


Figure 12: (a) Membrane topology of caveolin. (b) Different domains on caveolin (Williams & Lisanti, 2004).

The nature of caveolae

Unlike clathrin-coated vesicles, caveolae are not induced by binding of a ligand to its receptor but they are stationary and held in place by the cortical actin cytoskeleton underlying the plasma membrane (Fig. 13, step 1) (Pelkmans *et al.*, 2001; Thomsen *et al.*, 2002). Only upon specific signals they will detach from the membrane as an endocytic vesicle. Caveolae have a protein coat, though not as dense as the clathrin coat, that consists

mainly of caveolin-1 (caveolin-3 in muscle cells) (Rothberg *et al.*, 1992). Caveolins are integral membrane proteins with both N- and C-terminal ends directed into the cytosol and one transmembrane region that does not span the membrane (Fig. 12) (Dupree *et al.*, 1993; Monier *et al.*, 1995). They are located in the membrane in lipid raft-like domains. Lipid rafts are more rigid domains 'floating' in the plasma membrane, enriched in cholesterol and sphingolipids (glycosphingolipids and sphingomyelin). The presence of caveolins discriminates caveolae from the 'ordinary' rafts and is due to the high affinity of caveolin for sphingolipids and especially cholesterol (Murata *et al.*, 1995). In caveolae, every 14-16 caveolin monomers oligomerize to form the coat of the vesicle.

Induction of internalization

The internalization of Simian virus 40 (SV40), a DNA virus (*Papovaviridae*, polyomavirinae) of approximately 50 nm, is intensively studied and occurs via caveolae-mediated endocytosis. Based on the findings concerning the internalization of SV40, a model for caveolae-mediated endocytosis was proposed (Pelkmans & Helenius, 2002). After binding of a ligand to its receptor, this complex can diffuse laterally along the membrane until it is trapped in a caveola (Fig. 13, step 2) (Pelkmans *et al.*, 2001). The caveolae are linked to the cortical actin that needs to depolymerise to enable internalization. This occurs via tyrosine phosphorylation in proteins associated with the caveolae (Pelkmans *et al.*, 2002). *In vivo* Src phosphorylates caveolin but it is not known if this is necessary and/or sufficient to depolymerise the actin (Parton *et al.*, 1994). The released actin monomers assemble into a small actin patch surrounding the vesicle. This is followed by peaks of actin polymerization that result in the formation of actin tails that function as propellers to direct the vesicle into the cytosol (Fig. 13, step 3). Next to actin rearrangements, also dynamin is strictly necessary to enable vesicle closure and detachment from the plasma membrane (Pelkmans *et al.*, 2002). Once the vesicle is released, the actin tail is no longer necessary (Fig. 13, step 4) (Pelkmans & Helenius, 2002).

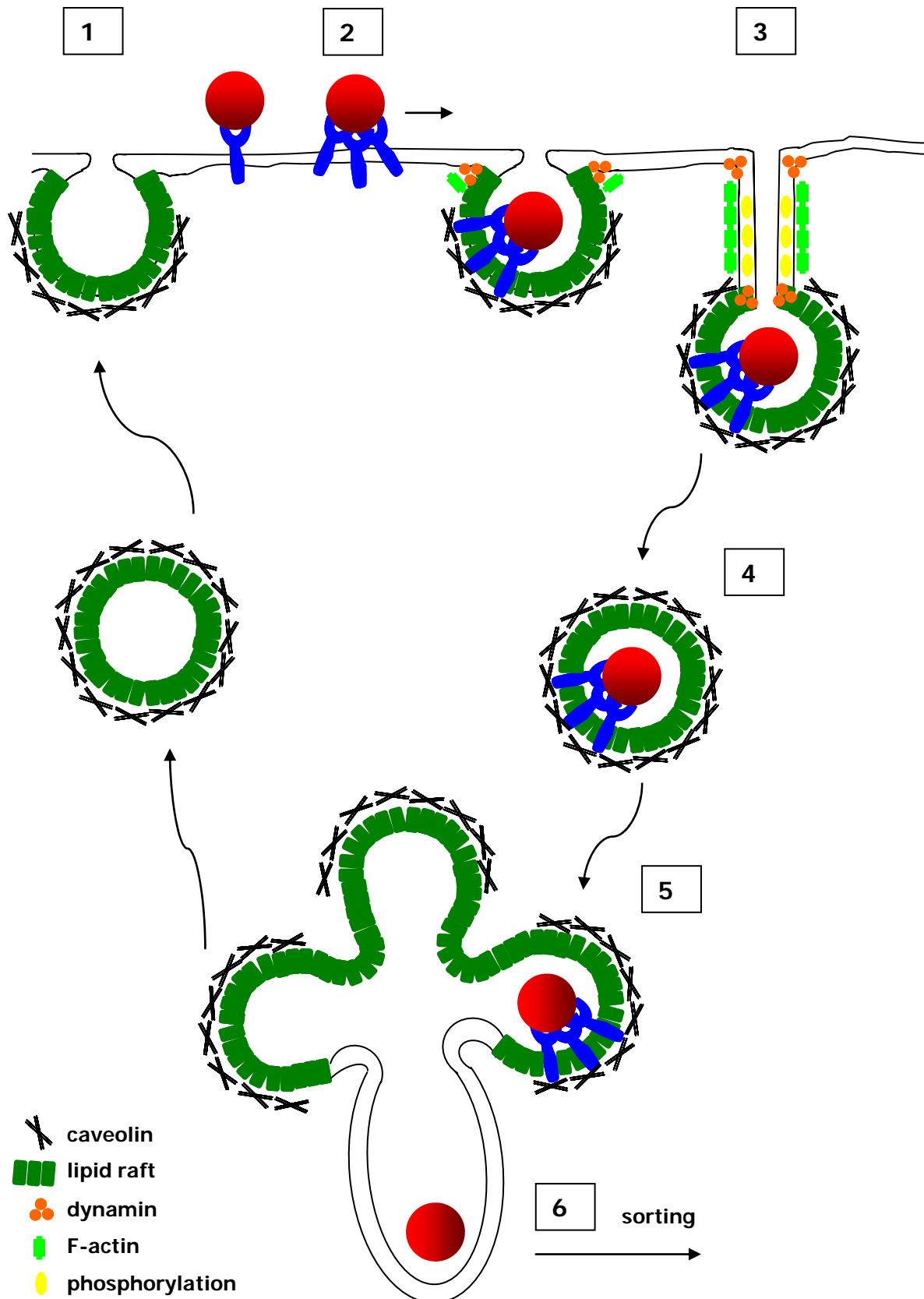


Figure 13: Caveolae-mediated internalization. 1 Caveolae are not induced but stationary present in the plasma membrane. 2 Binding of ligand to receptor and diffusion of the complex along the membrane. 3 Dynamin recruitment and actin rearrangements to induce internalization. 4 Released vesicle. 5 Fusion with the caveosome. 6 A caveolin-free vesicle is split off the caveosome and sorted to the ER (adapted from Pelkmans & Helenius, 2003).

Intracellular fate of the internalized vesicle

The vesicles released in the cytosol transfer their cargo to larger 'caveosomes' by membrane fusion (Fig. 13, step 5) (Pelkmans *et al.*, 2001). Just like caveolae, membranes of caveosomes contain caveolin-1, cholesterol and sphingolipids. Their pH is neutral and they are consistently present and distributed throughout the cytosol. After fusion of several caveolae into the caveosome, its structure becomes more dynamic and caveolin-free vesicles are split off and cargo is sorted to the ER via transport over the microtubules (Fig. 13, step 6).

Inhibition of caveolae-mediated internalization

Caveolae-mediated internalization is often indirectly blocked by disrupting lipid rafts. This is done by sterol-binding or -extracting drugs like filipin and nystatin or methyl- β -cyclodextrin (Schnitzer *et al.*, 1994). Although not specific, this approach is very effective. Again, all proteins involved in the cascade described above can be used as targets. For example, blocking tyrosine kinases by inhibitors like genistein blocks internalization through caveolae (Parton *et al.*, 1994; Parton & Richards, 2003).

Clathrin- and caveolae-independent endocytosis

Little is known about the mechanisms underlying clathrin- and caveolae-independent pathways. However, probably they fulfil unique functions and the internalization is tightly regulated, as is the intracellular destination of the internalized ligands (Conner & Schmid, 2003). In what follows, the independent pathways will be described based on some selected examples. The independent pathways are identified based on characteristics like (i) the dependency on dynamin, (ii) the association with lipid rafts and (iii) the dependency on Rho GTPases. Most likely, as these pathways are further characterized, more characteristics will be necessary to differentiate between independent pathways.

Dynamin-dependent and cholesterol depletion sensitive pathway

Both the β -subunit of the IL-2 receptor (IL-2R) and the common cytokine receptor γ (γ_c) are internalized through a clathrin- and caveolae-independent pathway that depends on dynamin and is associated with lipid rafts (Kirkham & Parton, 2005). Binding of IL-2 to the IL-2R increases the association between the receptor and rafts. The internalization is regulated by the Rho GTPases RhoA and Rac1 (Lamaze *et al.*, 2001). The γ_c receptor is a subunit shared by several cytokine receptors, namely IL-2, -4, -7, -9, -15 and -21 receptors (Schluns & Lefrancois, 2003). The internalization of the γ_c cytokine receptor is further characterized by

recruitment and polymerization of actin and activity of the Rho GTPase RhoA (Sauvonnet *et al.*, 2005).

A dynamin-dependent and cholesterol depletion sensitive pathway is also (mis)used by rotavirus (*Reoviridae*) to gain entry into an epithelial cell line. It is not known if this pathway is regulated by Rho GTPases (Sanchez-San Martin *et al.*, 2004).

Dynamin-independent and cholesterol depletion sensitive pathway

Endocytosis of the glycosylphosphatidylinositol (GPI) -anchored folate receptor can occur via clathrin-mediated internalization. But this is not sufficient for effective cytoplasmic folate delivery (Ritter *et al.*, 1995). Efficient folate delivery results from internalization via a clathrin- and caveolae-independent pathway that does not rely on dynamin (Sabharanjak & Mayor, 2004). Further, the pathway is associated with lipid rafts via the GPI-anchor. After a couple of minutes, endocytosed proteins are detected in GPI-anchored protein enriched endocytic compartments (GEECs). Formation of these GEECs is regulated by the Rho GTPase Cdc42 (Sabharanjak *et al.*, 2002). The final destination of the compounds inside the GEECs is cell type dependent and varies from recycling compartments to late endosomes.

In cell lines that lack caveolae and in embryonic fibroblasts, SV40 is taken up by a clathrin- and caveolae-independent pathway that depends on cholesterol but not on dynamin (Damm *et al.*, 2005).

Dynamin-dependent and cholesterol depletion insensitive pathway

A possible example of a dynamin-dependent clathrin- and caveolae-independent pathway that does not depend on cholesterol is influenza virus entry in Hela cells. It is definitely a cholesterol-independent, clathrin- and caveolae-independent pathway but the dependency on dynamin has not been studied yet and was only suggested in 2002 by Sieczkarski & Whittaker based on dependency on dynamin of influenzavirus in another cell type, Mv-1 lung cells (Roy *et al.*, 2000). However, numerous viruses have been shown to use different pathways to enter different cell types, or even use different pathways in one cell. Therefore, dependency on dynamin should be further investigated before any conclusions can be made.

Dynamin-independent and cholesterol depletion insensitive pathway

The entry of murine polyomavirus strain RA in fibroblasts and kidney epithelial cells occurs via a clathrin- and caveolae-independent pathway that is independent from dynamin and cholesterol (Gilbert & Benjamin, 2000). A rearrangement of the actin microfilaments is

necessary for internalization. For intracellular transport to the nucleus, the virus relies on microtubules (Gilbert *et al.*, 2003).

Recently, it was shown that the antibody-induced internalization of surface-expressed antigens in FIPV-infected monocytes are internalized via a clathrin- and caveolae-independent pathway that does not require dynamin or rafts. Further, this pathway is independent of actin and Rho GTPases, and phosphatases and tyrosine kinases. It did however depend on serine/threonine kinases (Dewerchin *et al.*, 2008).

1.2.5 Internalization of coronaviruses

1.2.5.1 Group 1 coronaviruses

Feline coronaviruses (FCoV)

Together with the question whether FCoVs enter cells via direct fusion at the plasma membrane or fusion with the endosomal membranes after endocytosis, it is also questioned if uncoating of FCoV particles depends on low pH. Without resolving the first question and eliminating the possibility of direct fusion, Takano *et al.* claimed that acidification of the endosome is necessary for FIPV infection of monocytes (2008). However, Regan *et al.* claim that the pH drop in the endosome is necessary for infectious entry of eFCoV strain 79-1683, but not for FIPV strains 79-1146 and DF2 (2008). As such, the mystery around FCoV entry remains unresolved.

Naturally, the FCoV S-protein will fulfil an important role in the internalization process. In monocytes/macrophages, the M-protein might also be involved in some crucial interactions, as neutralizing antibodies against M-protein suggest involvement in a post-absorption step (Kida *et al.*, 2000).

The role of proteases in FCoV entry is another point of discussion. Regan *et al.* reported a cathepsin-mediated cleavage of the viral spike protein (2008). For eFCoV 79-1683, both cathepsin B and L can perform this cleavage, while FIPV 79-1146 and DF2 depend on cathepsin B activity. However, de Haan *et al.* claim independence from cathepsins for FIPV 79-1146, but report possible furin cleavage for some serotype I strains (2008).

Canine coronavirus (CCoV)

The internalization of canine coronavirus is not extensively studied. The exact internalization pathway is not known. It has been suggested that CCoV enters cells via endocytosis, as replication could be inhibited by chloroquine (Savarino *et al.*, 2003).

Human coronaviruses (HCoV)

HCoV-229E binds to APN in lipid rafts in human fibroblasts. At 37 °C the receptor/virus complexes redistribute to caveolae for virus entry through caveolae-mediated internalization (Nomura *et al.*, 2004). This was shown by co-localization studies between the virus and caveolins, disruption of the rafts by cholesterol depleting drugs and caveolin knockdown by RNA interference. Kawase *et al.* showed that also in Hela cells, HCoV-229E enters via endocytosis through an endosomal pathway (2009). Infection was profoundly blocked by lysosomotropic agents as well as by protease inhibitors. Cathepsin L is involved in the fusogenic activation of HCoV-229E S-protein in endosomal infection. It is however very likely that this is not the only protease involved (Kawase *et al.*, 2009).

The mechanism of internalization has not been studied for HCoV-NL63. As this virus uses the same receptor(s) as SARS-CoV, it might be suggested that it also uses the same internalization pathway(s). This is possible but uncertain as viruses have been shown to interact in different ways with identical binding partners triggering various processes. It has already been shown that SARS-CoV and NL63 probably engage ACE2 differentially. NL63-driven entry is also less dependent on a low pH environment and activity of endosomal proteases compared to infection with SARS-CoV (Hofmann *et al.*, 2006).

Porcine coronaviruses

The mechanism of TGEV internalization has been studied in a cell line stably expressing porcine APN. Electron microscopic analysis showed TGEV in endocytic pits and apical vesicles so the virus clearly enters via endocytosis and not via direct fusion at the plasma membrane. Acidification of the endosome is required for infection. Thickening of the membrane below adsorbed TGEV particles, visible on electron microscopic pictures, indicates that TGEV enters cells via clathrin-mediated endocytosis (Hansen *et al.*, 1998). Based on the finding that cholesterol is important for TGEV infection, it is suggested that TGEV entry may occur via lipid rafts (Ren *et al.*, 2008). Further research is necessary to confirm this hypothesis because the effect of methyl- β -cyclodextrin is not necessarily due to raft-association of the internalization process, especially not if CME can possibly occur.

The internalization mechanisms of PRCoV and PEDV have not been studied in detail.

For both bat coronavirus and rabbit coronavirus, the internalization pathway has not been studied.

1.2.5.2 Other important coronaviruses

Mouse hepatitis virus (MHV)

Although virus binding does not depend on cholesterol and the MHV receptor is not present in lipid rafts, virus entry was reduced by cholesterol depletion (Choi *et al.*, 2005). After virus binding independent from rafts, the virus particles shift to the raft-fraction of the membrane. This redistribution of spike proteins in the membrane and the interaction between lipid rafts and spikes may be necessary for internalization.

In the past, both endosomal and non-endosomal routes have been described for MHV entry (Nash & Buchmeier, 1997). The route of entry used, was said to depend upon the virus strain and the cell type of the host cell. Kooi *et al.*, suggested a minor role for the endocytic pathway and showed that MHV can induce direct fusion at pH 7.4 (1991). Binding of MHV (MHV-A59 and MHV-4) to its receptor can induce a conformational change in the spike protein into a fusogenic state, possibly ready to induce direct fusion at the plasma membrane (Matsuyama & Taguchi, 2002; Zelus *et al.*, 2003; Miura *et al.*, 2004). On the other hand, Krzystyniak and Dupuy reported that infection by MHV-3 is sensitive to lysosomotropic agents, suggesting virus entry via endocytosis (1984).

Recently, a novel study was set up to resolve the discussion on the route of entry of MHV strain A59. It was shown that virus uptake occurs via endocytosis (Eifart *et al.*, 2007). This is the natural consequence of the observed requirement for the low pH in endosomes to initiate fusion of the viral spike with the host cell membranes. The low pH-triggered conformational alterations in the S-ectodomain are irreversible because low pH-treatment of virus particles in the absence of target domains causes an irreversible loss of fusion activity. Protease activity is not required for MHV entry. As chlorpromazine is an efficient inhibitor of MHV entry, the virus is probably endocytosed via clathrin-mediated internalization (Eifart *et al.*, 2007). This is consistent with the dependency on cholesterol, as methyl- β -cyclodextrin is a potent inhibitor of CME.

Unlike for MHV-A59, endosomal protease activity is required for infection with MHV-2. Low pH activates cathepsins B and L, that are also involved in SARS-CoV infectivity, to mediate MHV-2 entry (Qiu *et al.*, 2006). These results might confirm the statement of Nash & Buchmeier that entry pathways vary between different virus strains and their target cells (1997). Further analysis of MHV-2 entry showed the involvement of clathrin but remarkably not of Eps15, a component that until now was always involved in CME (Pu & Zhang, 2008).

Infectious bronchitis virus (IBV)

Avian IBV uses an endocytic pathway for entry into cells. Cell fusion occurs in a low pH-dependent manner in the endosomes without activity of endosomal proteases (Chu *et al.*, 2006). The low pH-induced conformational change is reversible, unlike the conformational change of MHV spikes.

Severe acute respiratory syndrome virus (SARS-CoV)

SARS-CoV infection of Vero E6 cells (and 293T and Huh-7 cells) requires acidification of endosomes, indicating that the virus enters cells via endocytosis (Simmons *et al.*, 2004; Yang *et al.*, 2004). The ACE2 receptor is located in lipid rafts that probably also function as a platform to mediate SARS-CoV entry (Lu *et al.*, 2008). In (COS7) cells that are transfected with ACE2, the endocytosis entry pathway was further characterized using inhibitors and gene silencing and appeared to be clathrin-mediated. After CME, the virus relocates to EEA1-positive early endosomes (Inoue *et al.*, 2007). Wang *et al.* also studied the pathway of SARS-CoV internalization (2008). They used HEK293E cells transfected with ACE2 and Vero E6 cells for studies with inhibitors, expression of dominant-negative proteins, gene silencing and co-localization studies. They confirmed that endocytosis was associated with lipid rafts, brought the virus in early endosomes and depended on the pH drop in late endosomes. However, the pathway appeared independent from clathrin and caveolae. Next to low pH, activity of cathepsins is also required for successful SARS-CoV entry (Huang *et al.*, 2006). Recently, a novel mechanism of priming for the SARS-CoV S-protein has been reported. Sequential trypsin cleavage at two different sites, namely cleavage at the S1/S2 boundary and position 797 in S2, would enable the S-protein to mediate membrane fusion, presumably at the cell surface (Belouzard *et al.*, 2009).

1.3 REFERENCES

Addie, D. D., Schaap, I. A. T., Nicolson, L. & Jarret, O. (2003). Persistence and transmission of natural type I feline coronavirus infection. *J Gen Virol* **84**, 2735-2744.

Addie, D. D., Toth, S., Murray, G. D. & Jarret, O. (1995). Risk of feline infectious peritonitis in cats naturally infected with feline coronavirus. *Am J Vet Res* **56**, 429-433.

Aderem, A. & Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* **17**, 593-623.

Alvarez, C. P., Lasal, F., Carrillo, J., Muniz, O., Corbi, A. L. & Delgado, R. (2002). C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *J Virol* **76**, 6841-6844.

Araki, N., Johnson, M. T. & Swanson, J. A. (1996). A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* **135**, 1249-1260.

Babcock, G. J., Eshaki, D. J., Thomas Jr., W. D. & Ambrosino, D. M. (2004). Amino acids 270-510 of the severe acute respiratory syndrome coronavirus spike protein are required for interaction with receptor. *J Virol* **78**, 4552-4560.

Baker, S. C. (2008). Coronaviruses: molecular biology. In: *The encyclopedia of virology*. Mahy, B. (Ed.). Elsevier, New York, USA, 554-562.

Baric, R. S., Nelson, G. W., Fleming, J. O., Keck, J. G., Casteel, N. & Stohlman, S. A. (1988). Interactions between coronavirus nucleocapsid protein and viral RNAs: implications for viral transcription. *J Virol* **62**, 4280-4287.

Beattie, E. C., Carroll, R. C., Yu, X., Morishita, W., Yasuda, H., von Zastrow, M. & Malenka, R. C. (2000). Regulation of AMPA endocytosis by a signalling mechanism shared with LTD. *Nature Neurosci* **3**, 1291-1300.

Belouzard, S., Chu, V. C. & Whittaker, G. R. (2009). Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *PNAS* **106**, 5871-5876.

- Benbacer, L., Kut, E., Besnardeau, L., Laude, H. & Delmas, B. (1997).** Interspecies aminopeptidase N chimeras reveal species-specific receptor recognition by canine coronavirus, feline infectious peritonitis virus, and transmissible gastroenteritis virus. *J Virol* **71**, 734-737.
- Benetka, V., Kübber-Heiss, A., Kolodziejek, J., Nowotny, N., Hofmann-Parisot, M. & Möstl, K. (2004).** Prevalence of feline coronavirus types I and II in cats with histopathologically verified feline infectious peritonitis. *Vet Microbiol* **99**, 31-42.
- Bonavia, A., Zelus, B. D., Wentworth, D. E., Talbot, P. J. & Holmes, K. V. (2003).** Identification of a receptor-binding domain of the spike glycoprotein of human coronavirus HCoV-229E. *J Virol* **77**, 2530-2538.
- Bosch, B. J., van der Zee, R., de Haan, C. A. M. & Rottier, P. J. M. (2003).** The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J Virol* **77**, 8801-8811.
- Bredenbeek, P. J., Pachuk, C. J., Noten, A. F. H., Charité, J., Luytjes, W., Weiss, S. R. & Spaan, W. J. M. (1990).** The primary structure and expression of the 2nd open reading frame of the polymerase gene of the coronavirus MHV-A59 - A highly conserved polymerase is expressed by an efficient ribosomal frame shifting mechanism. *Nucleic Acids Res* **18**, 1825-1832.
- Breslin, J. J., Mork, I., Smith, M. K., Vogel, L. K., Hemmila, E. M., Bonavia, A., Talbot, P. J., Sjöström, H., Norén, O. & Holmes, K. V. (2003).** Human coronavirus 229E: receptor binding domain and neutralization by soluble receptor at 37 °C. *J Virol* **77**, 4435-4438.
- Brierley, I. (1995).** Ribosomal frameshifting on viral RNAs. *J Gen Virol* **76**, 1885-1892.
- Brierley, I., Bournsnel, M. E. G., Binns, M. M., Bilimoria, B., Blok, V. C., Brown, T. D. K. & Inglis, S. C. (1987).** An efficient ribosomal frame shifting signal in the polymerase-encoding region of the coronavirus IBV. *EMBO J* **6**, 3779-3785.
- Brockway, S. M., Clay, C. T., Lu, X. T. & Denison, M. R. (2003).** Characterization of the expression, intracellular localization, and replication complex association of the putative mouse hepatitis virus RNA-dependent RNA polymerase. *J Virol* **77**, 10515-10527.
- Brodsky, F. M., Chen, C.-Y., Kneuhl, C., Towler, M. C. & Wakeham, D. E. (2001).** Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu Rev Cell Dev Biol* **17**, 517-568.

Choi, K. S., Aizaki, H. & Lai, M. M. C. (2005). Murine coronavirus requires lipid rafts for virus entry and cell-cell fusion but not for virus release. *J Virol* **79**, 9862-9871.

Chu, V. C., McElroy, L. J., Aronson, J. M., Oura, T. J., Harbison, C. E., Bauman, B. E. & Whittaker, G. R. (2007). Feline aminopeptidase N is not a functional receptor for avian infectious bronchitis virus. *Virology* **4**, 20-29.

Chu, V. C., McElroy, L. J., Chu, V., Bauman, B. E. & Whittaker, G. R. (2006). The avian coronavirus infectious bronchitis virus undergoes direct low-pH-dependent fusion activation during entry in host cells. *J Virol* **80**, 3180-3188.

Compton, S. R., Rogers, D. B., Holmes, K. V., Fertsch, D., Remenick, J. & McGowan, J. J. (1987). In vitro replication of mouse hepatitis virus strain A59. *J Virol* **61**, 1814-1820.

Conner, S. D. & Schmid, S. L. (2003). Regulated portals of entry into the cell. *Nature* **422**, 37-44.

Cornelissen, E., Dewerchin, H. L., Van Hamme, E. & Nauwynck, H. J. (2009). Absence of antibody-dependent, complement-mediated lysis of feline infectious peritonitis virus-infected cells. *Virus Res* **144**, 285-289.

Cornelissen, E., Dewerchin, H. L., Van Hamme, E. & Nauwynck, H. J. (2007). Absence of surface expression of feline infectious peritonitis virus. *Vet Microbiol* **121**, 131-137.

Cremona, O., Di Paolo, G., Wenk, M. R., Lüthi, A., Kim, W. T., Takei, K., Daniell, L., Nemoto, Y., Shears, S. B., Flavell, R. A., Mc Cormick, D. A. & De Camilli, P. (1999). Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell* **99**, 179-188.

Damm, E., Pelkmans, L., Kartenbeck, J., Mezzacasa, A., Kurzchalia, T. & Helenius A. (2005). Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. *J Cell Biol* **168**, 477-488.

Danielsen, E. M. (1990). Biosynthesis of intestinal microvillar proteins. Dimerization of aminopeptidase N and lactase-phlorizin hydrolase. *Biochemistry* **29**, 305-308.

Danielsen, E. M. (1990). Perturbation of intestinal microvillar enzyme biosynthesis by amino acid analogs. *J Biol Chem* **265**, 14566-14571.

De Camilli, P. & Takei, K. (1996). Molecular mechanisms in synaptic vesicle endocytosis and recycling. *Neuron* **16**, 481-886.

De Camilli, P., Takei, K. & McPherson, P. S. (1995). The function of dynamin in endocytosis. *Curr Biol* **5**, 559-886.

De Groot-Mijnes, J., van Dun, J., van der Most, R. & de Groot, R. (2005). Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. *J Virol* **79**, 1036-1044.

De Haan, C. A., Haijema, B. J., Schellen, P., Wichers Schreur, P., te Lintelo, E., Vennema, H. & Rottier, P. J. M. (2008). Cleavage of group 1 coronavirus spike proteins: how furin cleavage is traded off against heparan sulphate binding upon cell culture adaptation. *J Virol* **82**, 6078-6083.

De Haan, C. A., Masters, P. S., Shen, X., Weiss, S. & Rottier, P. J. (2002). The Group-specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host. *Virology* **296**, 177-189.

Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L. K., Sjöström, H., Norén, O. & Laude, H. (1992). Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature* **357**, 417-419.

Delmas, B., Gelfi, J., Sjöström, H., Norén, O. & Laude, H. (1993). Further characterization of aminopeptidase N as a receptor for coronaviruses. *Adv Exp Med Biol* **342**, 293-298.

Denison, M. R., Spaan, W. J. M., van der Meer, Y., Gibson, C. A., Sims, A. C., Prentice, E. & Lu, S. T. (1999). The putative helicase of coronavirus Mouse hepatitis virus is processed from the replicase gene polyprotein and localizes in complexes that are active in viral RNA synthesis. *J Virol* **73**, 6862-6871.

Dewerchin, H. L. (2008). Characterization of putative immune evasion mechanisms of feline infectious peritonitis virus. PhD thesis, Ghent University, p181.

Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2006). Feline infectious peritonitis virus-infected monocytes internalize viral membrane-bound proteins upon antibody-addition. *J Gen Vir* **87**, 1685-1690.

Dewerchin, H. L., Cornelissen, E., Van Hamme, E. & Nauwynck, H. J. (2008). Surface-expressed viral proteins in feline infectious peritonitis virus-infected monocytes are internalized through a clathrin- and caveolae-independent pathway. *J Gen Vir* **89**, 2731-2740.

Di Fiore, P. P. & De Camilli, P. (2001). Endocytosis and signaling : an inseperable partnership. *Cell* **106**, 1-4.

Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V. & Simons, K. (1993). Caveolae and the sorting of the trans-Golgi network of epithelial cells. *EMBO J* **12**, 1597-1605.

Dveksler, G. S., Pensiero, M. N., Cardellichio, C. B., Williams, R. K., Jiang, G., Holmes, K. V. & Dieffenbach, C. W. (1991). Cloning of the mouse hepatitis virus (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. *J Virol* **65**, 6881-6891.

Dye, C. & Siddell, S. G. (2005). Genomic RNA sequence of feline coronavirus strain FIPV WSU-79/1146. *J Gen Virol* **86**, 2249-2253.

Dye, C., Temperton, N. & Siddell, S. G. (2007). Type I feline coronavirus spike glycoprotein fails to recognize aminopeptidase N as a functional receptor on feline cell lines. *J Gen Virol* **88**, 1753-1760.

Eifart, P., Ludwig, K., Böttcher, C., de Haan, C. A. M., Rottier, P. J. M., Korte, T. & Herrman, A. (2007). Role of endocytosis and low pH in murine hepatitis virus strain A59 cell entry. *J Virol* **81**, 10758-10768.

Fiscus, S. A. & Teramoto, Y. A. (1987). Antigenic comparison of feline coronavirus isolates: evidence for markedly different peplomer glycoproteins. *J Virol* **61**, 2607-2613.

Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. F. & van Kooyk, Y. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T-cells. *Cell* **100**, 587-597.

Gerber, J. D., Ingersoll, J. D., Gast, A. M., Christianson, K. K., Selzer, N. L., Landon, R. M., Pfeiffer, N. E., Sharpee, R. L. & Beckenhauer, W. H. (1990). Protection against feline infectious peritonitis by intranasal inoculation of a temperature-sensitive FIPV vaccine. *Vaccine* **8**, 536-542.

Gilbert, J. M. & Benjamin, T. L. (2000). Early steps of polyomavirus entry into cells. *J Virol* **74**, 8582-8588.

Gilbert, J. M., Goldberg, I. G. & Benjamin, T. L. (2003). Cell penetration and trafficking of polyomavirus. *J Virol* **77**, 2615-2622.

Glende, J., Schwegmann-Wessels, C., Al-Falah, M., Pfefferle, S., Qu, X., Deng, H., Drosten, C., Naim, H. Y. & Herrler, G. (2008). Importance of cholesterol-rich membrane microdomains in the interaction of the S protein of SARS-coronavirus with the cellular receptor angiotensin-converting enzyme 2. *Virology* **381**, 215-221.

Gold, E. S., Underhill, D. M., Morrissette, N. S., Guo, J., McNiven, M. A. & Aderem, A. (1999). Dynamin 2 is required for phagocytosis in macrophages. *J Exp Med* **190**, 1849-1856.

Gonzalez, J. M., Gomez-Puertas, P., Cavanagh, D., Gorbalenya, A. E. & Enjuanes, L. (2003). A comparative sequence analysis to revise the current taxonomy of the family *Coronaviridae*. *Arch Virol* **148**, 2207-2235.

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J. & Snijder, E. J. (2006). *Nidovirales*: evolving the largest RNA virus genome. *Virus Res* **117**, 17-37.

Gosert, R., Kanjanahaluethai, A., Egger, D., Bienz, K. & Baker, S. C. (2002). RNA replication of Mouse hepatitis virus takes place at double-membrane vesicles. *J Virol* **76**, 3697-3708.

Gunn-Moore, D., Gruffydd-Jones, T. & Harbour, D. (1998). Detection of feline coronaviruses by culture and reverse transcriptase polymerase chain reaction of blood samples from healthy cats and cats with clinical feline infectious peritonitis. *Vet Microbiol* **62**, 193-205.

Haagmans, B. L., Egberink, H. F. & Horzinek, M. C. (1996). Apoptosis and T-cell depletion during feline infectious peritonitis. *J Virol* **70**, 8977-8983.

Haijema, B. J., Rottier, P. & de Groot, R. J. (2007). Feline coronaviruses: a tale of two-faced types. In: *Coronaviruses, molecular and cellular biology*. Thiel, V. (Ed.). Caister Academic Press, Norfolk, UK, 183-203.

Haijema, B. J., Volders, H. & Rottier, P. (2004). Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. *J Virol* **78**, 3863-3871.

Halary, F., Amara, A., Lortat-Jacob, H., Messerle, M., Delaunay, T., Houles, C., Fieschi, F., Renzana-Seisdedos, F., Moreau, J. F., Chanet-Merville, J. (2002). Human cytomegalovirus

binding to DC-SIGN is required for dendritic cell infection and target cell *trans*-infection. *Immunity* **17**, 653-664.

Han, D. P., Lohani, M. & Cho, M. W. (2007). Specific asparagine-linked glycosylation sites are critical for DC-SIGN- and L-SIGN-mediated severe acute respiratory syndrome coronavirus entry. *J Virol* **81**, 12029-12039.

Hayashi, T., Watabe, Y., Nakayama, H. & Fujiwara, K. (1982). Enteritis due to feline infectious peritonitis virus. *Jpn J Vet Sci* **44**, 97-106.

Hegyí, A. & Kolb, A. F. (1998). Characterization of determinants involved in the feline infectious peritonitis virus receptor function of feline aminopeptidase N. *J Gen Virol* **79**, 1387-1391.

Herrewegh, A. A., de Groot, R. J., Cepica, A., Egberink, H., Horzinek, M. C. & Rottier, P. J. M. & (1995). Detection of feline coronavirus RNA in feces, tissues, and body fluids of naturally infected cats by reverse transcriptase PCR. *Clin Microbiol* **33**, 684-689.

Herrewegh, A. A., Smeenk, I., Horzinek, M. C., Rottier, P. J. M. & de Groot, R. J. (1998). Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. *J Virol* **72**, 4508-4514.

Hinshaw, J. E. (2000). Dynamin and its role in membrane fission. *Annu Rev Cell Dev Biol* **16**, 483-519.

Hinshaw, J. E. & Schmid, S. L. (1995). Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature* **374**, 190-192.

Hofmann, H., Pyrc, K., van der Hoek, L., Geier, M., Berkhout, B. & Pöhlmann, S. (2005). Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. *Proc Natl Acad Sci USA* **22**, 7988-7993.

Hofmann, H., Simmons, G., Rennekamp, A. J., Chaipan, C., Gramberg, T., Heck, E., Geier, M., Wegele, A., Marzi, A., Bates, P. & Pöhlmann, S. (2006). Highly conserved regions within the spike proteins of human coronavirus 229E and NL63 determine recognition of their respective cellular receptors. *J Virol* **80**, 8639-8652.

Hohdatsu, T., Izumiya, Y., Yokoyama, Y., Kida, K. & Koyama, H. (1998). Differences in virus receptor for type I and type II feline infectious peritonitis virus. *Arch Virol* **143**, 839-850.

Hohdatsu, T., Nakamura, M., Ishizuka, Y., Yamada, H. & Koyama, H. (1991). A study on the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection in feline macrophages by monoclonal antibodies. *Arch Virol* **120**, 207-217.

Hohdatsu, T., Okada, S. & Koyama, H. (1991). Characterization of monoclonal antibodies against feline infectious peritonitis virus type II and antigenic relationship between feline, porcine, and canine coronaviruses. *Arch Virol* **117**, 85-95.

Hohdatsu, T., Tokunaga, J. & Koyama, H. (1994). The role of IgG subclass of mouse monoclonal antibodies in antibody-dependent enhancement of feline infectious peritonitis virus infection of feline macrophages. *Arch Virol* **139**, 273-285.

Hofmann, H., Simmons, G., Rennekamp, A. J., Chaipan, C., Gramberg, T., Heck, E., Geier, M., Wegele, A., Marzi, A., Bates, P. & Pöhlmann, S. (2006). Highly conserved regions within the spike proteins of human coronavirus 229E and NL63 determine recognition of their respective cellular receptors. *J Virol* **80**, 8639-8652.

Huang, I. C., Bosch, B. J., Li, W., Farzan, M., Rottier, P. M. & Choe, H. (2006). SARS-CoV, but not HCoV-NL63, utilizes cathepsins to infect cells: viral entry. *Adv Exp Med Biol* **581**, 335-338.

Inoue, Y., Tanaka, N., Tanaka, Y., Inoue, S., Morita, K., Zhuang, M., Hattori, T. & Sugamura, K. (2007). Clathrin-dependent entry of severe acute respiratory syndrome coronavirus into target cells expressing ACE2 with the cytoplasmic tail deleted. *J Virol* **81**, 8722-8729.

Ishida, T., Shibana, A., Tanaka, S., Uchida, K. & Mochizuki, M. (2004). Use of recombinant feline interferon and glucocorticoid in the treatment of feline infectious peritonitis. *J Feline Med Surg* **6**, 107-109.

Jacobse-Geels, H., Daha, M. & Horzinek, M. C. (1982). Antibody, immune complexes and complement activity fluctuations in experimental feline infectious peritonitis. *Am J Vet Res* **43**, 666-670.

Jacobse-Geels, H., Daha, M. & Horzinek, M. C. (1980). Isolation and characterization of feline C3 and evidence for the immune complex pathogenesis of feline infectious peritonitis virus. *J Immunol* **125**, 1606-1610.

Jeffers, S. A., Hemmila, E. M. & Holmes, K. V. (2006). Human coronavirus 229E can use CD209L (L-SIGN) to enter cells. *Adv Exp Med Biol* **581**, 265-269.

Jeffers, S. A., Tusell, S. M., Gillim-Ross, L., Hemmila, E. M., Achenbach, J. E., Babcock, G. J., Thomas, W. D., Thackray, L. B., Young, M. D., Mason, R. J., Ambrosino, D. M., Wentworth, D. E., Demartini, J. C. & Holmes, K. V. (2004). CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci USA* **101**, 15748-15753.

Kawase, M., Shirato, K., Matsuyama, S. & Taguchi, F. (2009). Protease-mediated entry via the endosome of human coronavirus 229E. *J Virol* **83**, 712-721.

Khoo, U., Chan, K. Y. K., Chan, V. S. F. & Lin, C. L. S. (2008). DC-SIGN and L-SIGN: the SIGNS for infection. *J Mol Med* **86**, 861-874.

Kida, K., Hohdatsu, T., Kashimoto-Tokunaga, J. & Koyama, H. (2000). Neutralization of feline infectious peritonitis virus: preparation of monoclonal antibody that shows cell tropism in neutralizing activity after viral absorption into the cells. *Arch Virol* **145**, 1-12.

Kipar, A., Köhler, K., Leukert, W. & Reinacher, M. (2001). A comparison of lymphatic tissues from cats with spontaneous feline infectious peritonitis virus infection but no FIP, and cats with no infection. *J Comp Path* **125**, 182-191.

Kipar, A., May, H., Menger, S., Weber, M., Leukert, W. & Reinacher, M. (2005). Morphologic features and development of granulomatous vasculitis in feline infectious peritonitis. *Vet Pathol* **42**, 321-330.

Kirkham, M. & Parton, R. G. (2005). Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim Biophys Acta* **1746**, 349-363.

Kooi, C., Cervin, M. & Anderson, R. (1991). Differentiation of acid-pH-dependent and -nondependent entry pathways for mouse hepatitis virus. *Virology* **180**, 108-119.

Kozak, M. (1989). The scanning model for translation: an update. *J Cell Biol* **108**, 229-241.

Krzystyniak, K. & Dupuy, J. M. (1984). Entry of mouse hepatitis virus 3 into cells. *J Gen Virol* **65**, 227-231.

Kuiken, T., Fouchier, R. A., Schutten, M., Rimmelzwaan, G. F., van Amerongen, G., van Riel, D., Laman, J. D., de Jong, T., van Doornum, G., Lim, W., Ling, A. E., Chan, P. K., Tam, J. S., Zambon, M. C., Gopal, R., Drosten, C., van der Werf, S., Escriou, N., Manuguerra, J. C., Stöhr, K., Peiris, J. S. & Osterhaus, A. D. (2003). Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* **362**, 263-270.

Kummrow, M., Meli, M. L., Haessig, M., Goenczi, E., Poland, A., Pedersen, N. C., Hofmann-Lehmann, R. & Lutz, H. (2005). Feline coronavirus serotypes 1 and 2: seroprevalence and association with disease in Switzerland. *Clin Diagn Lab Immunol* **12**, 1209-1215.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A. & Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol Cell* **7**, 661-671.

Lanzrein, M., Schlegel, A. & Kempf, C. (1994). Entry and uncoating of enveloped viruses. *Biochem J* **302**, 313-320.

Lemmon, S. K. (2001). Clathrin uncoating : auxilin comes to life. *Curr Biol* **11**, 49-52.

Li, B. X., Ge, J. W. & Li, Y. J. (2007). Porcine aminopeptidase N is a functional receptor for the PEDV coronavirus. *Virology* **365**, 166-172.

Li, W., Moore, M. J., Vasilieva, N. Sui, J., Wong, S. K., Berne, M. A., Somasundaran, M., Sullivan, J. L., Luzuriaga, K., Greenough, T. C., Choe, H., Farzan, M. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450-454.

Li, W., Sui, J., Huang, I., J. H., Radoshitzky, S. R., Marasco, W. A., Choe, H. & Farzan, M. (2007). The S protein of human coronavirus NL63 and severe acute respiratory syndrome coronavirus bind overlapping regions of ACE2. *Virology* **367**, 367-374.

Lim, K. P. & Liu, D. X. (2001). The missing link in coronavirus assembly. *J Biol Chem* **276**, 17515-17523.

Lin, H., Feng, Y., Wong, G., Wang, L., Li, B., Zhao, X., Li, Y., Smaill, F. & Zhang, C. (2008). Identification of residues in the receptor-binding domain (RBD) of the spike protein of human coronavirus NL63 that are critical for the RBD-ACE2 receptor interaction. *J Gen Virol* **89**, 1015-1024.

Lozach, P. Y., Lortat-Jacob, H., De Lacroix De Lavalette, A., Staropoli, I., Fong, S., Amara, A., Houles, C., Fieschi S., Schwartz, O., Virelizier, J. L., Arenzana-Seisdedos, F. &

Altmeyer, R. (2003). DC-SIGN and L-SIGN are high-affinity binding receptors for hepatitis C virus glycoprotein E2. *J Biol Chem* **278**, 20358-20366.

Lu, Y., Liu, X. L. & Tam, J. P. (2008). Lipid rafts are involved in SARS-CoV entry into Vero E6 cells. *Biochem Biophys Res Comm* **369**, 344-349.

Luan, Y. & Xu, W. (2007). The structure and main functions of aminopeptidase N. *Curr Med Chem* **14**, 639-647.

Luytjes, W. (1995). Coronavirus gene expression - genome organization and protein synthesis. In: The coronaviridae. Siddell, S. G. (Ed.). Plenum Press, New York, USA, 33-54.

Madu, I. G., Chu, V. C., Lee, H., Regan, A. D., Bauman, B. E. & Whittaker, G. R. (2007). Heparan sulphate is a selective attachment factor for the avian coronavirus infectious bronchitis virus Beaudette. *Avian Dis* **51**, 45-51.

Maeda, J., Repass, J.F., Maeda, A. & Makino, S. (2001). Membrane topology of coronavirus E protein. *Virology* **281**, 163-169.

Marzi, A., Gramberg, T., Simmons, G., Moller, P., Rennekamp, A. J., Krumbiegel, M., Geier, M., Eisemann, J., Turza, N., Saunier, B., Steinkasserer, A., Becker, S., Bates, P., Hofmann, H. & Pöhlmann, S. (2004). DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus. *J Virol* **78**, 12090-12095.

Masters, P. S. (2006). The molecular biology of coronaviruses. *Adv Virus Res* **66**, 193-292.

Mathewson, A. C., Bishop, A., Yao, Y., Kemp, F., Ren, J., Chen, H., Xu, X., Berkhout, B., van der Hoek, L. & Jones, I. M. (2008). Interaction of severe acute respiratory syndrome-coronavirus and NL63 coronavirus spike proteins with angiotensin converting enzyme-2. *J Gen Virol* **89**, 2741-2745.

Matsuyama, S. & Taguchi, F. (2002). Receptor-induced conformational changes of murine coronavirus spike protein. *J Virol* **76**, 11819-11826.

Meli, M., Kipar, A., Müller, C., Jenal, K., Gönczi, E., Borel, N., Gunn-Moore, D., Chalmers, S., Lin, F., Reinacher, M. & Lutz, H. (2004). High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. *J Feline Med Surg* **6**, 69-81.

Mellman, I. & Steinman, R. M. (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106**, 255-258.

Miguel, B., Pharr, G. T. & Wang, G. (2002). The role of aminopeptidase N as a receptor for infectious bronchitis virus. *Arch Virol* **147**, 2047-2056.

Mina-Osorio, P. & Ortega, E. (2005). Aminopeptidase N (CD13) functionally interacts with FcγRs in human monocytes. *J Leukoc Biol* **77**, 1008-1017.

Mitchell, D. A., Fadden, A. J. & Drickamer, K. (2001). A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. *J Biol Chem* **276**, 28939-28945.

Miura, H. S., Nakagaki, K. & Taguchi, F. (2004). N-terminal domain of the murine coronavirus receptor CEACAM1 is responsible for fusogenic activation and conformational changes of the spike protein. *J Virol* **78**, 216-223.

Monier, S., Parton, R. G., Vogel, F., Behlke, J., Henske, A. & Kurzchalia, T. (1995). VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes *in vivo* and *in vitro*. *Mol Biol Cell* **6**, 911-927.

Murata, M., Peranen, J., Schreiner, R., Weiland, F., Kurzchalia, T. & Simons, K. (1995). VIP21/ caveolin is a cholesterol-binding protein. *Proc Natl Acad Sci USA* **92**, 10339-10343.

Nash, T. C. & Buchmeier, M. J. (1997). Entry of mouse hepatitis virus into cells by endosomal and nonendosomal pathways. *Virology* **233**, 1-8.

Navarrete Santos, A., Langner, J., Herrmann, M. & Riemann, D. (2000). Aminopeptidase N/CD13 is directly linked to signal transduction pathways in monocytes. *Cell Immunol* **201**, 22-32.

Nocek, B., Mulligan, R., Bargassa, M., Collart, F. & Joachimiak, A. (2007). Crystal structure of aminopeptidase N from human pathogen *Neisseria meningitidis*. *Proteins* **70**, 273-279.

Nomura, R., Kiyota, A., Suzaki, E., Kataoka, K., Ohe, Y., Miyamoto, K., Senda, T. & Fujimoto, T. (2004). Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. *J Virol* **78**, 8701-8708.

Oh, J. S., Song, D. S. & Park, B. K. (2003). Identification of a putative cellular receptor 150 kDa polypeptide for porcine epidemic diarrhea virus in porcine enterocytes. *J Vet Sci* **4**, 269-275.

Olsen, C. W., Corapi, W. V., Ngichabe, C. K., Baines, J. D. & Scott, F. W. (1992). Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody dependent enhancement of infection of feline macrophages. *J Virol* **66**, 956-965.

Opstelten, D. E., Raamsman, M. J. B., Wolfs, K., Horzinek, M. C. & Rottier, P. J. M. (1995). Envelope glycoprotein interactions in coronavirus assembly. *J Cell Biol* **131**, 339-349.

Ortego, J., Sola, I., Almazan, J., Ceriani, J. E., Riquelme, C., Balasch, M., Plana, J. & Enjuanes, L. (2003). Transmissible gastroenteritis virus coronavirus gene 7 is not essential but influences in vivo virus replication and virulence. *Virology* **308**, 13-22.

Othsuka, N., Yamada, Y. K. & Taguchi, F. (1996). Difference in virus-binding activity of two distinct receptor proteins of mouse hepatitis virus. *J Gen Virol* **77**, 1683-1692.

Paltrinieri, S., Parodi Cammarata, M., Cammarata, G. & Comazzi, S. (1998). Some aspects of humoral and cellular immunity in naturally occurring feline infectious peritonitis. *Vet Immunol Immunopathol* **65**, 205-220.

Paltrinieri, S., Ponti, W., Comazzi, S., Giordano, A. & Poli, G. (2003). Shifts in lymphocyte subsets in cats with feline infectious peritonitis (FIP): pathogenic role and diagnostic relevance. *Vet Immunol Immunopathol* **96**, 141-148.

Parton, R. G., Joggerst, B. & Simons, K. (1994). Regulated internalization of caveolae. *J Cell Biol* **127**, 1199-1215.

Parton, R. G. & Richards, A. A. (2003). Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. *Traffic* **4**, 724-738.

Pedersen, N. C., Allen, C. E. & Lyons, L. A. (2008). Pathogenesis of feline enteric coronavirus infection. *J Feline Med Surg* **10**, 529-541.

Pedersen, N. C., Black, J. W., Boyle, J. F., Evermann, J. F., McKeirnan, A. J. & Ott, R. L. (1984). Pathogenic differences between various feline coronavirus isolates. Coronaviruses; molecular biology and pathogenesis. *Adv Exp Med Biol* **173**, 365-380.

Pedersen, N. C. & Boyle, J. F. (1981). Immunologic phenomena in the effusive form of feline infectious peritonitis virus. *Am J Vet Res* **41**, 868-876.

Pedersen, N. C., Boyle, J. F., Floyd, K., Fudge, A. & Barker, J. (1981). An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. *Am J Vet Res* **42**, 368-377.

Pedersen, N. C., Evermann, J. F., McKeirnan, A. J. & Ott, R. L. (1984). Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. *Am J Vet Res* **45**, 2580-2585.

Pelkmans, L. & Helenius, A. (2002). Endocytosis via caveolae. *Traffic* **3**, 311-320.

Pelkmans, L. & Helenius, A. (2003). Insider information: what viruses tell us about endocytosis. *Curr Opin Cell Biol* **15**, 414-422.

Pelkmans, L., Kartenbeck, J. & Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol* **3**, 473-483.

Pelkmans, L., Püntener, D. & Helenius, A. (2002). SV-40 induced internalization of caveolae involves local actin polymerization and dynamin-recruitment. *Science* **296**, 535-539.

Phonhok, Y. & Rosenthal, K. S. (1991). Stabilization of clathrin coated vesicles by amantadine, tromantadine and other hydrophobic amines. *FEBS Lett* **281**, 188-190.

Pöhlmann, S., Zhang, F., Baribaud, F., Chen, Z., Leslie, G. J., Lin, G., Granelli-Piperno, A., Doms, R. W., Rice, C. M. & McKeating, J. A. (2003). Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J Virol* **77**, 4070-4080.

Poland, A. M., Vennema, H., Foley, J. E. & Pedersen, N. C. (1996). Two related strains of feline infectious peritonitis virus isolated from immunocompromised cats infected with a feline enteric coronavirus. *J Clin Microbiol* **34**, 3180-3184.

Pu, Y. & Zhang, X. (2008). Mouse hepatitis virus type 2 enters cells through a clathrin-mediated endocytic pathway independent of Eps15. *J Virol* **82**, 8112-8123.

Qiu, Z., Hingley, S. T., Simmons, G., Yu, C., Das Sarma, J., Bates, P. & Weiss, S. R. (2006). Endosomal proteolysis by cathepsins is necessary for murine coronavirus mouse hepatitis virus type 2 spike-mediated entry. *J Virol* **80**, 5768-5776.

Racoosin, E. L. & Swanson, J. A. (1993). Macropinosome maturation and fusion with tubular lysosomes in macrophages. *J Cell Biol* **121**, 1011-1020.

Rahman, S. A. E., El-Kenawy, A. A., Neumann, U., Herrler, G. & Winter, C. (2009). Comparative analysis of the sialic acid binding activity and the tropism for the respiratory epithelium of four different strains of avian infectious bronchitis virus. *Avian Pathol* **38**, 41-45.

Rawlings, N. D. & Barrett, A. J. (1999). MEROPS: the peptidase database. *Nucl Acids Res* **27**, 325-331.

Regan, A. D., Shraybaman, R., Cohen, R. D. & Whittaker, G. R. (2008). Differential role for low pH and cathepsin-mediated cleavage of the viral spike protein during entry of serotype II feline coronaviruses. *Vet Microbiol* **132**, 235-248.

Regan, A. D. & Whittaker, G. R. (2008). Utilization of DC-SIGN for entry of feline coronaviruses into host cells. *J Virol* **82**, 11992-11996.

Ren, X., Glende, J., Yin, J., Schwegmann-Wessels, C. & Herrler, G. (2008). Importance of cholesterol for infection of cells by transmissible gastroenteritis virus. *Virus Res* **137**, 220-224.

Ridley, A. J. (2001). Rho proteins: linking signalling with membrane trafficking. *Traffic* **2**, 303-310.

Ritter, T. E., Fajardo, O., Matsue, H., Anderson, R. G. & Lacey, S. W. (1995). Folate receptors targeted to clathrin-coated pits can not regulate vitamin uptake. *Proc Natl Acad Sci USA* **92**, 3824-3828.

Ritz, S. J., Egberink, H. & Hartmann, K. (2007). Influence of feline interferon-omega on the survival time and quality of life of cats with feline infectious peritonitis. *J Vet Intern Med* **21**, 1193-1197.

Rodal, S. K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B. & Sandvig, K. (1999). Extraction of cholesterol with methyl- β -cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell* **10**, 961-974.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R. & Anderson, R. G. (1992). Caveolin, a protein component of caveolae membrane coats. *Cell* **68**, 673-682.

Rottier, P. J. M., Nakamura, K., Schellen, P., Volders, H. & Haijema, B. J. (2005). Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. *J Virol* **79**, 14122-14130.

Rottier, P. J. M. & Rose, J. K. (1987). Coronavirus E1 glycoprotein expressed from cloned cDNA localizes in the golgi region. *J Virol* **61**, 2042-2045.

Rottier, P. J. M., Welling, G. W., Welling-Wester, S., Niesters, H. G. M., Lenstra, J. A. & Van der Zeijst, B. A. M. (1986). Predicted membrane topology of the coronavirus protein E1. *Biochemistry* **25**, 1335-1339.

Roy, A.-M. M., Parker, J. S., Parrish, C. R. & Whittaker, G. R. (2000). Early stages of influenza virus entry into Mv-1 lung cells: involvement of dynamin. *Virology* **267**, 17-28.

Sabharanjak, S. & Mayor, S. (2004). Folate receptor endocytosis and trafficking. *Adv Drug Deliv Rev* **56**, 1099-1109.

Sabharanjak, S., Sharma, P., Parton, R. G. & Mayor, S. (2002). GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell* **2**, 411-423.

Sanchez-San Martin, C., Lopez, T., Arias, C. F. & Lopez, S. (2004). Characterization of rotavirus cell entry. *J Virol* **78**, 2310-2318.

Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I. (1996). Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J* **15**, 6241-6250.

Sauvonnet, N., Dujeancourt, A. & Dautry-Varsat, A. (2005). Cortactin and dynamin are required for the clathrin-independent endocytosis of γ c cytokine receptor. *J Cell Biol* **168**, 155-163.

Savarino, A., Boelaert, J., Cassone, A., Majori, G. & Cauda, R. (2003). Effects of chloroquine on viral infections: an old drug against today's diseases? *Infect Dis* **3**, 722-727.

Sawicki, S. G. & Sawicki, D. L. (1995). Coronaviruses use discontinuous extension for synthesis of subgenome-length negative strands. *Adv Exp Med Biol* **380**, 499-506.

Sawicki, S. G., Sawicki, D. L. & Siddell, S. G. (2007). A contemporary view of coronavirus transcription. *J Virol* **81**, 20-29.

Schluns, K. S. & Lefrancois, L. (2003). Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* **3**, 269-279.

Schmid, S. L. (1997). Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu Rev Biochem* **66**, 511-548.

Schnitzer, J. E., Oh, P., Pinney, E. & Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis and capillary permeability of select macromolecules. *J Cell Biol* **127**, 1217-1232.

Schultze, B., Cavanagh, D. & Herrler, G. (1992). Neuraminidase treatment of avian infectious bronchitis coronavirus reveals a hemagglutinating activity that is dependent on sialic acid-containing receptors on erythrocytes. *Virology* **189**, 792-794.

Seto, E. S., Bellen, H. J. & Lloyd, T. E. (2002). When cell biology meets development: endocytic regulation of signalling pathways. *Genes Dev* **16**, 1314-1336.

Siddell, S. G. & Snijder, E. J. (2008). An introduction to Nidoviruses. In: Nidoviruses. Perlman, S., Gallagher, T. & Snijder, E. J. (Eds). ASM Press, Washington D.C., 1-13.

Sieczkarski, S. B. & Whittaker, G. R. (2002). Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. *J Virol* **76**, 10455-10464.

Simmons, G., Reeves, J. D., Rennekamp, A. J., Amberg, S. M., Piefer, A. J. & Bates, P. (2004). Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. *Proc Natl Acad Sci USA* **101**, 4240-4245.

Simpson, F., Hussain, N. K., Qualmann, B., Kelly, R. B., Kay, B. K., McPherson, P. S. & Schmid, S. L. (1999). SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nat Cell Biol* **1**, 119-124.

Sjöström, H. & Norén, O. (1982). Changes of the quaternary structure of microvillus aminopeptidase in the membrane. *Eur J Biochem* **122**, 245-250.

Sjöström, H., Norén, O. & Olsen, J. (2000). Structure and function of aminopeptidase N. *Adv Exp Med Biol* **477**, 25-34.

Slepnev, V. I., Ochoa, G. C., Butler, M. H. & De Camilli, P. (2000). Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites. *J Biol Chem* **275**, 17583-17589.

Smith, A. E. & Helenius, A. (2004). How viruses enter animal cells. *Science* **304**, 237-241.

Snijder, E. J., Bredenbeek, P. J., Dobbe, J. C., Thiel, V., Ziebuhr, J., Poon, L. L., Guan, Y., Rozanov, M. Spaan, W. J. & Gorbalenya, A. E. (2003). Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. *J Mol Biol* **331**, 991-1004.

Snijder, E. J., van der Meer, Y., Zevenhoven-Dobbe, J., Onderwater, J. J. M., van der Meulen, J., Koerten, H. K. & Mommaas, A. M. (2006). Ultrastructure and origin of membrane vesicles associated with the severe acute respiratory syndrome coronavirus replication complex. *J Virol* **80**, 5927-5940.

Snyder, G. A., Colonna, M. & Sun, P. D. (2005). The structure of DC-SIGNR with a portion of its repeat domain lends insights to modelling of the receptor tetramer. *J Mol Biol* **347**, 979-989.

Söderberg, C., Giugni, T. D., Zaia, J. A., Larsson, S., Wahlberg, J. M. & Möller, E. (1993). CD13 (human aminopeptidase N) mediates human cytomegalovirus infection. *J Virol* **67**, 6576-6585.

Spaan, W. J. M., Cavanagh, D. & Horzinek, M. C. (1988). Coronaviruses: structure and genome expression. *J Gen Virol* **69**, 2939-2952.

Spector, I., Shochet, N. R., Blasberger, D. & Kashman, Y. (1989). Latrunculins - novel marine macrolides that disrupt microfilament organization and affect cell growth. *Cell Motil Cytoskeleton* **13**, 127-144.

Stertz, S., Reichelt, M., Spiegel, M., Kuri, T., Martinez-Sobrido, L., Garcia-Sastre, A., Weber, F. & Kochs, G. (2007). The intracellular sites of early replication and budding of SARS-coronavirus. *Virology* **361**, 304-315.

Stoddart C. A. & Scott, F. W. (1989). Intrinsic resistance of feline peritoneal macrophages to coronavirus infection correlates with in vivo virulence. *J Virol* **63**, 436-440.

Stohlman, S. A., Fleming, J. O., Patton, C. D. & Lai, M. M. C. (1983). Synthesis and subcellular localization of the murine coronavirus nucleocapsid protein. *Virology* **130**, 527-532.

Sturman, L. S., Holmes, K. V. & Behnke, J. (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *J Virol* **33**, 449-462.

Swanson, J. A. & Watts, C. (1995). Macropinocytosis. *Trends Cell Biol* **5**, 424-428.

Takano, T., Hohdatsu, T., Toda, A., Tanabe, M. & Koyama, H. (2007). TNF-alpha, produced by feline infectious peritonitis virus (FIPV)-infected macrophages, upregulates expression of type II FIPV receptor feline aminopeptidase N in feline macrophages. *Virology* **364**, 64-72.

Takano, T., Katada, Y., Moritoh, S., Ogasawara, M., Satoh, K., Satoh, R., Tanabe, M. & Hohdatsu, T. (2008). Analysis of the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection: aminopeptidase N is not important and a process of acidification of the endosome is necessary. *J Gen Virol* **89**, 1025-1029.

Tassaneeritthep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke J., Sun, W., Eller, M. A., Pattanapanyasat, K., Sarasombath, S., Birx, D. L., Steinman, R. M., Schlesinger, S. & Marovich, M. A. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med* **197**, 823-829.

Tebar, F., Bohlander, S. K. & Sorkin, A. (1999). Clathrin assembly lymphoid myeloid leukemia (CALM) protein: localization in endocytic-coated pits, interactions with clathrin, and the impact of overexpression on clathrin-mediated traffic. *Mol Biol Cell* **10**, 2687-2702.

Thomsen, P., Roepstorff, K., Stahlhut, M. & van Deurs, B. (2002). Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Mol Biol Cell* **13**, 238-250.

Thorp, E. B. & Gallagher, T. M. (2004). Requirements for CEACAMs and cholesterol during murine coronavirus cell entry. *J Virol* **78**, 2682-2692.

Tresnan, D. B., Levis, R. & Holmes, K. V. (1996). Feline aminopeptidase N serves as a receptor for feline, canine, porcine and human coronaviruses in serogroup I. *J Virol* **70**, 8669-8674.

- Tusell, S. M., Schittone, S. A. & Holmes, K. V. (2007).** Mutational analysis of aminopeptidase N, a receptor for several group 1 coronaviruses, identifies key determinants of viral host range. *J Virol* **81**, 1261-1273.
- Vennema, H., Heijnen, L., Rottier, P. J. M., Horzinek, M. C. & Spaan, W. J. M. (1992).** A novel glycoprotein of feline infectious peritonitis coronavirus contains a KDEL-like endoplasmic reticulum retention signal. *J Virol* **66**, 4951-4956.
- Vennema, H., Poland, A., Foley, J. & Pedersen, N. C. (1998).** Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. *Virology* **243**, 150-157.
- Voyles, B. A. (2002).** Getting in: attachment, penetration and uncoating. In: The biology of viruses. Voyles, B. A. (Ed.). McGraw-Hill, New York, USA, 58-66.
- Wang, H., Yang, P., Liu, K., Guo, F., Zhang, Y., Zhang, G. & Jiang, C. (2008).** SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. *Cell Res* **18**, 290-301.
- Wang, L., Rothberg, K. G. & Anderson, R. G. W. (1993).** Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J Cell Biol* **123**, 1107-1117.
- Weingartl, H. M. & Derbyshire, J. B. (1994).** Evidence for a putative second receptor for porcine transmissible gastroenteritis virus on the villous enterocytes of newborn pigs. *J Virol* **68**, 7253-7259.
- Weiss, R. C., Cox, N. R. & Boudreaux, M. K. (1993).** Toxicologic effects of ribavirin in cats. *J Vet Pharmacol Ther* **16**, 301-316.
- Weiss, R. C., Cox, N. R. & Ostrom-Ram, T. (1990).** Effect of interferon or *Propionibacterium acnes* on the course of experimentally induced feline infectious peritonitis in specific pathogen free and random source cats. *Am J Vet Res* **51**, 726-733.
- Weiss, R. C. & Scott, F. W. (1981).** Pathogenesis of feline infectious peritonitis: nature and development of viremia. *Am J Vet Res* **42**, 382-390.
- Wentworth, D. E. & Holmes, K. V. (2001).** Molecular determinants of species specificity in the coronavirus receptor aminopeptidase N (CD13): influence of N-linked glycosylation. *J Virol* **75**, 9741-9752.

Wilde, A. & Brodsky, F. M. (1996). In vivo phosphorylation of adaptors regulates their interaction with clathrin. *J Cell Biol* **135**, 635-645.

Williams, T. M. & Lisanti, M. P. (2004). The caveolin proteins. *Genome Biol* **5**, 214-222.

Winter, C., Herrler, G. & Neumann, U. (2008). Infection of the tracheal epithelium by infectious bronchitis virus is sialic acid dependent. *Microbes Infect* **10**, 367-373.

Winter, C., Schwegmann-Weßels, C., Cavanagh, D., Neumann, U. & Herrler, G. (2006). Sialic acid is a receptor determinant for infection of cells by avian infectious bronchitis virus. *J Gen Virol* **87**, 1209-1216.

Wong, S. K., Li, W., Moore, M. J., Choe, H. & Farzan, M. (2004). A 193-amino-acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J Biol Chem* **279**, 3197-3201.

Yang, Z. Y., Huang, Y., Ganesh, L., Leung, K., Kong, W. P., Schwartz, O., Subbarao, K. & Nabel, G. J. (2004). pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. *J Virol* **78**, 5642-5650.

Yeager, C. L., Ashmun, R. A., Williams, R. K., Cardellicchio, C. B., Shapiro, L. H., Look, A. T. & Holmes, K. V. (1992). Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* **357**, 420-422.

Zelus, B. D., Schickli, J. H., Blau, D. M., Weiss, S. R. & Holmes, K. V. (2003). Conformational changes in the spike glycoprotein of murine coronavirus are induced at 37 °C either by soluble murine CEACAM1 receptors or by pH 8. *J Virol* **77**, 830-840.

Ziebuhr, J., Snijder, E. J. & Gorbalenya, A. E. (2000). Virus-encoded proteinases and proteolytic processing in the Nidovirales. *J Gen Virol* **81**, 853-879.

Zuñiga, S., Sola, I., Moreno, J. L., Sabella, P., Plana-Duran, J. & Enjuanes, L. (2007). Coronavirus nucleocapsid protein is an RNA chaperone. *Virology* **357**, 215-227.

Chapter 2

AIMS

Feline infectious peritonitis (FIP) is the most important infectious cause of death in cats. One to 5 % of the European cat population is a potential victim of this infection. At present, no means are available to heal cats. Furthermore, there is no effective vaccine available for prevention. Expanding the knowledge on FIP virus (FIPV) pathogenesis is indispensable in the search for a vaccine and treatment.

The process of viral entry is an attractive target for the development of new therapeutic agents. For example in the battle against human immunodeficiency virus 1 infections, this approach already led to the development of promising agents (Cooley & Lewin, 2003). This study was set up to unravel the process of FIPV entry and identify possible targets for drug development. At the start of this research, little was known about FIPV entry. It was even questioned whether FIPV entry is mediated via direct fusion at the plasma membrane or via fusion with endosomal membranes after endocytosis.

Viral entry consists of two major events. First, the virus binds to its receptor on the plasma membrane, leading to viral attachment. In a second step, bound virus particles pass the plasma membrane either via direct fusion or via endocytosis, which results in virus internalization. Internalization via endocytosis is followed by an extra uncoating step to release the viral RNA in the cytosol.

The first study was aimed at providing insights in how attachment and internalization are regulated in time and to quantify virus binding and internalization over time in monocytes, the target cell for FIPV *in vivo*. Different virus strains and different cats were included. It was also checked if cell lines were valid models to study FIPV entry. This study was set up to reveal whether FIPV enters target cells via direct fusion or via endocytosis, or a combination of both (chapter 3), and to provide basic information that was needed for the experimental set-up for the following studies that focus on attachment and internalization separately.

In chapter 4, the use of potential receptors for FIPV at different stages of the entry process was evaluated. This study was performed to provide new insights in the virus-receptor interactions upon viral contact with the host cell, the monocyte.

In a third study, the mechanism of internalization was further analysed in monocytes. Therefore, a variety of techniques was included, ranging from inhibition experiments, lentiviral transduction to express dominant-negative proteins in monocytes and co-localization studies (chapter 5). This study could identify cellular proteins that are necessary for FIPV entry in monocytes.

Finally, the release of the genome in the cytosol, necessary for genome replication, transcription and translation, was studied. A kinetical study of this process was performed to supply data on the efficiency of uncoating. Further, it was checked which triggers might induce release of the FIPV genome into the cytosol in monocytes (chapter 6).

REFERENCES

Cooley, L. A., & Lewin, S. R. (2003). HIV-1 cell entry and advances in viral entry inhibitor therapy. *J Clin Virol* **26**, 121-132.

**KINETIC ANALYSIS OF ATTACHMENT AND INTERNALIZATION
OF FELINE INFECTIOUS PERITONITIS VIRUS**

Evelien Van Hamme, Hannah L. Dewerchin, Els Cornelissen & Hans J. Nauwynck

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ABSTRACT

In this study, kinetics of attachment and internalization of feline infectious peritonitis virus (FIPV) serotype I strain Black and serotype II strain 79-1146, were determined in feline monocytes from two cats and in Crandell feline kidney (CrFK) cells. Attached FIPV I (Black) particles were observed on almost all monocytes. Within 1 hour, 17 particles were bound per cell and within 1 minute, 89 % of the bound particles were internalized. For FIPV II (79-1146), attachment was observed on 66 % and 95 % of all monocytes from the two cats. After 1 hour, respectively 5 and 20 particles were bound per cell (all cells considered). Within 1 minute, 60 % of the bound particles were internalized. Internalization in monocytes was efficient and proceeded via endocytosis. In CrFK cells, attachment and internalization were less efficient, especially for FIPV I (Black), so this cell line is not suitable for studying FIPV entry.

INTRODUCTION

Feline infectious peritonitis virus (FIPV) is a coronavirus belonging to the order of the *Nidovirales* (Cavanagh, 1997; Cowley *et al.*, 2000; Gorbalenya *et al.*, 2006). Based on comparative sequence analysis, human coronavirus strain 229E (HCoV-229E), porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhoea virus (PEDV), canine coronavirus (CCoV) and feline coronaviruses (FCoV) are grouped in phylogenetic group 1 (González *et al.*, 2003; Spaan *et al.*, 2005; Gorbalenya *et al.*, 2006). Feline coronaviruses are divided into two serotypes: serotype I and the less prevalent serotype II (Pedersen *et al.*, 1984; Hohdatsu *et al.*, 1992). Type II strains are closely related to CCoV and TGEV (Pedersen *et al.*, 1983) and are thought to be recombinants of type I strains and CCoV (Vennema *et al.*, 1995). Unlike type I strains, type II strains grow easily in cell culture, such as Crandell feline kidney (CrFK) cells, and are therefore used frequently in FIPV studies.

To infect a target cell, a virus particle proceeds through a multistep entry process. Each step is pre-programmed and tightly regulated in time and space (Smith & Helenius, 2004). The first step in the viral entry process is the attachment of viral proteins to receptors on the surface of the target cell. Some receptors function solely as attachment receptors that recruit fitting ligands for the internalizing receptors. Others attach and internalize particles in a single step process. Attachment and internalization have not been studied in detail for most coronaviruses (Holmes & Compton, 1995). Aminopeptidase N (APN), also called CD13, is a 150 kDa class II metalloprotease. This glycoprotein serves as a receptor for several group 1 coronaviruses (Tresnan *et al.*, 1996), including HCoV-229E (Yeager *et al.*, 1992), porcine respiratory coronavirus (PRCoV) and TGEV (Delmas *et al.*, 1992, 1993, 1994). For both serotype I and II FCoVs, stably expressed APN cDNA isolated from the *Felis catus* whole fetus (fcwf) cell line could induce susceptibility in FCoV-resistant cells. Although strains of both serotypes were able to infect these cells, the efficiency of infection was different (Tresnan *et al.*, 1996). Hohdatsu *et al.* (1998) showed that only serotype II strains of FCoV use feline APN (fAPN) as a receptor for internalization into feline cell lines, like CrFK and fcwf cells. Infection of bone marrow-derived macrophages with FIPV II (79-1146) could be restricted from approximately 33 % to 3 % of the cells by a monoclonal antibody against fAPN (Rottier *et al.*, 2005). Whether fAPN is the receptor for FIPV II on *in vivo* target cells, feline blood monocytes, is not known. The receptor for FIPV I has not been identified.

Viruses may enter cells via two pathways. Viral envelopes may fuse with the plasma membrane or with endosomal membranes after entering cells via endocytosis. Several group 1 coronaviruses enter cells via endocytosis. HCoV-229E enters human fibroblasts through

caveolae-mediated endocytosis (Nomura *et al.*, 2004). TGEV causes infection in Madin-Darby canine kidney (MDCK) cells, transfected with porcine APN after receptor-mediated endocytosis (Hansen *et al.*, 1998). CCoV also uses endocytosis to enter target cells (Savarino *et al.*, 2003). Mouse hepatitis virus (MHV), a group 2 coronavirus, enters cells by both endosomal and non-endosomal pathways. The pathway of internalization was shown to depend upon the strain of virus and the nature of the cell being infected (Kooi *et al.*, 1991; Nash & Buchmeier, 1997). Internalization of FIPV has not been studied in either primary cells or cell lines. Whether fusion at the plasma membrane or endocytosis occurs, is not known.

In this study, kinetics of attachment and internalization were studied for FIPV in feline blood monocytes, the primary target cells, and compared with those in CrFK cells, used frequently as a model cell line for FCoV-cell interactions. In addition to the routinely used serotype II strain, 79-1146, a serotype I strain, Black, was included. Further, it was investigated whether FIPV enters monocytes and CrFK cells via fusion with the plasma membrane or via endocytosis.

METHODS

Cells and virus

Monocytes were isolated from blood collected from two FCoV-, feline leukemia virus- and feline immunodeficiency virus-negative cats as described previously (Dewerchin *et al.*, 2005). The obtained monocytes were resuspended in RPMI 1640 (Gibco-Invitrogen) medium, supplemented with 10 % foetal bovine serum (FBS), 0.3 mg glutamine ml⁻¹, 100 U penicillin ml⁻¹, 0.1 mg streptomycin ml⁻¹, 0.1 mg kanamycin ml⁻¹, 10 U heparin ml⁻¹, 1 mM sodium pyruvate and 1 % non-essential amino acids 100x (Gibco-Invitrogen). Cells were seeded at a concentration of 2 x 10⁶ cells ml⁻¹ in 24-well dishes with cell culture coating (Nunc A/S) and cultivated at 37 °C with 5 % CO₂. The adherent cells consisted of 86 ± 7 % monocytes [assessed with monocyte marker DH59B; Veterinary Medical Research and Development (VMRD)]. Experiments were performed at 36 hours post seeding.

Type I FIPV strain Black (Black, 1980) and type II strain 79-1146 (McKeirnan *et al.*, 1981) were kindly provided by Dr Egberink (Utrecht University, the Netherlands). FIPV strain Black was passaged on fcwf cells and FIPV strain 79-1146 on CrFK cells.

Virus purification and biotinylation

FIPV I (Black) infected fcwf cells and culture fluids were subjected to three freeze thaw cycles. Batches of culture fluids from FIPV II (79-1146) infected CrFK cells were collected. For both serotypes, the obtained suspensions were clarified by centrifugation at $3000 \times g$ for 15 minutes. Purification and biotinylation were performed as described by Delputte *et al.* (2002) and Delputte & Nauwynck (2004). The supernatants were purified by ultracentrifugation with a Beckman Sw41 rotor (Beckman Coulter) on a 30 % sucrose cushion at $10^5 \times g$ for 165 minutes. Pellets were resuspended in phosphate buffered saline (PBS) and centrifuged at $14000 \times g$ for 30 minutes to remove cellular debris and virus aggregates. The purified virus was labelled with biotin by incubation of the virus suspension with a 10 g l^{-1} solution of Sulfo-NHS-LC-Biotin (Pierce, Perbio Science) in PBS in a 20 to 1 ratio for 90 minutes at $4 \text{ }^\circ\text{C}$. Tris buffer was added to a final concentration of 10 mM and 1 % FBS was used as cryoprotectant for storage at $-70 \text{ }^\circ\text{C}$. Experiments were performed by incubating $200 \text{ }\mu\text{g}$ of purified viral particle suspension with $10^{4.4}$ monocytes or 10^6 CrFK cells. To confirm whether the biotinylated particles were indeed virions, a co-localization assay was carried out. Two stainings were performed: viral proteins were detected using polyclonal anti-FIPV-FITC (VMRD) and biotin was visualized with streptavidin-Texas Red (Molecular Probes). The concurrence of anti-FIPV polyclonal antibodies and conjugated streptavidin was confirmed (Fig. 1). Further, it was shown that the biotinylation procedure did not influence the kinetics of attachment and internalization (data not shown).

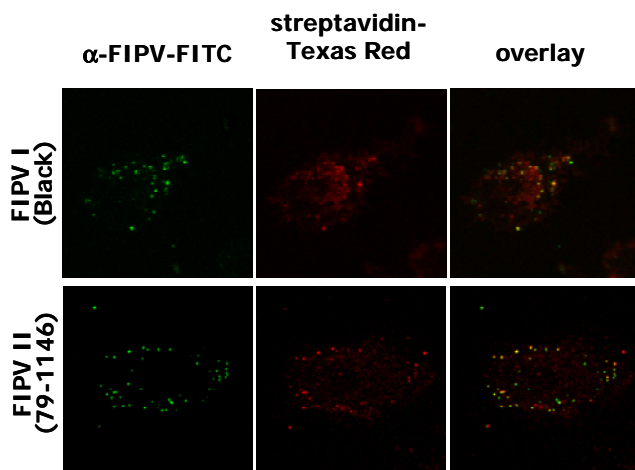


Figure 1: Co-localization of biotinylated particles and virions. Confocal images of monocytes incubated with biotinylated FIPV particles at $4 \text{ }^\circ\text{C}$. FIPV particles are visualized by α -FIPV-FITC, biotin was visualized by streptavidin-Texas Red.

Quantification of biotinylated particles in suspension

For quantification, 2 μl of virus suspension, diluted 10^{-3} , 10^{-4} and 10^{-5} in PBS, was applied to streptavidin-coated slides (Streptavidin Cover Slips, Xenopore) and left to dry for 45 minutes. Biotinylated particles were stained with FITC-labelled streptavidin (Molecular Probes), diluted 1/500 in PBS, and incubated for 1 hour at 37 °C. The slides were washed with PBS and finally mounted on a microscope slide using glycerine-PBS in a 9 to 1 ratio with 2.5 % 1,4-diazabicyclo(2,2,2)octane (Janssen Chimica). The number of particles in the suspension was calculated after manually counting fluorescent particles.

It was shown that 200 μg of purified viral particle suspension contains 7.9×10^9 FIPV I (Black) particles and 5.0×10^{10} FIPV II (79-1146) particles. This amount of virus suspension was applied to $10^{4.4}$ monocytes or 10^6 CrFK cells throughout the experiments.

Kinetics of attachment

Cells were chilled on ice (4 °C) for 15 minutes and inoculated with biotinylated FIPV I (Black) or II (79-1146) at 4 °C. At different time points, cells were washed with PBS and fixed with 1 % formaldehyde. Attached particles were visualized using FITC-labelled streptavidin (Molecular Probes). Cell nuclei were stained with Hoechst 33342 (Molecular Probes). Finally, coverslips were mounted on microscope slides.

Kinetics of internalization

Cells were chilled on ice, inoculated with biotinylated FIPV I (Black) or II (79-1146) and then incubated until the maximum number of attached particles was reached. Cells were washed to remove unbound virus particles. Then, plates were transferred to 37 °C to enable virus uptake. Cells were fixed at different time points with 1 % formaldehyde. Particles attached to the outside of the cell were visualized using Texas Red-labelled streptavidin (Molecular Probes). After permeabilizing cells with 0.1 % Triton X-100, internalized particles were stained with FITC-labelled streptavidin. Finally, the cell nuclei were stained and coverslips were mounted on microscope slides.

Using ethidium monoazide bromide (EMA), cell vitality was determined for cells subjected to the manipulations described above before fixation. For the monocytes, the vitality was 99.3 % and for the CrFK cells, 97.8 %.

Occurrence of internalization via fusion with the plasma membrane

Stainings were performed on monocytes inoculated with FIPV I (Black) or FIPV II (79-1146) and CrFK cells inoculated with FIPV II (79-1146) to determine whether the biotinylated

material at the plasma membrane after virus uptake via endocytosis, consisted of intact virions or viral envelopes, left behind after fusion. Cells were chilled on ice, inoculated with FIPV I (Black) and FIPV II (79-1146) and incubated at 4 °C. When attachment was completed, cells were transferred to 37 °C as described for the determination of the kinetics of internalization. Cells were fixed when maximal uptake was reached and remaining material at the plasma membrane was stained with polyclonal, FITC-labelled, anti-FIPV antibodies (VMRD). After permeabilization of the cells with 0.1 % Triton X-100, co-localization with mouse anti nucleocapsid antibodies was studied after staining with the monoclonal antibody E22-2 (kindly provided by Dr Hohdatsu, Kitasato University, Japan) and visualization with goat anti-mouse-Texas Red (Molecular Probes).

Microscopy and statistics

Analyses of virus attachment and internalization were performed by means of a DM IRB inverted microscope (Leica Microsystems). Images were obtained using a Leica TCS SP2 laser scanning spectral confocal system linked to a DM IRB inverted microscope (Leica). Argon and He/Ne lasers were used for exciting FITC and Texas Red fluorochromes, respectively. Leica confocal software was used for image acquisition.

Triplicate assays were performed and compared using the Wilcoxon Signed Ranks test from the SPSS software package (version 12.0, SPSS).

RESULTS

Kinetics of attachment

Monocytes - Kinetics of attachment on monocytes from two cats were determined with both serotypes. The courses of the obtained kinetics are shown in Figure 2b. The number of particles attached at a certain time point is the average of the number of attached particles for 50 randomly chosen cells. Both cells with and without bound particles on their plasma membrane were included. For each cell, the entire plasma membrane was screened for the presence of particles.

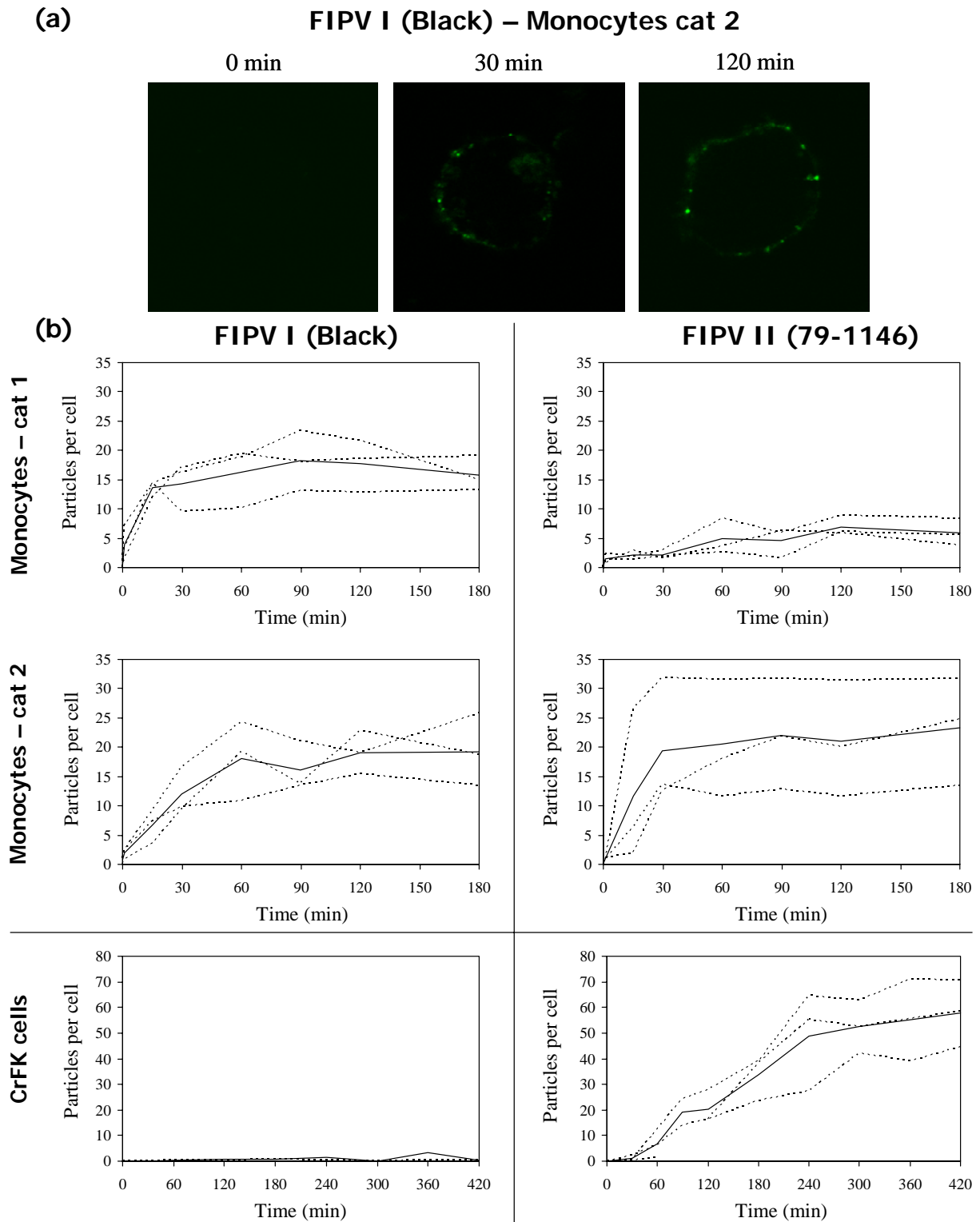


Figure 2: Kinetics of attachment of FIPV I (Black) and FIPV II (79-1146) to monocytes of cat 1 and cat 2 and CrFK cells. Cells were inoculated with biotinylated FIPV and incubated at 4 °C. At designated timepoints virus was visualized using streptavidin-FITC. (a) Confocal images of single sections through cells. Green dots represent bound virus particles. (b) ---- courses determined in 3 independently performed experiments; — mean course based on the 3 independently performed experiments.

FIPV I (Black) – After monocytes were incubated with FIPV I (Black) particles at 4 °C, bound virus was observed on the membrane of almost all monocytes (98.8 % of the monocytes for cat 1 and 99.5 % for cat 2). During the first 15 minutes of incubation, the number of attached FIPV I (Black) particles to monocytes of cat 1, gradually increased to 14 ± 1 bound particles per cell. Longer incubation times did not increase the amount of virus that bound noticeably. For cat 2, similar kinetics of attachment were obtained. A slightly slower increase persisted for 1 hour to 18 ± 7 bound particles per cell. From 1 hour on, no further increase was observed and attachment maintained at the same level.

FIPV II (79-1146) – Bound FIPV II (79-1146) particles were observed on the membrane of 66.2 % of the monocytes isolated from cat 1. During the first hour of incubation, the number of attached particles slowly increased to 5 ± 3 particles per cell (all cells considered). Despite longer incubation times (up to 3 hours), no more particles bound to the cell surface. In contrast, almost all monocytes of cat 2 (95.0 %) attached FIPV II (79-1146) particles at their plasma membrane. Within the first 30 minutes of incubation on ice, the number of attached particles strongly increased to 19 ± 10 bound particles per cell. From 30 minutes on, attachment maintained at the same level. Clearly, cat 2 monocytes reached a higher level of attached FIPV II (79-1146) particles than cat 1 monocytes.

CrFK cells - Kinetics of attachment of FIPV particles on the surface of CrFK cells are shown in Figure 2b for FIPV I (Black) and FIPV II (79-1146). The number of particles attached at different time points was determined as described for the monocytes.

FIPV I (Black) - Attachment of FIPV I (Black) particles was restricted to 15.4 % of the CrFK cells. After two hours of incubation on ice only 1 ± 1 particle bound per cell (all cells considered). Despite longer incubation times no further increase of this number was observed.

FIPV II (79-1146) - In contrast to the results for FIPV I (Black), almost all cells (99.8 %) had bound FIPV II (79-1146) particles on their plasma membrane. Also, more particles were attached, although the kinetics were slow. During 4 hours of incubation, the number of bound particles increased gradually to 49 ± 19 particles per cell. Longer incubation times increased the amount of bound virus slightly.

In Table 1, a comparison was made between the number of particles added to the cells and the resulting level of attachment for the different virus strains and cell types. Virus particles were present in excess in all experiments, therefore it might be suggested that all possible FIPV binding sites were taken. This could be confirmed by evaluating attachment after incubation with viral particle suspensions of varying concentrations.

Kinetics of internalization

Kinetics of internalization of FIPV were determined by incubating virus particles with the cells on ice until maximum attachment was reached, i.e. 1 hour for incubation of monocytes with both strains, and 2 hours and 5 hours for incubation of CrFK cells with respectively FIPV I (Black) and FIPV II (79-1146) (Fig. 2). Then, plates were transferred to 37 °C (= time point zero for the kinetics of internalization).

The kinetics of internalization of FIPV are shown in Figure 3. The percentages shown were calculated by analyzing 10 cells for the presence of particles in the cytosol and on the surface of the cell. Only cells that had attached and/or internalized particles were taken into account. For each cell, the entire cell volume was screened for particles.

Monocytes - The kinetics of internalization determined in monocytes are shown in Figure 3b.

FIPV I (Black) – The kinetics of internalization for FIPV I (Black) were similar for both cats. Almost all monocytes that bound FIPV I (Black) virions internalized virions (98.9 % of the monocytes that had bound particles for cat 1 and 98.5 % for cat 2). Remarkably, immobilization of the cells at 4 °C could not completely inhibit internalization as there were already particles internalized at time point zero. Within the first minute after the start of virus uptake, already 89 ± 6 % of the bound particles were internalized per cell. This level of internalization was roughly maintained during longer incubation times, though a slight decrease to 85 ± 13 % could be observed after one hour of incubation.

FIPV II (79-1146) – As for FIPV I (Black), internalization of FIPV II (79-1146) was mediated by approximately all monocytes that had bound virus particles (92.5 % of the analyzed monocytes isolated from cat 1 and all analyzed monocytes from cat 2) and kinetics were similar for both cats. After one minute, an average of 60 ± 9 % of the bound virions were internalized per cell. However, internalization further increased gradually up to 1 hour of incubation at 37 °C. Then, an average of 90 ± 7 % of the bound particles were taken up per cell.

CrFK cells - Kinetics of internalization of FIPV particles in CrFK cells are shown in Figure 3b.

FIPV I (Black) – Unlike for monocytes, uptake was restricted in CrFK cells to 20.8 % of the monocytes that had bound virus or only 3.2 % of all cells. No internalization was observed after the first minute of incubation at 37 °C, but during the first 2 hours the kinetics gradually mounted to the maximal level of internalization where 52 % of the bound particles per cell were internalized. During longer incubation times, internalization maintained at the same level. The percentage of internalized particles per cell varied between 0 and 100 % as often only 1 particle was bound to the cell, so only 1 particle could be internalized.

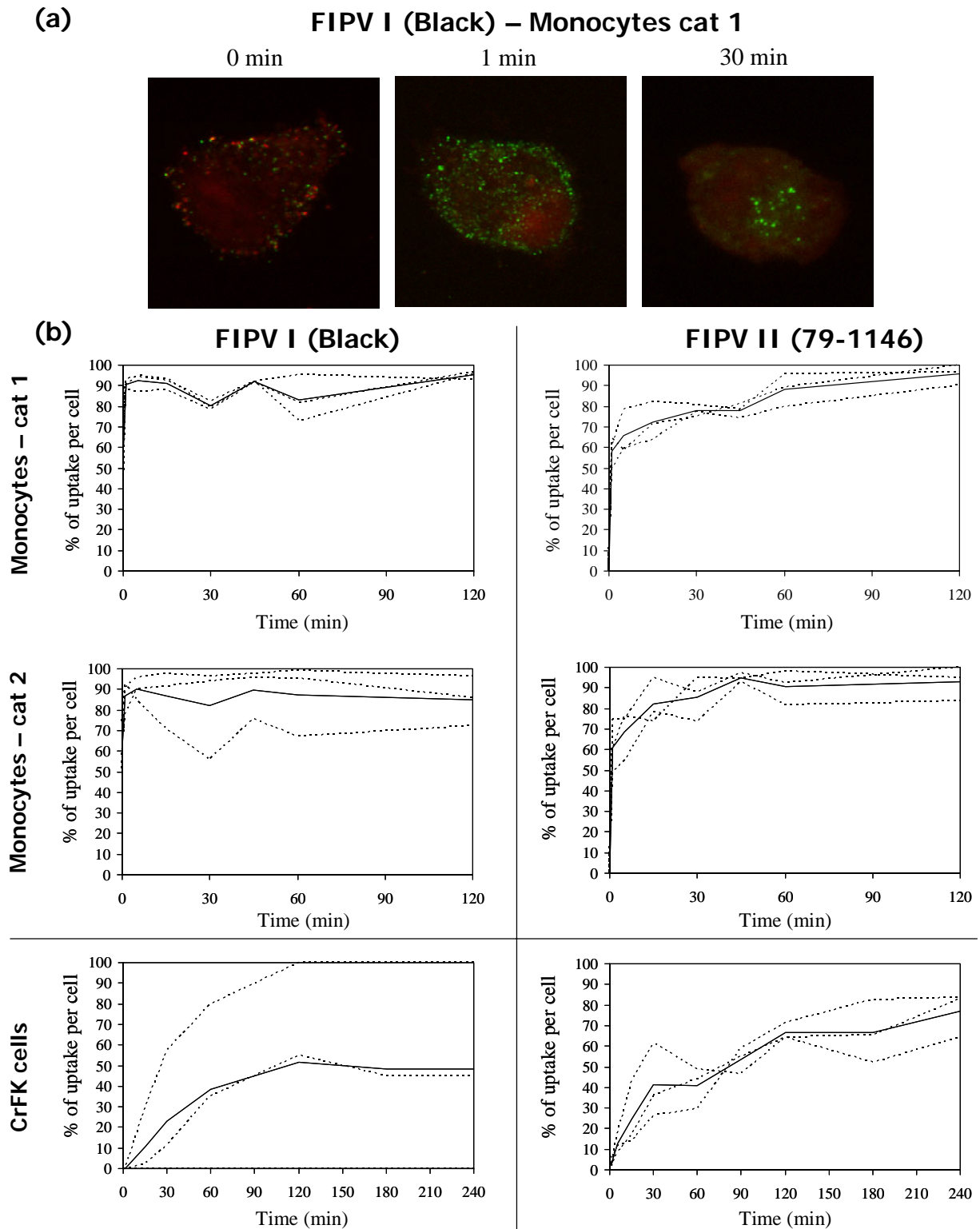


Figure 3: Kinetics of internalization of FIPV I (Black) and FIPV II (79-1146) in monocytes of cat 1 and cat 2 and CrFK cells. Cells were inoculated with biotinylated FIPV and incubated at 4 °C until saturation of attachment. Plates were then transferred to 37 °C. At designated timepoints virus at the plasma membrane was visualized using streptavidin-TR and - after permeabilization - internalized virus was visualized using streptavidin-FITC. (a) Confocal images of single sections through cells. Red dots represent virus particles at the cell surface, exclusively green dots internalized virus particles. (b) ---- courses determined in 3 independently performed experiments; — mean course based on the 3 independently performed experiments.

FIPV II (79-1146) – All cells were capable of internalizing FIPV II (79-1146) virions. Within the first minute, no more than 3 ± 2 % of the bound particles were observed inside the cell. But, again, the percentage of internalization per cell increased gradually, and reached 67 ± 4 % after 2 hours of incubation at 37 °C. Longer incubation times only slightly increased the percentage of internalization per cell.

Table 1. Comparison of the number of FIPV particles added to the cells and the number of particles attached after incubation at 4°C until saturation

Virus strain	Particles added	Cell type	Bound particles
FIPV I (Black)	7.9×10^9	CrFK	2.00×10^6
		Monocytes cat 1	7.31×10^6
		Monocytes cat 2	7.74×10^6
FIPV II (79-1146)	5.0×10^{10}	CrFK	1.44×10^8
		Monocytes cat 1	2.58×10^6
		Monocytes cat 2	9.46×10^6

Occurrence of internalization via fusion with the plasma membrane

The kinetics of internalization indicate that the majority of bound FIPV particles enters cells via endocytosis. But virus uptake via endocytosis did not reach 100 % in monocytes, nor in CrFK cells. A small fraction of biotinylated material remained at the outside of the cell. This could be bound virus particles, unable to enter the cell, or biotinylated envelopes that were left behind after fusion with the host cell membrane. A staining was performed to determine the nature of what was left at the plasma membrane after internalization was completed. Cells were therefore fixed after 1 hour and 2 hours of incubation at 37 °C for respectively monocytes and CrFK cells. The results are shown in Figure 4. It was obvious that all the biotinylated FIPV material at the plasma membrane co-localized with FIPV nucleocapsid proteins. Thus, the biotinylated material consisted of intact virions, unable to enter the cell. This implies that, under the present experimental conditions, both FIPV strains enter monocytes exclusively via endocytosis and so does FIPV II (79-1146) in CrFK cells. It can be stated that fusion at the plasma membrane is not involved in FIPV entry.

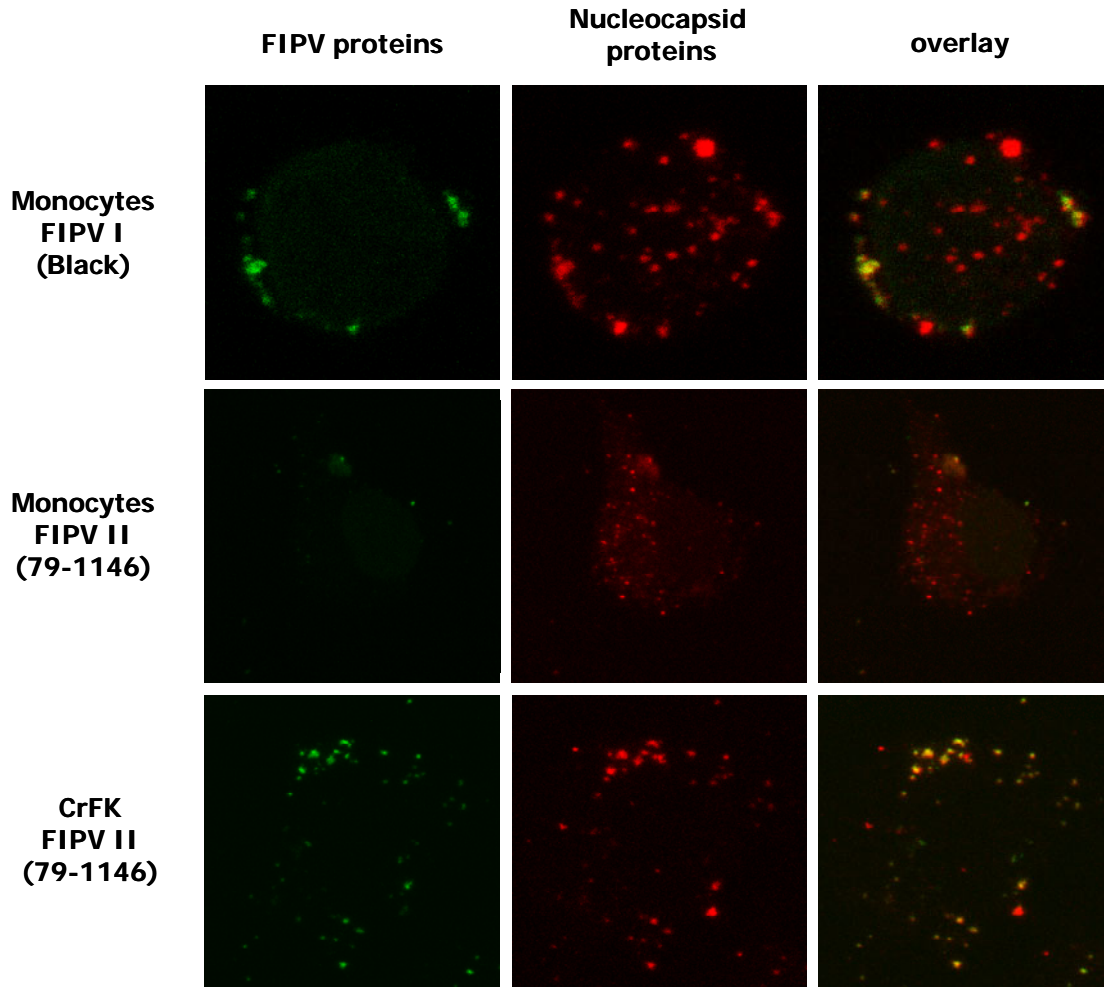


Figure 4: Co-localization of non-internalized biotinylated material and FIPV nucleocapsid protein. Confocal images of sections through monocytes and CrFK cells incubated at 4 °C, transferred to and left at 37 °C until uptake was completed. Biotinylated material was stained with α -FIPV-FITC, nucleocapsid protein with mAb E22-2 and goat α -mouse-TR.

DISCUSSION

Feline infectious peritonitis (FIP) is a fatal viral infection. No effective treatment is commercially available as several important questions concerning the pathogenesis of FIP remain unanswered. Especially virus-cell interactions need to be studied in greater detail in order to obtain insights on possible targets for drug development. Interfering with the internalization process of viruses is a new strategy for development of antiviral compounds as has been described for e.g. human immunodeficiency virus, hepatitis C virus and dengue virus (Altmeyer, 2004; Este, 2003; Le Calvez *et al.*, 2004). In this study, the initial steps in

FIPV infection were analyzed for the first time in the FIPV target cell by determining kinetics of attachment and internalization, using biotinylated virus.

Feline blood monocytes are the target cells *in vivo* for feline infectious peritonitis virus, therefore these cells were used in the experiments to approximate the natural situation as much as possible. Two virus strains were included: the FIPV serotype I strain Black and the routinely used serotype II strain 79-1146.

The kinetics of attachment for FIPV I (Black) were quite similar for both cats. However, the kinetics of attachment of FIPV II (79-1146) determined for the two cats were significantly different (Wilcoxon Signed Ranks (WSR): $p=0.025$). This difference could be the consequence of variable expression levels of one of the cellular components involved in attachment, e.g. a specific serotype II FIPV receptor, between the two cats. Another difference in kinetics of attachment was observed between FIPV I (Black) and FIPV II (79-1146) for the monocytes of cat 1 (WSR: $p=0.012$) and cat 2 (WSR: $p=0.036$). This could indicate that type I and II strains use different receptors on monocytes and that the serotype II receptor may be differentially expressed in between cats, while apparently the serotype I receptor is not. Differences in receptor use between type I and II have already been demonstrated in cell culture (Hohdatsu *et al.*, 1998). As only two cats were considered, these data cannot lead to general conclusions. Further research will focus on the receptors of FIPV in monocytes, which will allow interpretation of the present findings.

Internalization in monocytes was very efficient for both serotypes. The efficiency of TGEV internalization in the MDCK cell line is evenly efficient as that of FIPV in monocytes. TGEV is another group 1 coronavirus. TGEV virions can be observed in endocytic pits and apical vesicles, by electron microscopy, after 3 to 10 minutes of incubation at 38 °C with MDCK cells, stably expressing the receptor, porcine aminopeptidase N (Hansen *et al.*, 1998). For HCoV-229E, most particles are internalized in human fibroblasts within the first hour of incubation at 37 °C, but a fraction is still bound to the cell membrane upon 3 hours of incubation (Nomura *et al.*, 2004). Similar results as those for FIPV are obtained for the attachment and internalization of porcine reproductive and respiratory syndrome virus (PRRSV), another member of the *Nidovirales*, in macrophages (Nauwynck *et al.*, 1999). Attachment of biotinylated PRRSV reaches saturation after 1 hour. After 1 minute, confocal images showed uptake of virus particles and after 1 to 2 hours the uptake was completed. The kinetics determined for FIPV led to the same conclusion (Nauwynck *et al.*, 1999).

PRRSV particles start to accumulate in endosomes approximately 1 or 2 hours after the start of the virus uptake. For FIPV, more intense and larger fluorescent spots were already

observed in confocal images at 15 minutes after the start of virus uptake. TGEV virions start to accumulate in endosomes, already 10 minutes after initiation of endocytosis (Hansen *et al.*, 1998).

Another mutual observation between PRRSV and FIPV is the decreasing number of particles inside the cell at later time points in the kinetics: for FIPV within 1 hour (data not shown), for PRRSV after 3 to 5 hours (Nauwynck *et al.*, 1999). The decreasing number of particles explains the apparent drop in internalization described for FIPV I (Black) where the percentages of uptake per cell were higher after 1 minute than after 1 hour. The decrease in particle number is probably the result of particle disassembly. This has also been described for TGEV where genome penetration is situated around 1 hour after the onset of internalization (Hansen *et al.*, 1998).

Replication of FIPV is limited to a small fraction (less than 1 % for FIPV 79-1146) of monocytes (Dewerchin & Cornelissen, 2005) and peritoneal macrophages (Morahan *et al.*, 1985; Stoddart & Scott, 1989). However, virus binding and uptake took place in almost all monocytes. Thus, the mechanism behind the resistance of most monocytes/macrophages must lie in an inhibition of genome release and/or genome translation. Further research will clarify which step in virus replication is blocked in cells resistant to FIPV infection. Replication of PRRSV in alveolar macrophages is also restricted in a step after binding and internalization. Although bound particles and internalization were observed in almost all alveolar macrophages, viral PRRSV antigens could be detected in only 12.3 % of the cells (Duan *et al.*, 1997; Nauwynck *et al.*, 1999).

Attachment and internalization of FIPV I (Black) virions to the plasma membrane of CrFK cells were very inefficient. It is possible that no specific mechanism is involved. Particles may accidentally co-internalize with other compounds. The ability of FIPV I (Black) to induce infection of CrFK cells was studied by inoculating cells with the same concentration of virus as used for determining the kinetics, and a subsequent incubation for 12 hours at 37 °C. Staining with polyclonal anti-FIPV antibodies revealed that only 0.003 % of the cells were infected (data not shown). Infectivity of FIPV I (Black) in CrFK cells is the subject of contradictory literature. According to Black (1980), FIPV strain Black is able to infect and cause cytopathogenic effects (CPE) in CrFK cells 3 to 5 days post inoculation. Possibly plaques are being formed at later time points post inoculation due to cell-to-cell spread of the virus by the few primary infected cells that were observed 12 hours post inoculation. On the other hand, Hohdatsu *et al.* (1998) reported that CrFK cells were not susceptible to

infection with FIPV I (Black) as no CPE, nor virus specific antigens could be detected 3 days post inoculation. It is possible that the susceptibility of CrFK cells of different laboratories may differ due to genetic changes during long term subpassaging of the cell lines.

CrFK cells are often used as a model cell line for FIPV serotype II infection studies. However, internalization of FIPV II (79-1146) was slower and less efficient than in monocytes. Thus, FIPV seems to interact differently with CrFK cells than with monocytes. This confirms earlier observations where inoculation of CrFK cells with FIPV II (79-1146) resulted in a high percentage of infected cells, but low production of newly synthesized virions. In contrast, inoculation of monocytes led to a low percentage of infected cells, but to large amounts of newly produced virions (Dewerchin *et al.*, 2005).

Viruses may enter cells via two pathways. The envelope of the virion may directly fuse with the plasma membrane or the envelope may fuse with the endosomal membrane after entering the cell via endocytosis. The pathway used depends upon the virus strain and the nature of the cell being infected (Kooi *et al.*, 1991; Nash & Buchmeier, 1997). Certain viruses even use both pathways in a particular cell type (Schaeffer *et al.*, 2004). The results of this study indicate that bound FIPV particles enter target cells exclusively via endocytosis. Several other group 1 coronaviruses have also been shown to enter cells via endocytosis, e.g. HCoV-229E, TGEV and CCoV. Further research will focus on determining the characteristics of the endocytic pathway that is used by the FIPV serotype I and II virions in monocytes.

In this study, kinetics of attachment and internalization of FIPV in its primary target cell, the blood monocyte, were studied in detail for the first time. Attachment and especially internalization proceeded very efficiently for the strains of both serotypes. Bound particles entered target cells via endocytosis and not via fusion. Although infection is restricted to a small fraction of monocytes, virus uptake took place in the majority of cells, indicating that blocking of infection occurs at the level of genome release or translation/transcription. In contrast, attachment and internalization in CrFK cells were shown to proceed inefficiently, especially for FIPV I (Black) virions. It is concluded that this cell line is not suitable for studying FIPV entry into target cells.

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REFERENCES

- Altmeyer, R. (2004).** Virus attachment and entry offer numerous targets for antiviral therapy. *Curr Pharm Des* **10(30)**, 3701-3712.
- Black, J. W. (1980).** Recovery and in vitro cultivation of a coronavirus from laboratory-induced cases of feline infectious peritonitis (FIP). *Vet Med Small Anim Clin* **75**, 811-814.
- Cavanagh, D. (1997).** *Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*. *Arch Virol* **142**, 629-633.
- Cowley, J. A., Dimmock, C. M., Spann, K. M. & Walker, P.J. (2000).** Gill-associated virus of *Penaeus monodon* prawns: an invertebrate virus with ORF1a and ORF1b genes related to arteri- and coronaviruses. *J Gen Virol* **81**, 1473-1484.
- Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L. K., Sjöström, H., Norén, O. & Laude, H. (1992).** Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature* **357**, 417-419.
- Delmas, B., Gelfi, J., Kut, E., Sjöström, H., Norén, O. & Laude, H. (1994).** Determinants essential for the transmissible gastroenteritis virus-receptor interaction reside within a domain of aminopeptidase N that is distinct from the enzymatic site. *J Virol* **68**, 5216-5224.
- Delmas, B., Gelfi, J., Sjöström, H., Norén, O. & Laude, H. (1993).** Further characterization of aminopeptidase N as a receptor for coronaviruses. *Adv Exp Med Biol* **342**, 293-298.
- Delputte, P. L., Vanderheijden, N., Nauwynck, H. J. & Pensaert, M. B. (2002).** Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *J Virol* **76**, 4312-4320.
- Delputte, P. L. & Nauwynck, H. J. (2004).** Porcine arterivirus infection of alveolar macrophages is mediated by sialic acid on the virus. *J Virol* **78**, 8094-8101.
- Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2005).** Replication of feline coronaviruses in peripheral blood monocytes. *Arch Virol* **150**, 2483-2500.

Duan, X., Nauwynck, H. J. & Pensaert, M. B. (1997). Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). *Arch Virol* **142**, 2483-2497.

Este, J. A. (2003). Virus entry as a target for anti-HIV intervention. *Curr Med Chem* **10**, 1617-1632.

González, J. M., Gomez-Puertas, P., Cavanagh, D., Gorbalenya, A. E. & Enjuanes, L. (2003). A comparative sequence analysis to revise the current taxonomy of the family *Coronaviridae*. *Arch Virol* **148**, 2207-2235.

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J. & Snijder, E. J. (2006). *Nidovirales*: Evolving the largest RNA virus genome. *Virus Res* **117**, 17-37.

Hansen, G. H., Delmas, B., Besnardeau, L., Vogel, L. K., Sjöström, H., Laude, H. & Norén, O. (1998). The coronavirus transmissible gastroenteritis virus causes infection after receptor-mediated endocytosis and acid-dependent fusion with an intracellular compartment. *J Virol* **72**, 527-534.

Hohdatsu, T., Okada, S., Ishizuka, Y., Yamada, H. & Koyoma, H. (1992). The prevalence of types I and II feline coronavirus infections in cats. *J Vet Med Sci* **54**, 557-562.

Hohdatsu, T., Izumiya, Y. & Yokoyama, Y. (1998). Differences in virus receptor for type I and type II feline infectious peritonitis virus. *Arch Virol* **143**(5), 839-850.

Holmes, K. V. & Compton, S. R. (1995). Coronavirus receptors. In *The Coronaviridae*, pp. 55-72. Edited by S. G. Siddell. New York: Plenum Press.

Kooi, C., Cervin, M. & Anderson, R. (1991). Differentiation of acid-pH-dependent and -nondependent entry pathways for mouse hepatitis virus. *Virology* **180**, 108-119.

Le Calvez, H., Yu, M. & Fang, F. (2004). Biochemical prevention and treatment of viral infections – A new paradigm in medicine for infectious diseases. *Virology* **1**, 12-18.

McKeirnan, A. J., Evermann, J. F., Hargis, A., Miller, L. M. & Ott, R. L. (1981). Isolation of feline coronaviruses from two cats with different disease manifestations. *Feline Pract* **11**, 16-20.

Morahan, P. S., Connor, J. R. & Leary, K. R. (1985). Viruses and the versatile macrophage. *Br Med Bull* **41**, 15-21.

Nash, T. C. & Buchmeier, M. J. (1997). Entry of mouse hepatitis virus into cells by endosomal and nonendosomal pathways. *Virology* **233**, 1-8.

Nauwynck, H. J., Duan, X., Favoreel, H., Van Oostveldt, P. & Pensaert, M. (1999). Entry of porcine reproductive and respiratory syndrome virus into alveolar macrophages via receptor-mediated endocytosis. *J Gen Virol* **80**, 297-305.

Nomura, R., Kiyota, A., Suzaki, E., Kataoka, K., Ohe, Y., Miyamoto, K., Senda, T. & Fujimoto, T. (2004). Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. *J Virol* **78**, 8701-8708.

Pedersen, N. C., Black, J. W., Boyle, J. F., Evermann, J. F., McKeirnan, A. J. & Ott, R. L. (1983). Pathogenic differences between various feline coronavirus isolates. In *Molecular biology and pathogenesis of coronaviruses*, pp. 365-380. London: Plenum press.

Pedersen, N. C., Evermann, J. F., Alison, J., McKeirnan, A. J. & Ott, R. L. (1984). Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. *Am J Vet Res* **45**, 2580-2585.

Rottier, P. J. M., Nakamura, K., Schellen, P., Volders, H. & Haijema, B. J. (2005). Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. *J Virol* **79**, 14122-14130.

Savarino, A., Boelaert, J. R., Cassone, A., Majori, G. & Cauda, R. (2003). Effects of chloroquine on viral infections: an old drug against today's diseases? *Lancet Infect Dis* **3**, 722-727.

Schaeffer, E., Soros, V. B. & Greene, W. C. (2004). Compensatory link between fusion and endocytosis of human immunodeficiency virus type I in human CD4 T lymphocytes. *J Virol* **78**, 1375-1383.

Smith, A. E. & Helenius, A. (2004). How viruses enter animal cells. *Science* **304**, 237-242.

Spaan, W. J. M., Brian, D., Cavanagh, D., de Groot, R. J., Enjuanes, L., Gorbalenya, A. E., Holmes, K. V., Masters, P. S., Rottier, P. & other authors (2005). Family *Coronaviridae*. In *Virus taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 947-964. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. Amsterdam: Elsevier.

Stoddart, M. E. & Scott, F. W. (1989). Intrinsic resistance of feline infectious peritoneal macrophages to coronavirus infection correlates with in vivo virulence. *J Virol* **63**, 436-440.

Tresnan, D. B., Levis, R. & Holmes, K. V. (1996). Feline aminopeptidase N serves as a receptor for feline, canine, porcine and human coronaviruses in serogroup I. *J Virol* **70**, 8669-8674.

Vennema, H., Poland, A., Floyd, H. K. & Pedersen, N. C. (1995). A comparison of the genomes of FECVs and FIPVs: what they tell us about the relationships between feline coronaviruses and their evolution. *Feline Pract* **64**, 40-44.

Yeager, C. L., Ashmun, R. A., Williams, R. K., Cardellichio, C. B., Shapiro, L. H., Look, A. T. & Holmes, K. V. (1992). Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* **357**, 420-422.

**INTRIGUING INTERPLAY BETWEEN AMINOPEPTIDASE N AND
DC-SIGN IN FELINE INFECTIOUS PERITONITIS VIRUS TYPE II
ENTRY IN PRIMARY FELINE MONOCYTES**

Evelien Van Hamme, Hannah L. Dewerchin, Els Cornelissen & Hans J. Nauwynck

In preparation

ABSTRACT

Two potential receptors have been described for feline coronaviruses (FCoV). Feline APN (fAPN) can serve as a receptor for serotype II, but not serotype I, FCoVs in cell lines. Secondly, FCoVs can use dendritic cell-specific intercellular adhesion molecule grabbing non-integrin (DC-SIGN) for entry into host cells. In this study, the interplay between APN and DC-SIGN as receptors for FIPV was analysed in Crandell feline kidney (CrFK) cells and in monocytes. First, the effect of blocking the potential receptor(s) on attachment, internalization and infection was evaluated and secondly, the level of co-localization of FIPV and the receptors was determined. It was found that blocking fAPN completely inhibited binding to and infection of CrFKs and that FIPV co-localized with fAPN. In contrast, on monocytes, binding and infection were only reduced by 53 % and 44 % respectively and only 60 % of bound FIPV co-localized with fAPN. Transduction of human DC-SIGN was found to render CHO cells susceptible to binding and internalization of FIPV but not to infection. Blocking feline DC-SIGN (fDC-SIGN) did not influence binding and infection of monocytes and there was no co-localization. However, blocking fDC-SIGN caused a further inhibition of infection from 56 % up to 18 % infection in APN-blocked monocytes. APN is capable of binding and internalizing FIPV and APN-mediated entry leads to infection in monocytes. However, this is not the exclusive receptor for FIPV on monocytes, unlike in CrFK cells. fDC-SIGN is not directly required for attachment or internalization in monocytes, but important for infection via an alternative pathway independent of APN. Finally, this study indicates that caution should be taken when using certain cell lines for receptor and entry studies.

INTRODUCTION

Feline coronaviruses belong to coronavirus subgroup 1 and occur in two pathotypes: the often unapparent enteric feline coronavirus (eFCoV) and the deadly feline infectious peritonitis virus (FIPV). Each pathotype comprises two serotypes. Serotype I causes most natural infections, whereas serotype II is mostly used for research purposes because it grows better in culture (Pedersen *et al.*, 1984).

The process of viral entry is an attractive target for the development of new therapeutic agents. To this end, a lot of research has been focussing on entry processes for a range of viruses (Cooley & Lewin, 2003; Timpe & McKeating, 2009). This has led to the development of a number of promising agents, for example in the treatment of HIV-1 (Cooley & Lewin, 2003). Over the last couple of years, the knowledge on the entry of feline coronaviruses in host cells has expanded. After binding to the receptor(s), the virus is internalized in monocytes through endocytosis, more specifically via a clathrin- and caveolae-independent pathway using dynamin (Van Hamme *et al.*, 2007, 2008). According to Regan *et al.* (2008), subsequent escape from endosomes is mediated by cathepsin B for FIPV strains 79-1146 and DF2, while for the enteric strain 79-1683 low pH in endocytic compartments and cathepsin L need to assist cathepsin B. In contrast, de Haan *et al.* (2008) observed that infection with FIPV 79-1146 appears to be insensitive to cathepsin inhibitors.

Two potential receptors have been described for the feline coronaviruses. The first one is aminopeptidase N (APN), also designated CD13, a ubiquitous and multifunctional glycoprotein of approximately 110 kDa and 967 amino acids (Luan & Xu, 2007). It is a type II metalloprotease that contains seven domains (Sjöström *et al.*, 2000). Domains V to VII can interact to form non-covalently linked homodimers (Sjöström *et al.*, 2000). Feline APN (fAPN) serves as a receptor for feline, canine, porcine and human coronaviruses in coronavirus subgroup 1 (Tresnan *et al.*, 1996). However, only serotype II and not serotype I FCoV strains are able to recognize fAPN (Hohdatsu *et al.*, 1998; Dye *et al.*, 2007). Binding of serotype II strains to fAPN from cell lines can be blocked completely by the monoclonal antibody R-G-4 that binds to a region on fAPN between aa 251 to 582, i.e. in domains V or VI (Hohdatsu *et al.*, 1998; Tusell *et al.*, 2007). The attachment of FIPV to fAPN was mainly studied on cell lines and by means of a fAPN cDNA clone originating from the fcwf-4 cell line. Secondly, it has been described that feline coronaviruses use dendritic cell (DC)-specific intercellular adhesion molecule (ICAM) grabbing non-integrin (DC-SIGN, CD209) for entry into Crandell feline kidney (CrFK) cells, mouse 3T3 cells transfected with DC-SIGN and monocytes (Regan & Whittaker, 2008). DC-SIGN is a C-type lectin, which implies that it

recognizes its ligands, high-mannose oligosaccharides, through Ca^{2+} -dependent carbohydrate-recognition domains (Drickamer, 1999). Many viruses, such as HIV, Ebola and hepatitis C, use DC-SIGN or the homologue L-SIGN, expressed in liver and lymph nodes, to augment infection (Geijtenbeek *et al.*, 2000; Alvarez *et al.*, 2002; Lozach *et al.*, 2003; Pöhlmann *et al.*, 2003). Some coronaviruses also interact with these lectins. For SARS-CoV, DC-SIGN and L-SIGN can enhance infection of cells that co-express the major SARS receptor, ACE2 (Marzi *et al.*, 2004; Jeffers *et al.*, 2004; Yang *et al.*, 2004). DC-SIGN, but not L-SIGN, can similarly augment infection with human coronavirus (HCoV) NL63, that also relies on ACE2 for infectious entry (Hofmann *et al.*, 2006). Further, L-SIGN expressed in non-susceptible cells can bind HCoV-229E (Jeffers *et al.*, 2006). For the feline coronaviruses, expression of human DC-SIGN in CrFK cells induced an increase of infection that was blocked by mannan, a competitor of DC-SIGN binding. Further, infection of feline monocytes, the *in vivo* target cell, was strongly reduced by mannan (Regan & Whittaker, 2008).

In this study, the role of the candidate receptors was determined in the distinct steps of the multi-step entry process in primary target cells, blood monocytes. The effects of blocking fAPN and fDC-SIGN in monocytes were studied for the processes of attachment, internalization and infection by FIPV 79-1146. In addition, co-localization studies were performed between FIPV and these potential receptors. The aim of this study was to understand how FIPV interacts with its receptors on the primary FIPV target cells *in vivo*, the blood monocytes.

METHODS

Cells and virus

Feline blood monocytes were isolated from blood collected from a feline coronavirus-, feline leukaemia virus- and feline immunodeficiency virus-negative cat and cultured as described before (Dewerchin *et al.*, 2005). CrFK cells were purchased from the ATCC. CHO control cells and CHO transfectants stably expressing wild-type human DC-SIGN (de Witte *et al.*, 2006) were a gift of Dr Geijtenbeek (Department of Molecular Cell Biology and Immunology, VU University Medical Centre Amsterdam, Amsterdam, The Netherlands). FIPV serotype II strain WSU 79-1146 (FIPV II) was a kind gift of Dr Egberink (Department of Infectious Diseases and Immunology, Utrecht, The Netherlands) and passaged on CrFK cells (McKeirnan *et al.*, 1981).

Antibodies

The monoclonal antibody R-G-4 (mAb R-G-4) directed against feline aminopeptidase N (fAPN) (Hohdatsu *et al.*, 1998) was a kind gift of Dr Hohdatsu (Department of Veterinary Infectious Diseases, Towada, Japan). The monoclonal antibody 25-2B against fAPN, also designated CD13, was purchased from Veterinary Medical Research and Development (VMRD, Pullman, USA). A polyclonal mouse antibody raised against full-length human DC-SIGN, also designated CD209, was purchased from Abnova (Taipei, Taiwan). mAb DH59B (VMRD) was used to stain CD172a in the plasma membrane of monocytes. Immunoglobulin G1 13D12 against pseudorabies virus gD was produced in the laboratory (Nauwynck & Pensaert, 1995) and was used as an irrelevant isotype-matched control antibody. Polyclonal anti-FIPV antibodies against FIPV serotype II strain WSU 79-1146 were a kind gift of Dr Rottier (Department of Infectious Diseases and Immunology, Utrecht, The Netherlands).

Inhibition assays

The experimental design to study attachment and internalization separately and the time points used to evaluate the potency to inhibit these processes, were determined based on data obtained earlier on the kinetics of attachment and internalization of FIPV (Van Hamme *et al.*, 2007).

Attachment inhibition assay. Cells were washed at 68 h post-seeding and pre-incubated at 4 °C for 60 minutes with mAb R-G-4 (25 mg/ml) and/or mannan (50 mg/ml), or an irrelevant isotype-matched mAb. After pre-treatment, FIPV was added to the cells at a multiplicity of infection (moi) of 1 (resulting concentrations: 8 mg/ml antibody and/or 16 mg/ml mannan). Cells and virus were incubated further at 4 °C for 1.5 h (monocytes) or 3 h (CrFK and CHO cells). Then, cells were washed with ice-cold RPMI 1640 and fixed with formaldehyde (1 %). Bound particles were stained with anti-FIPV-biotin, followed by streptavidin-FITC (Molecular Probes-Invitrogen, Merelbeke, Belgium). After mounting of the coverslips, attachment was quantified per cell by counting the number of bound particles for at least 20 cells.

Entry inhibition assay. Cells were washed at 68 h post-seeding and pre-incubated at 37 °C for 60 minutes with mAb R-G-4 and/or mannan, or an irrelevant isotype-matched mAb. After pre-treatment, FIPV was added to the cells at an moi of 1. Cells and virus were incubated at 37 °C for 10 minutes (monocytes) or 1 h (CrFK and CHO cells). Then, cells were washed with ice-cold RPMI 1640 and fixed with formaldehyde (1 %). Bound particles were

stained with anti-FIPV-biotin, followed by streptavidin-Texas Red (Molecular Probes-Invitrogen, Merelbeke, Belgium). After permeabilization with Triton X-100 (0.1 %), internalized particles were stained with anti-FIPV-biotin, followed by streptavidin-FITC. After mounting of the coverslips, internalization was quantified per cell as the ratio of internalized virus particles to the total number of cell associated particles. At least ten cells were analyzed and all particles over the entire volume of the cell were monitored.

Infection inhibition assay. At 56 h post-seeding, cells were washed with RPMI 1640 and pre-incubated for 1 h at 37 °C with mAb R-G-4 and/or mannan, or an irrelevant isotype-matched mAb. After pre-treatment, FIPV was added to the cells at an moi of 1. Then, after 1 h the inoculum was replaced by medium supplemented with the antibodies or mannan, at the same concentrations as for pre-treatment. The cells were incubated for another 11 h at 37 °C. Finally, cells were washed, fixed and permeabilized. Permeabilization was followed by 1 h of incubation at 37 °C with anti-FIPV-FITC and 10 minutes with Hoechst 33342 (Molecular Probes). Coverslips were mounted onto microscope slides and analyzed by confocal microscopy. Cells with cytoplasmic expression of viral proteins were scored as infected cells. All cells on the coverslips were evaluated.

Co-localization assays

Monocytes, CrFK cells and CHO cells were washed at 68 h post-seeding and chilled at 4 °C for 20 minutes. Then, cells were inoculated with FIPV at an moi of 1. Cells and virus were incubated further at 4 °C for 1.5 h. Then, cells were washed with ice-cold RPMI 1640 and fixed with formaldehyde (1 %). Bound particles were stained with anti-FIPV-biotin, followed by streptavidin-FITC (Molecular Probes-Invitrogen, Merelbeke, Belgium). Slides were incubated with mAb 25-2B to stain fAPN, or with pAb anti-DC-SIGN antibodies to stain DC-SIGN. The mAb DH59B was used to visualize CD172a (VMRD). As a conjugate, Texas red-labelled goat anti-mouse antibodies were used (Molecular Probes). Coverslips were mounted onto microscope slides and analyzed by confocal microscopy.

Microscopy and statistics

Infection assays were analyzed by a DM IRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). Attachment, internalization and co-localization assays were analyzed with a Leica TCS SP2 laser scanning spectral confocal system linked to a DM IRB inverted microscope (Leica). Argon and He/Ne lasers were used for exciting FITC and Texas red fluorochromes, respectively. Leica confocal software was used for image acquisition.

Triplicate assays were performed and compared with the Mann-Whitney U test using the SPSS software package (version 12.0, SPSS Inc. Chicago, Illinois, USA). P values < 0.05 were considered significantly different.

RESULTS

Expression of the potential receptors fAPN and DC-SIGN on monocytes

fAPN was present on all monocytes and it was expressed in the plasma membrane as well as in the cytosol. This was assessed by stainings with mAb 25-2B before and after permeabilization (Fig. 1). It was, however, remarkable that the R-G-4 epitope was only abundantly present extracellularly on approximately 10 % of the monocytes. After permeabilization, the R-G-4 epitope could be detected in all cells (Fig. 1). In contrast, on CrFK cells, the R-G-4 epitope was extracellularly present on all cells (data not shown). This suggests that the expression pattern and/or the conformation of APN is cell type dependent. Furthermore, it was shown that the extracellular expression of the R-G-4 epitope on monocytes increased over time, from no cells with extracellular R-G-4 epitope expression on day 0, up to 16 % of cells with extracellular expression at day 5 post seeding (data not shown). For DC-SIGN, practically all monocytes expressed a substantial amount of DC-SIGN in their plasma membrane. DC-SIGN was also present intracellular (Fig. 1).

Evaluation of the role of fAPN in FIPV entry

The role of fAPN as an entry mediator for FIPV was evaluated in CrFK cells and in monocytes by means of inhibition studies with mAb R-G-4 and co-localization studies between FIPV and fAPN stained with mAb 25-2B.

fAPN is the major receptor for infectious entry on CrFK cells. On CrFK cells, (pre-)treatment with mAb R-G-4 completely (and significantly) blocked binding of FIPV (Fig. 2). Further, viral particles were not observed in CrFK cells treated with this mAb due to the fact that there was no virus binding (Fig. 3). This is clear from the picture in Figure 3 where no bound particles (in red) are visible. As infection was also blocked completely (and significantly) by this antibody (Fig. 4), it is safe to say that internalization after binding to fAPN leads to infection. All the particles bound to the cell surface co-localized with the epitope bound by mAb 25-2B and therefore co-localized with fAPN (Fig. 5). It is clear that fAPN is a necessary receptor for FIPV infection in the CrFK cell line.

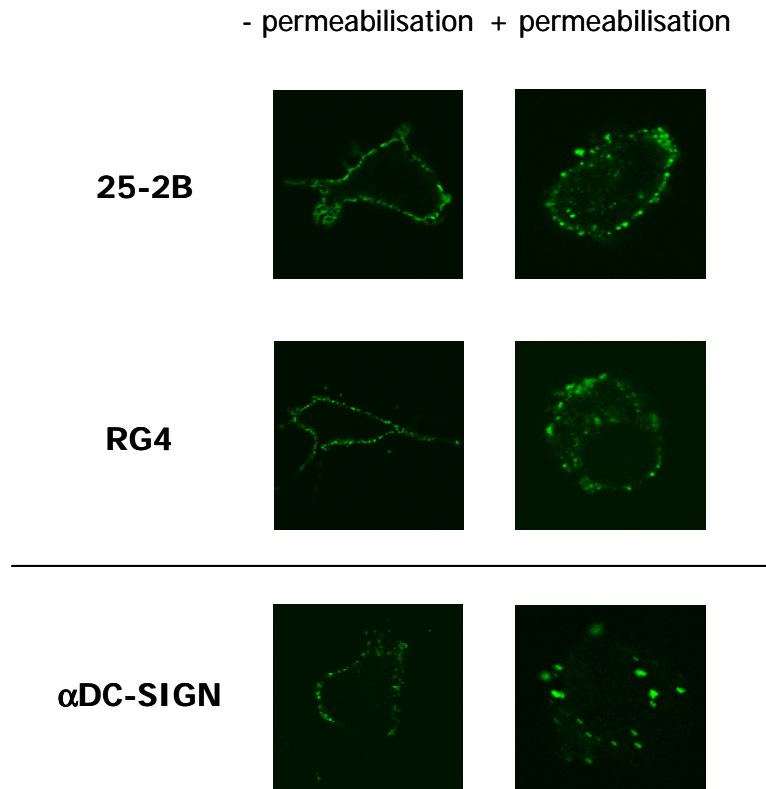


Figure 1: Expression of fAPN and fDC-SIGN on blood monocytes. fAPN on monocytes was stained with two mAbs on the plasma membrane and/or in the cell. DC-SIGN in the plasma membrane of monocytes was stained with a pAb.

fAPN is a receptor for infectious entry on monocytes. After incubation of cells with mAb R-G-4, attachment of FIPV was significantly reduced to 46.6 % of attachment on control cells (Fig. 2). Despite the fact that there was a big reduction by the mAb, this inhibition was not complete like observed for the CrFK cells. The internalization of bound FIPV in the presence of R-G-4 in monocytes was unaffected (Fig. 3). This means that the percentage of the virions that still bound to monocytes in the presence of mAb R-G-4 that was internalized, was similar to the percentage of particles that was internalized in the absence of inhibition. Particles that got internalized could also productively infect the monocyte as infection can be significantly reduced to 56.2 % of the control through administration of mAb R-G-4. Co-localization studies showed that 59.9 ± 16.2 % of the FIPV particles that bound to monocytes, co-localize with fAPN (Fig. 5). This amount of co-localization was significantly higher than what was expected based on coincidence (27.8 ± 5.2 %), as shown by the co-localization staining between FIPV and CD172a, an irrelevant surface protein. This confirms the involvement of fAPN in virus binding to monocytes. However, a relatively high percentage of binding and infection with FIPV is still unaccounted

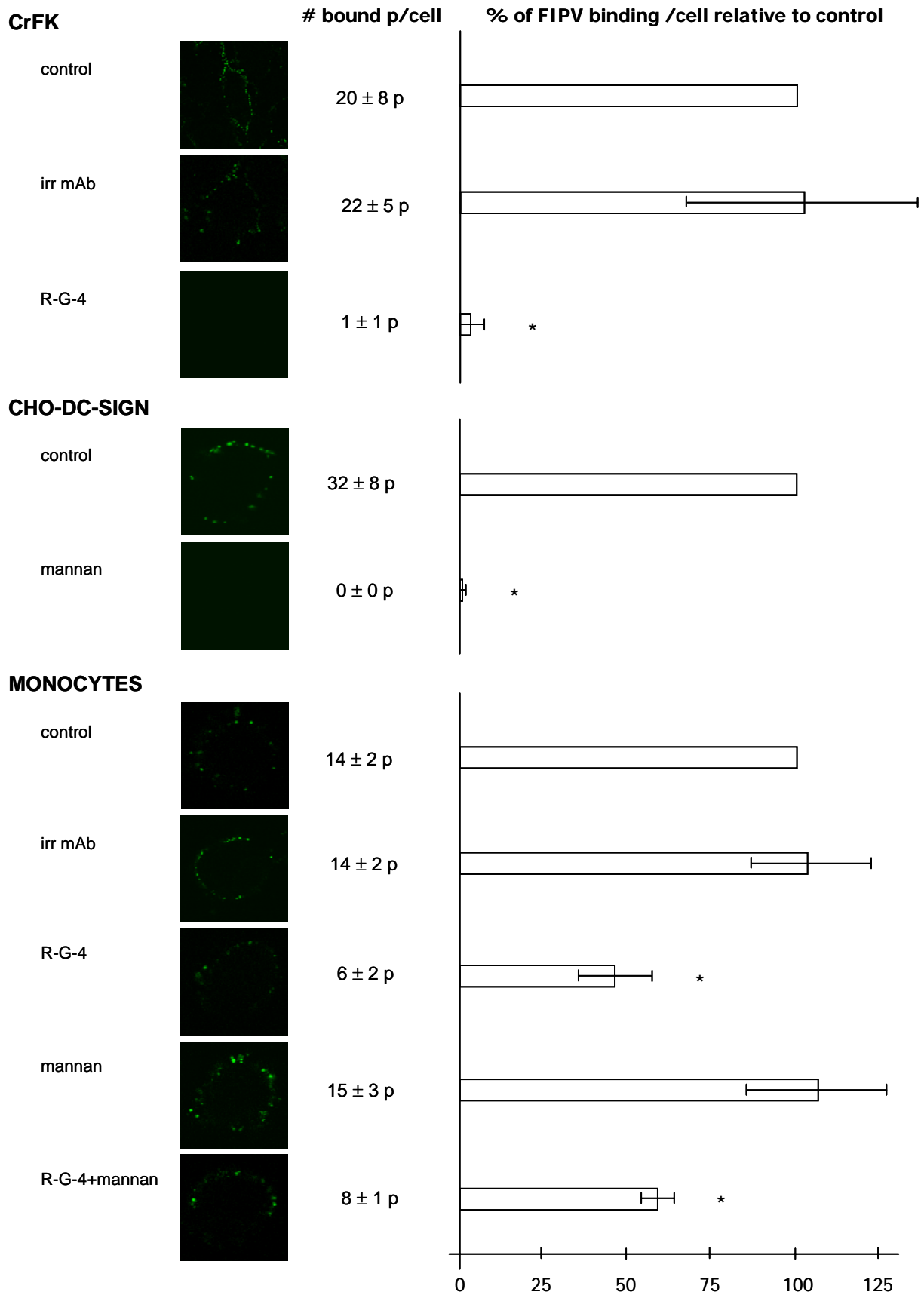


Figure 2: Attachment of FIPV on CrFK cells, CHO-DC-SIGN cells and monocytes in the presence of mAb R-G-4 and/or mannan. Confocal images of single sections through cells are shown. Green dots represent bound virus particles. Next to each image the number of particles bound per cell under

the described conditions is indicated. The graphs illustrate FIPV binding per cell in the presence of an irrelevant mAb (irr mAb), mAb R-G-4 and/or mannan relative to binding in control cells. Data represent means \pm standard deviations of triplicate assays. 'p' stands for particles. * marks values that are significantly different from the control.

for, indicating that fAPN is a receptor for FIPV but not the sole receptor. Moreover, these results show that the entry events in CrFK cells do not reflect what occurs in monocytes.

Evaluation of the role of DC-SIGN in FIPV entry

The role of DC-SIGN as an entry mediator for FIPV was evaluated on wild type CHO cells, CHO cells stably transduced with human DC-SIGN and in monocytes by means of inhibition studies with mannan and co-localization studies between FIPV antigens and feline DC-SIGN stained with pAb anti-DC-SIGN.

Human DC-SIGN (hDC-SIGN) can bind and internalize FIPV. Wild type CHO cells do not bind or internalize FIPV (data not shown). However, after stable transduction with hDC-SIGN, virus binds efficiently to all cells (Fig. 2) and is also able to enter these cells (Fig. 3). As virus attachment on the cells can be completely (and significantly) blocked by mannan, hDC-SIGN is the virus binding receptor on these cells. This is confirmed by the full co-localization between FIPV and hDC-SIGN on these cells (Fig. 5). The small number of particles that bound to cells in the presence of mannan were not internalized in the cells, indicating that DC-SIGN is responsible for both binding and internalization in CHO-DC-SIGN cells. The cells are however not susceptible to infection, which means that hDC-SIGN can not mediate infectious entry in these cells.

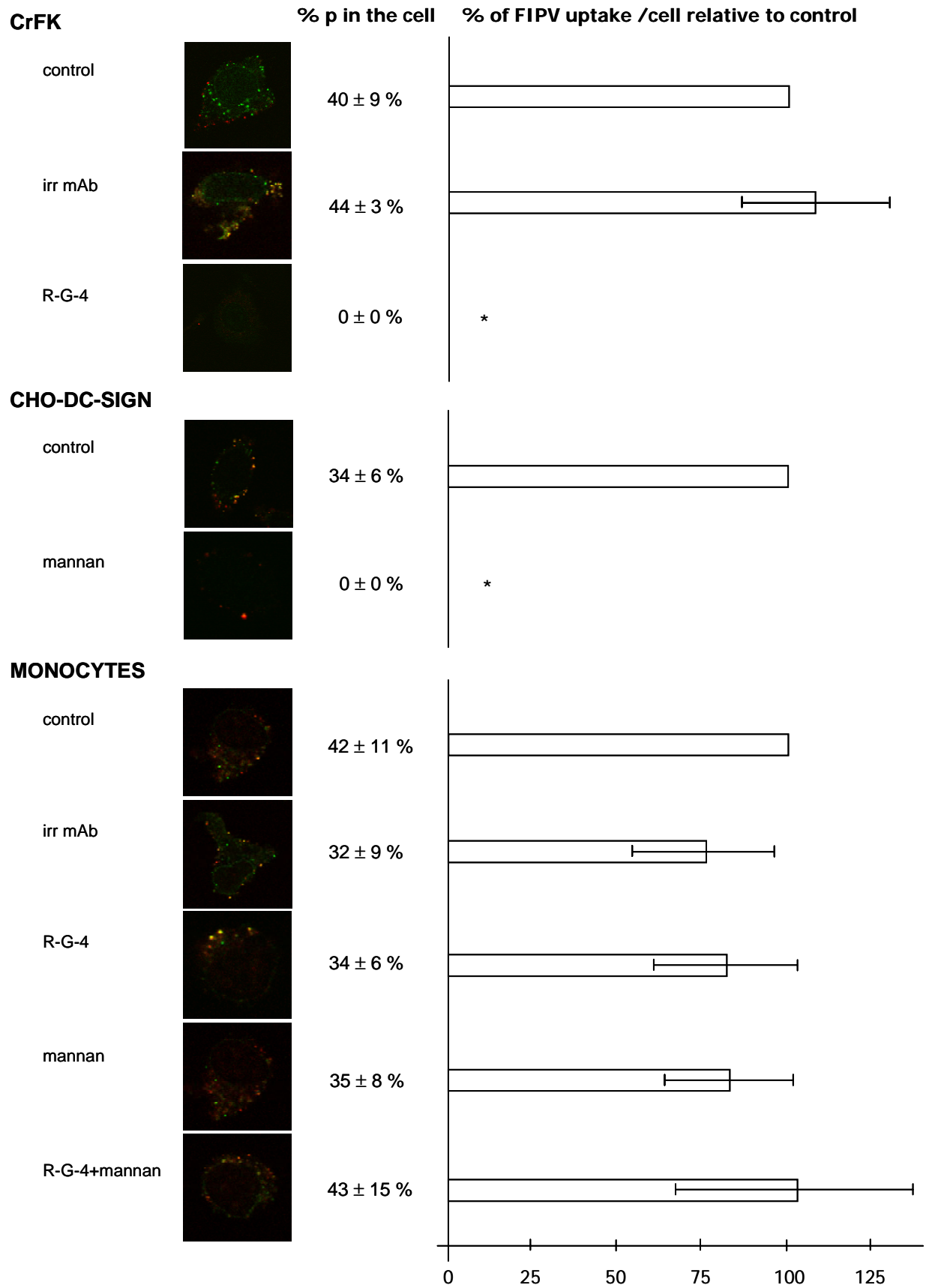


Figure 3: Internalization of FIPV in CrFK cells, CHO-DC-SIGN cells and monocytes in the presence of mAb R-G-4 and/or mannan. Confocal images of single sections through cells are shown. All red dots

represent bound virus particles that are not internalized, while exclusively green dots represent internalized virus particles. Next to each image the percentage of internalized particles (p) per cell under the described conditions is indicated. The graphs illustrate the internalization per cell in the presence of an irrelevant Ab (irr mAb), mAb R-G-4 and/or mannan relative to internalization in control cells. Data represent means \pm standard deviations of triplicate assays. * marks values that are significantly different from the control.

Role of feline DC-SIGN (fDC-SIGN) in binding and internalization of FIPV in target cells. As hDC-SIGN can bind and internalize FIPV particles, the role of fDC-SIGN, naturally present on the *in vivo* target cells for FIPV, was evaluated in monocytes. Figure 2 showed that blocking fDC-SIGN with mannan had no effect on virus binding. This was further confirmed by the fact that combining mannan and R-G-4 did not significantly increase the inhibition of attachment caused by R-G-4. Accordingly, internalization could not be influenced by the presence of mannan alone or a combination of mAb R-G-4 and mannan (Fig. 3). For infection, like for attachment and internalization, no inhibiting effect was observed when only mannan was added. This suggests that fDC-SIGN is not involved in the entry process of FIPV in primary cells and that the binding capacities of hDC-SIGN observed in cell lines do not reflect the situation in the natural target cells. The results of the co-localization study between FIPV and fDC-SIGN on monocytes supported this conclusion as the observed co-localization of 28.9 ± 4.5 % did not significantly exceed the level of 'background' co-localization (27.8 ± 5.2 %; Fig. 5). However, when both mAb R-G-4 and mannan were added to the cells, infection was reduced significantly more efficiently than when only aminopeptidase N was blocked: a reduction to 17.8 % of infection in control cells compared to 56.2 % when only APN is blocked. Based on these results, it appears that fDC-SIGN does seem to have a role in the infection process, even though the underlying mechanism remains elusive thus far.

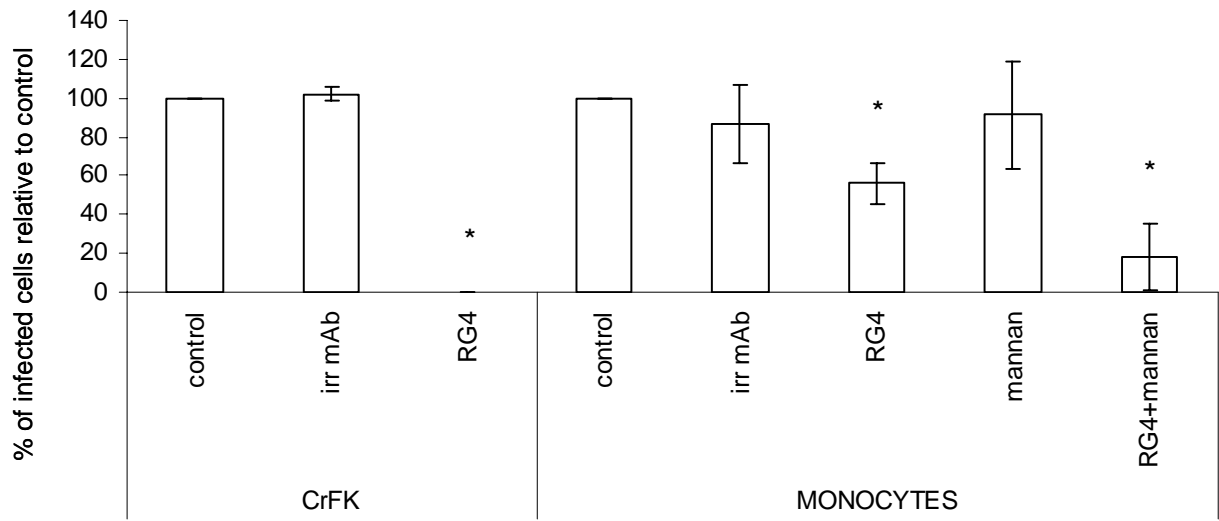


Figure 4: Graph illustrating the percentage of infected cells in the presence of an irrelevant mAb (irr mAb), mAb R-G-4 and/or mannan relative to the percentage of infected control cells. Data represent means \pm standard deviations of triplicate assays. * marks results that are significantly different from the control.

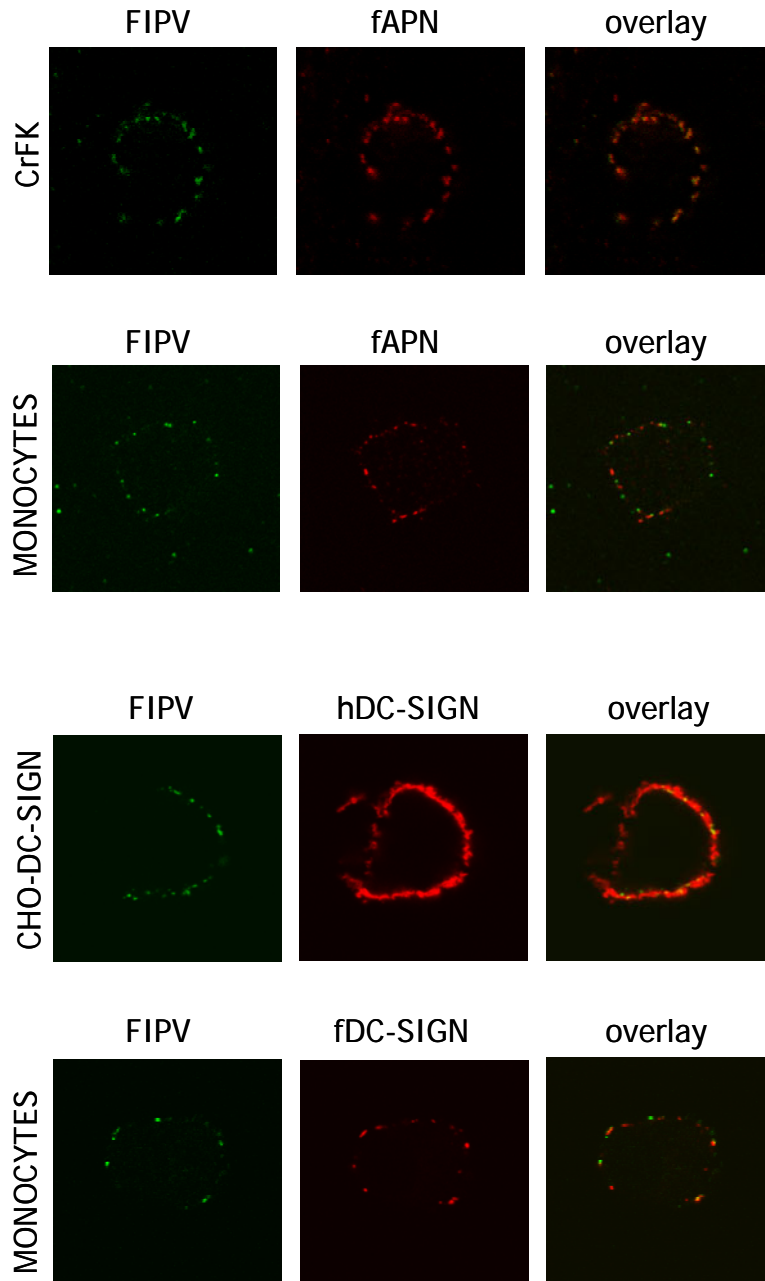


Figure 5: Co-localization of FIPV with membrane proteins. Co-localization stainings are performed after attachment of FIPV to the cells at 4 °C. FIPV was stained with biotinylated anti-FIPV antibodies and streptavidin-FITC. fAPN was stained with mAb 25-2B, and hDC-SIGN and fDC-SIGN with pAbs. All these Abs were visualized with goat anti-mouse-Texas Red antibodies. The figure shows confocal images of single sections through cells.

DISCUSSION

The main goal of this study was to evaluate the role and interplay of two described receptors for FIPV, aminopeptidase N (APN) and DC-SIGN, in its natural host cell, the monocyte. This was pursued through a combination of inhibition assays at different stages of the entry process, infection inhibition assays and co-localization studies.

Feline aminopeptidase N (fAPN) was shown before to be a receptor for type II FIPV on cell lines (Tresnan *et al.*, 1996; Hohdatsu *et al.*, 1998; Dye *et al.*, 2007). Our results confirmed and extended these findings showing that fAPN is a necessary receptor for FIPV II on CrFK cells and the major attachment receptor on these cells. Although it was not possible to confirm that fAPN is also involved in the subsequent internalization of the virus in CrFK cells, as virus did not bind in the presence of mAb R-G-4 (directed against fAPN), it is most likely as FIPV was found to co-localize with fAPN inside these cells (data not shown). For human coronavirus 229E, that uses human APN as a receptor, it was also indirectly shown that APN is involved in internalization. The virus was shown to co-localize with caveolin inside human fibroblast cells, and caveolin was shown to co-localize with APN inside the cells (Nomura *et al.*, 2004).

On feline monocytes, fAPN also acts as a virus binding receptor. However, it is not the only receptor as virus binding could not be completely blocked by anti-fAPN antibodies (mAb R-G-4). The internalization experiments indicated that the fraction of the virus bound to the other unknown receptor, that is internalized, is similar to the fraction of bound virus, that is internalized when fAPN is available. Further, binding to fAPN can lead to infection as infection was reduced by mAb R-G-4. Because blocking fAPN did not completely prevent infection, it is most likely that internalization through the other receptor also leads to infection.

Hence, entry in CrFK cells provides useful information about the FIPV-fAPN interaction but does not reflect the complete picture of what happens during virus entry in monocytes. This is in line with previous findings that indicate that CrFK cells are of limited use as a model cell line to study interactions of FIPV with target cells (Dewerchin *et al.*, 2005; Van Hamme *et al.*, 2007).

The virus binding and internalizing capacities of human DC-SIGN (hDC-SIGN) became evident based on the fact that transduction of CHO cells with hDC-SIGN rendered them capable of binding and internalizing FIPV. However, internalization through hDC-SIGN did

not lead to infection in these cells. This might be due to the absence of a factor necessary for escape of the virions from endosomes as internalization through hDC-SIGN targets the endosomal/lysosomal pathway (Engering *et al.*, 2002). Unfortunately, these experiments could not be performed with cells transduced with fDC-SIGN, as cloning and characterization of the feline homologue has yet to be achieved. This also has hampered research on another feline virus, feline immunodeficiency virus (FIV), where interactions between FIV surface glycoproteins and hDC-SIGN were shown and the authors remained cautious with conclusions towards fDC-SIGN (de Parseval *et al.*, 2004). Nevertheless, the experiments with the CHO-hDC-SIGN cells provide a positive control for the set-up of the inhibition and co-localization assays.

Even though it would be very interesting to evaluate the FIPV binding and internalizing capacities of fDC-SIGN by expressing this protein in non-susceptible cells, there are already strong indications that fDC-SIGN is not necessary for virus binding and internalization in its natural host cell, the monocyte. Blocking fDC-SIGN with mannan on monocytes did not affect FIPV binding and internalization. Also infection of monocytes was not affected by blocking DC-SIGN with mannan. Additionally, when fAPN is blocked, FIPV binding does not transfer to fDC-SIGN because even then virus binding is not reduced by mannan. It seems clear that fDC-SIGN is not the unknown receptor for FIPV that is responsible for the fraction of binding and infection that remains after blocking fAPN. However, treating fAPN-blocked cells with mannan, did cause a supplemental reduction of infection. The involvement of DC-SIGN is most likely situated in a step after internalization because when both fAPN and fDC-SIGN are blocked, there is still efficient internalization of bound particles.

Having listed the results from this study, a comparison can be made with the results obtained by Regan & Whittaker (2008). They induced susceptibility to infection in unsusceptible cells (mouse 3T3 cells) after transfection with hDC-SIGN. In our study, virus internalization could be induced in transfected CHO cells, but infection could not be. A possible explanation is that CHO cells lack a factor necessary to release the FIPV genome from intracellular vesicles, while this factor is present in the mouse cells used by Regan & Whittaker. Further, Regan & Whittaker found that transfection of hDC-SIGN in CrFK cells enhanced infection of these cells. This could be explained by increased virus binding and internalization. Infection of monocytes could also be blocked with mannan, according to Regan & Whittaker. In our hands, no inhibition of infection was observed after treating monocytes with mannan. There is no obvious explanation for the different outcome of this experiment. Maybe multiple passaging in the laboratory, induced culture adaptations in the virus that influence virus-receptor interactions.

The data obtained in this study provide the following insights in FIPV type II entry in monocytes. Virus can bind to fAPN and will probably be internalized into the cell via this receptor. A particle internalized via this pathway can lead to infection of the cell. A second, unknown receptor can also bind and internalize FIPV. This pathway probably also leads to infection. fDC-SIGN is not the unknown receptor, but can fulfil a role in infection via this unknown receptor in a post-internalization step. Hypothetically, fDC-SIGN might be involved in genome release of virus that enters cells via the unknown receptor.

The experiments showed that blocking fDC-SIGN did not affect infection. This might suggest that the fAPN-mediated pathway can compensate for fDC-SIGN malfunction or unavailability.

The receptor for serotype I FIPV in monocytes is still unknown. Probably, FIPV type I does not use APN (Hohdatsu *et al.*, 1998; Dye *et al.*, 2007). Possibly, the pathway for type II using the unknown receptor with/without DC-SIGN might also be the pathway used by type I FIPV. This will be studied in the future by comparing binding of FIPV type I to monocytes, with binding of FIPV type II in the presence of antibodies against APN on these monocytes. Further, the effect of blocking DC-SIGN with mannan, on infection with FIPV type I in monocytes will be studied.

An interesting observation was that the epitope recognized by mAb R-G-4 was extracellularly present on all CrFK cells, but only on a small fraction of monocytes. Some possible explanations might be that the epitope could no longer be recognized by mAb R-G-4 on the majority of monocytes because the epitope is masked by glycosylation of sites near the R-G-4 epitope or by the presence of (other) proteins interacting with APN.

Because the R-G-4 binding epitope might be different between monocytes and CrFK cells, the R-G-4 mAb could be less capable of blocking potential virus binding to monocytes. However the virus binding sequences might be intact. This could explain the difference in blocking potential of the mAb between CrFK cells and monocytes. In that case, virus binding to fAPN would be underestimated on monocytes. However, the co-localization assay seems to confirm that not all bound virus is attached to fAPN.

It would be very interesting to identify the differences between APN expressed on cell lines and on primary monocytes and to determine if the FIPV-APN interaction occurs at the same epitopes of APN in these different cell types.

Taken together, in this study, the role of aminopeptidase N and DC-SIGN as receptors for FIPV were analysed for the first time in the *in vivo* target cell, the monocyte, at different

stages of entry and infection. fAPN is capable of binding and internalizing FIPV in these cells and fAPN-mediated entry leads to infection. However, this is not the exclusive receptor for FIPV on monocytes, like it appears to be in CrFK cells. DC-SIGN can also play a role during FIPV entry in monocytes. However, DC-SIGN is not directly required for attachment or internalization, but is probably involved in infection via an alternative pathway independent of fAPN. Besides elucidating some aspects about FIPV entry in monocytes, these new insights reveal how much is unknown about FIPV entry. This study is again a reminder that results of studies obtained in cell culture should be analysed with care, knowing that viruses can use different receptors and a variety of cellular proteins to gain entry into different cells.

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REFERENCES

- Alvarez, C. P., Lasal, F., Carrillo, J., Muniz, O., Corbi, A. L. & Delgado, R. (2002).** C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *J Virol* **76**, 6841-6844.
- Cooley, L. A., & Lewin, S. R. (2003).** HIV-1 cell entry and advances in viral entry inhibitor therapy. *J Clin Virol* **26**, 121-132.
- de Haan, C. A. M., Haijema, B. J., Schellen, P., Wichgers Schreur, P., te Lintelo, E., Vennema, H. & Rottier, P. J. M. (2008).** Cleavage of group 1 coronavirus spike proteins: how furin cleavage is traded off against heparan sulfate binding upon cell culture adaptation. *J Virol* **82**, 6078-6083.
- de Parseval, A., Su, S. V., Elder, J. H. & Lee, B. (2004).** Specific interaction of feline immunodeficiency virus surface glycoprotein with human DC-SIGN. *J Virol* **78**, 2597-2600.
- Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2005).** Replication of feline coronaviruses in peripheral blood monocytes. *Arch Virol* **150**, 2483-2500.
- De Witte, L., Abt, M., Schneider-Schaulies, S., van Kooyk, Y. & Geijtenbeek, T. B. H. (2006).** Measles virus targets DC-SIGN to enhance dendritic cell infection. *J Virol* **80**, 3477-3486.
- Drickamer, K. (1999).** C-type lectin-like domains. *Curr Opin Struct Biol* **9**, 585-590.
- Dye, C., Temperton, N. & Siddell, S. G. (2007).** Type I feline coronavirus spike glycoprotein fails to recognize aminopeptidase N as a functional receptor on feline cell lines. *J Gen Virol* **88**, 1753-1760.
- Engering, A., Gijtenbeek, T. B. H., van Vliet, S. J., Wijers, M., van Liempt, E., Demareux, N., Lanzavecchia, A., Fransen, J., Figdor, C. G., Piguet, V. & van Kooyk, Y. (2002).** The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol* **168**, 2118-2126.
- Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. F. & van Kooyk, Y. (2000).** DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T-cells. *Cell* **100**, 587-597.

Hohdatsu, T., Izumiya, Y., Yokoyama, Y., Kida, K. & Koyama, H. (1998). Differences in virus receptor for type I and type II feline infectious peritonitis virus. *Arch Virol* **143**, 839-850.

Hofmann, H., Simmons, G., Rennekamp, A. J., Chaipan, C., Gramberg, T., Heck, E., Geier, M., Wegele, A., Marzi, A., Bates, P. & Pöhlmann, S. (2006). Highly conserved regions within the spike proteins of human coronavirus 229E and NL63 determine recognition of their respective cellular receptors. *J Virol* **80**, 8639-8652.

Jeffers, S. A., Hemmila, E. M. & Holmes, K. V. (2006). Human coronavirus 229E can use CD209L (L-SIGN) to enter cells. *Adv Exp Med Biol* **581**, 265-269.

Jeffers, S. A., Tusell, S. M., Gillim-Ross, L., Hemmila, E. M., Achenbach, J. E., Babcock, G. J., Thomas, W. D., Thackray, L. B., Young, M. D., Mason, R. J., Ambrosino, D. M., Wentworth, D. E., Demartini, J. C. & Holmes, K. V. (2004). CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci USA* **101**, 15748-15753.

Lozach, P. Y., Lortat-Jacob, H., De Lacroix De Lavalette, A., Staropoli, I., Foug, S., Amara, A., Houles, C., Fieschi S., Schwartz, O., Virelizier, J. L., Arenzana-Seisdedos, F. & Altmeyer, R. (2003). DC-SIGN and L-SIGN are high-affinity binding receptors for hepatitis C virus glycoprotein E2. *J Biol Chem* **278**, 20358-20366.

Luan, Y. & Xu, W. (2007). The structure and main functions of aminopeptidase N. *Curr Med Chem* **14**, 639-647.

Marzi, A., Gramberg, T., Simmons, G., Moller, P., Rennekamp, A. J., Krumbiegel, M., Geier, M., Eisemann, J., Turza, N., Saunier, B., Steinkasserer, A., Becker, S., Bates, P., Hofmann, H. & Pöhlmann, S. (2004). DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus. *J Virol* **78**, 12090-12095.

Nauwynck, H. J. & Pensaert, M. B. (1995). Effect of specific antibodies on the cell associated spread of pseudorabies virus in monolayers of different cell types. *Arch Virol* **140**, 1137-1146.

Pedersen, N. C., Evermann, J. F., Mc Keirnan, A. J. & Ott, R. L. (1984). Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. *Am J Vet Res* **45**, 2580-2585.

Pöhlmann, S., Zhang, F., Baribaud, F., Chen, Z., Leslie, G. J., Lin, G., Granelli-Piperno, A., Doms, R. W., Rice, C. M. & McKeating, J. A. (2003). Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J Virol* **77**, 4070-4080.

Regan, A. D., Shraybman, R., Cohen, R. D. & Whittaker, G. R. (2008). Differential role of low pH and cathepsin-mediated cleavage of the viral spike protein during entry of serotype II coronaviruses. *Vet Microbiol* **132**, 235-248.

Regan, A. D. & Whittaker, G. R. (2008). Utilization of DC-SIGN for entry of feline coronaviruses into host cells. *J Virol* **82**, 11992-11996.

Sjöström, H., Norén, O. & Olsen, J. (2000). Structure and function of aminopeptidase N. *Adv Exp Med Biol* **477**, 25-34.

Timpe, J. M. & McKeating, J. A. (2008). Hepatitis C virus entry: possible targets for therapy. *Gut* **57**, 1728-1737.

Tresnan, D. B., Levis, R. & Holmes, K. V. (1996). Feline aminopeptidase N serves as a receptor for feline, canine, porcine and human coronaviruses in serogroup I. *J Virol* **70**, 8669-8674.

Tusell, S. M., Schittone, S. A. & Holmes, K. V. (2007). Mutational analysis of aminopeptidase N, a receptor for several group 1 coronaviruses, identifies key determinants of viral host range. *J Virol* **81**, 1261-1273.

Van Hamme, E., Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2007). Attachment and internalization of feline infectious peritonitis virus in feline blood monocytes and Crandell feline kidney cells. *J Gen Virol* **88**, 2527-2535.

Van Hamme, E., Dewerchin, H. L., Cornelissen, E., Verhasselt, B. & Nauwynck, H. J. (2008). Clathrin- and caveolae-independent entry of feline infectious peritonitis virus in monocytes depends on dynamin. *J Gen Virol* **89**, 2147-2156.

Yang, Z. Y., Huang, Y., Ganesh, L., Leung, K., Kong, W. P., Schwartz, O., Subbarao, K. & Nabel, G. J. (2004). pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. *J Virol* **78**, 5642-5650.

**THE MECHANISM OF FELINE INFECTIOUS PERITONITIS VIRUS
TYPE II INTERNALIZATION IN MONOCYTES**

Evelien Van Hamme, Hannah L. Dewerchin, Els Cornelissen, Bruno Verhasselt & Hans J.
Nauwynck

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ABSTRACT

Feline infectious peritonitis virus (FIPV), a coronavirus that causes a lethal chronic disease in cats, enters feline monocytes via endocytosis. In this study, the pathway of internalization is characterized by evaluating the effect of chemical inhibitors and/or expression of dominant-negative (DN) proteins on the percentage of internalized virions per cell and infection. Further, co-localization studies were performed to determine the involvement of certain cellular internalization proteins. FIPV is not internalized through a clathrin-mediated pathway, as chlorpromazine, amantadine and DN eps15 did not influence virus uptake and FIPV did not co-localize with clathrin. The caveolae-mediated pathway could be excluded based on the inability of genistein and DN caveolin-1 to inhibit virus uptake and lack of co-localization between FIPV and caveolin-1. Dynamin inhibitory peptide and DN dynamin effectively inhibited virus internalization. The inhibitor strongly reduced uptake to 20.3 ± 1.1 % of uptake in untreated cells. In the presence of DN dynamin, uptake was 58.7 ± 3.9 % relative to uptake in untransduced cells. Internalization of FIPV was slightly reduced to 85.0 ± 1.4 % and 87.4 ± 6.1 % of internalization in control cells by the sterol-binding drugs nystatin and methyl- β -cyclodextrin, respectively. Rho GTPases were inhibited by *Clostridium difficile* toxin B, but no effect was observed. These results were confirmed with infection studies showing that infection was not influenced by chlorpromazine, amantadine and genistein but was significantly reduced by dynamin inhibition and nystatin. In conclusion, these results indicate that FIPV enters monocytes through a clathrin- and caveolae-independent pathway that strongly depends on dynamin and is slightly sensitive to cholesterol depletion.

INTRODUCTION

Viral entry occurs through a number of successive steps allowing the virus to bring its genome inside the cell. The virus either fuses with the host cell membrane or utilizes the endocytosis machinery of the cell. Endocytosis can be divided in two categories: phagocytosis of large cargo and pinocytosis of smaller cargo (Conner & Schmid, 2003). Phagocytosis is an active and highly regulated process involving specific cell surface receptors and signalling cascades mediated by Rho GTPases (Hall & Nobes, 2000; Conner & Schmid, 2003). Pinocytosis can occur through several pathways. The cargo and its receptor determine which pinocytic pathway will be used (Conner & Schmid, 2003). A first possible route for pinocytosis is 'macropinocytosis' (Swanson & Watts, 1995). Other pathways are named after the main structural compound involved: 'clathrin-mediated endocytosis' (Brodsky *et al.*, 2001) and 'caveolae-mediated endocytosis' (Pelkmans & Helenius, 2002). Finally, there is a group called 'clathrin- and caveolae-independent pathways'. They are differentiated based on their dependency on cholesterol and/or association with lipid rafts, dynamin and Rho GTPases (Nichols & Lippincott-Schwartz, 2001; Conner & Schmid, 2003). Viruses are specialized in misusing these endocytic pathways to gain entry into the cell (Sieczkarski & Whittaker, 2002a; Pelkmans & Helenius, 2003).

Recently, we have demonstrated that feline infectious peritonitis virus (FIPV), a devastating coronavirus in cats, enters its host cell, the monocyte, via endocytosis (Van Hamme *et al.*, 2007). The pathway used is unknown. Transmissible gastro-enteritis virus (TGEV), canine coronavirus and human coronavirus 229E (HCoV-229E) are coronaviruses that - like FIPV - belong to phylogenetic group 1. They have all been shown to enter cells via endocytosis (Hansen *et al.*, 1998; Savarino *et al.*, 2003; Nomura *et al.*, 2004). The pathways used for internalization have not been studied, except for HCoV-229E that enters human fibroblast cells through the caveolae-mediated pathway (Nomura *et al.*, 2004). Severe acute respiratory syndrome (SARS), an emerging disease in humans, is caused by a coronavirus belonging to phylogenetic group 2 (Kim *et al.*, 2006). Due to its threat, many studies have been done to better understand how the virus interacts with its host and host cells. It was shown that SARS virus enters HepG2 cells expressing ACE2 via pH-dependent endocytosis through clathrin-coated vesicles (Yang *et al.*, 2004; Inoue *et al.*, 2007). More recently, SARS virus was shown to enter Vero E6 cells through receptor-mediated, clathrin- and caveolae-independent endocytosis, likely involving lipid rafts (Wang *et al.*, 2008). The entry of murine coronavirus, also belonging to group 2, is extensively studied. Although entry via non-endosomal routes has been suggested, recent publications assign a major role for

cholesterol-dependent endocytosis, possibly through clathrin-coated pits, in murine cells (Thorp & Gallagher, 2004; Choi *et al.*, 2005; Eifart *et al.*, 2007).

In this study, the mechanism of internalization of FIPV in monocytes was determined through chemical inhibition of internalization, transduction of monocytes with constructs to induce expression of dominant-negative (DN) proteins that hinder some pathways and co-localization studies. Our results indicate that FIPV is internalized through a clathrin- and caveolae-independent pathway that strongly depends on dynamin and is slightly cholesterol depletion sensitive.

METHODS

Cells and virus

Monocytes were isolated from blood, collected from feline coronavirus-, feline leukaemia virus- and feline immunodeficiency virus-negative cats as described previously (Dewerchin *et al.*, 2005) and seeded on glass coverslips in medium [RPMI 1640 enriched with 10 % fetal bovine serum, 0.3 mg glutamine ml⁻¹, 1mM sodium pyruvate and 1 % non-essential amino-acids 100x (GIBCO-Invitrogen)]. Cells consisted of 86 ± 7 % monocytes. FIPV serotype II strain 79-1146 was kindly provided by Dr Egberink (Faculty of Veterinary Medicine, Utrecht University, The Netherlands) and passaged in a feline cell line (CrFK) (McKeirnan, *et al.*, 1981). Batches of culture fluids were purified by ultracentrifugation and purified virus was biotinylated as described previously (Van Hamme *et al.*, 2007). Purified viral particle suspension (200 µg) was applied to 10^{4.4} monocytes throughout the experiments. This amount of suspension contains 5.0 x 10¹⁰ virus particles of which 1.9 x 10⁵ particles are infectious (Van Hamme *et al.*, 2007). This suspension was used exclusively in entry assays, not in the infection and co-localization studies.

Entry inhibition assay

Monocytes were washed extensively at 68 h post-seeding and pre-incubated at 37 °C for 30 minutes with one of the following compounds dissolved in RPMI 1640 (Gibco-Invitrogen) (all products were purchased from Sigma-Aldrich; unless stated otherwise): 0.1 µM wortmannin, 20 µM latrunculin B (ICN Biochemicals), 2 µM chlorpromazine, 500 µM amantadine, 50 µg nystatin ml⁻¹, 10 mM methyl-β-cyclodextrin, 50 µg genistein ml⁻¹, 40 and 80 µM dynamin inhibitory peptide (dip) (batch 5 - Tocris Cookson) or 0.74 nM *Clostridium difficile* toxin B. Working concentrations were optimized qualitatively in internalization assays with control

ligands while insuring that viability of the cells was always over 99%. After pre-treatment, monocytes were inoculated with biotinylated FIPV in the presence of the compound used for pre-treatment. Cells and virus were incubated at 37 °C for 1 h. Then, cells were briefly washed with ice-cold RPMI 1640 and fixed with formaldehyde (1 %). Bound particles were stained with streptavidin-Texas red (Molecular Probes) and after permeabilization with Triton X-100 (0.1 %) internalized particles were stained with streptavidin-fluorescein isothiocyanate (FITC) (Molecular Probes). After mounting of the coverslips, internalization was quantified per cell as the ratio of internalized virus particles to the total number of cell-associated particles. At least ten cells were analysed and all particles over the entire volume of the cell were monitored. The procedure described above, from seeding of the cells up to the analysis, was performed three times.

To assess the effectiveness of all agents, internalization of biotinylated transferrin (Sigma-Aldrich), FITC-labelled albumin and fluorescent 1µm FluoSpheres (Molecular Probes) was studied in the presence of appropriate inhibitors. After fixation and permeabilization, biotinylated transferrin was visualized with streptavidin-FITC and in the cells incubated with fluorescent beads, cortical actin was stained using falloidin-Texas red (Molecular Probes). Coverslips were mounted on microscope slides and analysed by confocal microscopy. Cells were scored as internalizing cells if all control ligands were internalized. For each experiment 50-100 cells were analysed and experiments were performed three times.

Plasmid constructions and production of lentiviral supernatants

Transfer vectors were prepared by deletion of green fluorescent protein (GFP) from the TRIPΔU3-CMV-GFP-WPRE vector (=TRIPΔU3-CMV-WPRE). The enhanced GFP (EGFP) tagged DN eps15 construct, named DIII and the EGFP tagged control construct D3Δ2, a kind gift from Dr Benmerah (Benmerah *et al.*, 1998), were excised from pEGFP-C2 and cloned into TRIPΔU3-CMV-WPRE. EGFP tagged wild type (WT) and DN caveolin-1, a kind gift from Dr Helenius (Kurzchalia *et al.*, 1992; Pelkmans *et al.*, 2001), and WT and DN dynamin 2(aa), a kind gift from Dr McNiven (Cao *et al.*, 1998; 2000) are also cloned into TRIPΔU3-CMV-WPRE. Biological activity of the constructs in the original plasmids and after transfer into pTRIPΔU3-CMV-WPRE was tested in CrFK cells.

pMD.G and p8.91 were used as envelope and packaging plasmids, respectively, as described previously (Stove *et al.*, 2005). At 70 % confluency, 293FT cells (Invitrogen) were co-transfected with 1.66 µg packaging plasmids, 3.33 µg envelope plasmids and 3.33 µg transfer plasmids using a calcium phosphate transfection kit (Invitrogen). Lentiviral supernatants were harvested after 40 h (Stove *et al.*, 2005).

Inhibition of virus internalization pathways through lentiviral gene transfer

At 3 h post-seeding, cells were washed and medium was replaced by lentiviral supernatants. At 24 h post-seeding, cells were washed and fresh medium was added. Internalization assays were performed at 68 h post-seeding. After washing the cells, biotinylated FIPV was added and incubated with the cells for 1 h at 37 °C. Then, cells were briefly washed with ice-cold RPMI 1640 and fixed with formaldehyde (1 %). Bound particles were stained with streptavidin-Alexa Fluor 350 (Molecular Probes) and after permeabilization with Triton X-100 (0.1 %) internalized particles were stained with streptavidin-Texas red. Coverslips were mounted and the level of internalization was quantified as described above.

For the controls, transduced cells were incubated with biotinylated transferrin or biotinylated cholera toxin B (Sigma-Aldrich). After fixation and permeabilization, ligands were visualized with streptavidin-Texas red. Coverslips were mounted onto microscope slides and analysed by confocal microscopy. Cells were scored as internalizing cells if all control ligands were internalized.

Co-localization with clathrin

At 68 h post-seeding, cells were washed with RPMI 1640 (37 °C) and incubated with non-biotinylated FIPV at an m.o.i. of 5 for 0, 5, 15 and 45 minutes at 37 °C. Then, cells were washed with RPMI 1640 and fixed. Cells were washed again, first with RPMI 1640 followed by Tris-buffered saline (TBS; 20 mM Tris/HCl, 150 mM NaCl, pH 7.5) with 4.5 % sucrose and 2 % inactivated goat serum (TBS-GS) and permeabilized with methanol for 30 s at -20°C (Racoosin & Swanson, 1994). Clathrin was stained with anti-clathrin heavy-chain IgM antibodies (ICN Biochemicals), diluted 1 : 50 in PBS supplemented with 0.3 % gelatine (PBS-G) (Van de Walle *et al.*, 2001; Misinzo *et al.*, 2005). Afterwards, cells were washed in TBS-GS and incubated for 1 h at 37 °C with biotin-labelled goat anti-mouse IgM antibodies (Santa Cruz Biotechnology) diluted 1 : 100 in PBS-G. Then, cells were washed and incubated with streptavidin-Texas red (Molecular Probes) diluted 1 : 50 in PBS-G. After washing, FIPV was stained with anti-FIPV-FITC [Veterinary Medical Research Development (VMRD)]. Coverslips were mounted on microscope slides and analysed by confocal microscopy.

Co-localization with caveolin-1

At 68 h post-seeding, cells were washed with RPMI 1640 (37 °C) and incubated with non-biotinylated FIPV at an m.o.i. of 5 for 0, 5, 15 and 45 minutes at 37 °C. Then, cells were washed with RPMI 1640 and fixed. Cells were permeabilized with Triton X-100 (0.1 %) and

washed with PBS. Caveolin-1 was stained with polyclonal rabbit anti-caveolin-1 antibodies (Abcam), diluted 1 : 200 in PBS, by incubation for 1 h at 37 °C. Afterwards, cells were washed and incubated for 1 h at 37 °C with Texas red-labelled goat anti-rabbit antibodies (Molecular Probes) diluted 1 : 100 in PBS. After washing, FIPV was stained with anti-FIPV-FITC. Coverslips were mounted onto microscope slides and analysed by confocal microscopy.

Infection inhibition assay

At 56 h post-seeding, cells were washed with RPMI 1640 and pre-incubated for 30 minutes at 37 °C with inhibitory compounds (wortmannin, latrunculin B, chlorpromazine, amantadine, nystatin, genistein, dip and toxin B) dissolved in RPMI 1640. Then, cells were inoculated with non-biotinylated FIPV (m.o.i. of 1 except for dip and the corresponding control: m.o.i. of 0.1 of FIPV grown in serum-free medium) in the presence of the inhibitors in RPMI and incubated at 37 °C. After 1 h the inoculum was washed off and cells were treated with a trypsin/EDTA (0.25 %/0.02 % in RPMI) solution for 5 minutes at 37 °C to remove bound, non-internalized virus particles from the plasma membrane. Figure 1 shows the effective removal of non-internalized virus particles by this method. Thereafter, cells were extensively washed with RPMI 1640 and medium was added. After 11 h of incubation at 37 °C, cells were washed, fixed and permeabilized. Permeabilization was followed by 1 h of incubation at 37 °C with anti-FIPV-FITC and 10 minutes with Hoechst 33342 (Molecular Probes). Coverslips were mounted onto microscope slides and analysed by confocal microscopy. Cells with cytoplasmic expression of viral proteins were scored as infected cells. All cells on the coverslips were evaluated.

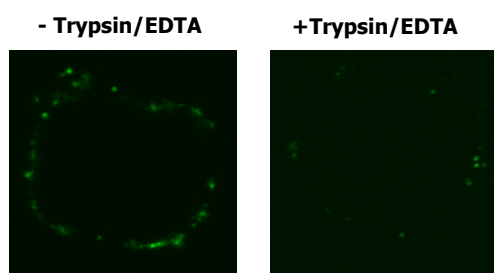


Figure 1: Confocal images of single sections through monocytes. Green signals represent attached FIPV virions without and with trypsin/EDTA treatment.

Microscopy and statistics

Internalization and infection assays were analysed by a DM IRB inverted microscope (Leica Microsystems). Images of internalization assays were obtained with a Leica TCS SP2 laser scanning spectral confocal system linked to a DM IRB inverted microscope (Leica). Argon

and He/Ne lasers were used for exciting FITC and Texas red fluorochromes, respectively. Leica confocal software was used for image acquisition.

Triplicate assays were performed and compared using the Mann-Whitney U test from the SPSS software package (version 12.0, SPSS). $P \leq 0.05$ were considered significantly different.

RESULTS

FIPV entry does not occur via phagocytosis nor macropinocytosis

Wortmannin and latrunculin B interfere with phagocytosis and macropinocytosis through their action on phosphatidyl-inositol-3 kinases and actin respectively (Spector *et al.*, 1983; Araki *et al.*, 1996). The influence of these compounds on the internalization of FIPV in monocytes was studied. Wortmannin and latrunculin B reduced the uptake of control ligands (fluorescent beads) significantly to 36.6 ± 4.0 % and 17.2 ± 3.8 %, respectively, relative to the untreated controls. The internalization of FIPV was unaffected: 104.7 ± 9.9 % for wortmannin and 100.1 ± 9.0 % for latrunculin B relative to the untreated controls (Fig. 2).

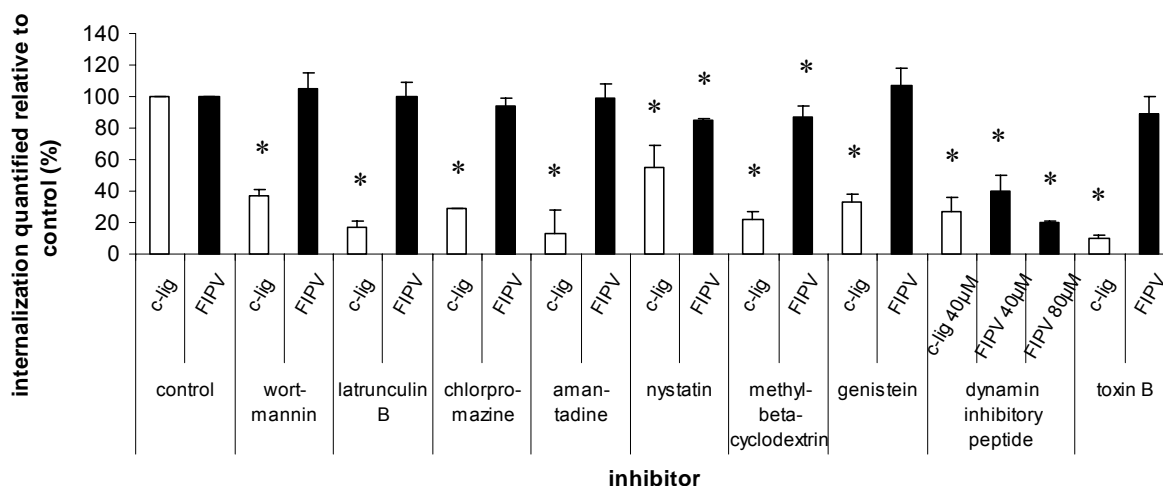


Figure 2: The effect of various inhibitors on the internalization of control ligands and biotinylated FIPV in monocytes. Cells were pre-treated with the inhibitors for 30 minutes at 37°C. Respective control ligands ('c-lig') or FIPV were added to the cells and subsequently incubated with the cells in the presence of the inhibitor for 1h at 37°C. After fixation and staining, internalization was quantified. Percentages shown are relative to internalization of control ligands or FIPV without inhibitor (control). Data represent means \pm standard deviations of triplicate assays. * marks values that are significantly different from the control.

FIPV entry is mediated by a clathrin-independent pathway

The internalization of several ligands into the cell occurs through clathrin-coated pits. To determine whether FIPV enters monocytes through this pathway, cells were treated with chlorpromazine and amantadine, both are potent inhibitors of clathrin-mediated internalization (Phonphok & Rosenthal, 1991; Wang *et al.*, 1993). Transferrin uptake occurs constitutively via clathrin-mediated endocytosis, and was therefore used as a control (Harding *et al.*, 1983). After treatment of monocytes with chlorpromazine and amantadine, transferrin uptake was reduced significantly to 28.9 ± 0.4 % and 13.0 ± 15.4 % respectively, of the control. The internalization of FIPV, however, remained at the same level (94.0 ± 5.0 % and 99.3 ± 9.2 % of the control for chlorpromazine and amantadine, respectively), despite the presence of inhibitors (Fig. 2).

Secondly, monocytes were transduced with a control construct (D3Δ2) or with DN eps15 (DIII). Eps15 protein is crucial for clathrin-mediated endocytosis (Benmerah *et al.*, 1998). To assess the effectiveness of the constructs, internalization of transferrin was studied in transduced cells: in cells with D3Δ2, the uptake was 103.3 ± 21.7 % relative to the uptake in untransduced control cells; in DIII transduced cells, the uptake was 47.9 ± 8.5 % relative to the control. Thus, the uptake of transferrin was reduced significantly relative to the uptake in D3Δ2 transduced cells. The internalization of FIPV showed no reduction: in cells with D3Δ2, the uptake was 98.3 ± 0.7 % relative to the uptake in untransduced control cells; in DIII transduced cells, the uptake was 90.3 ± 10.6 % relative to the control. Thus, transduction with dominant negative eps15 did not influence the cell's ability to internalize FIPV (Fig. 3, 1A-B).

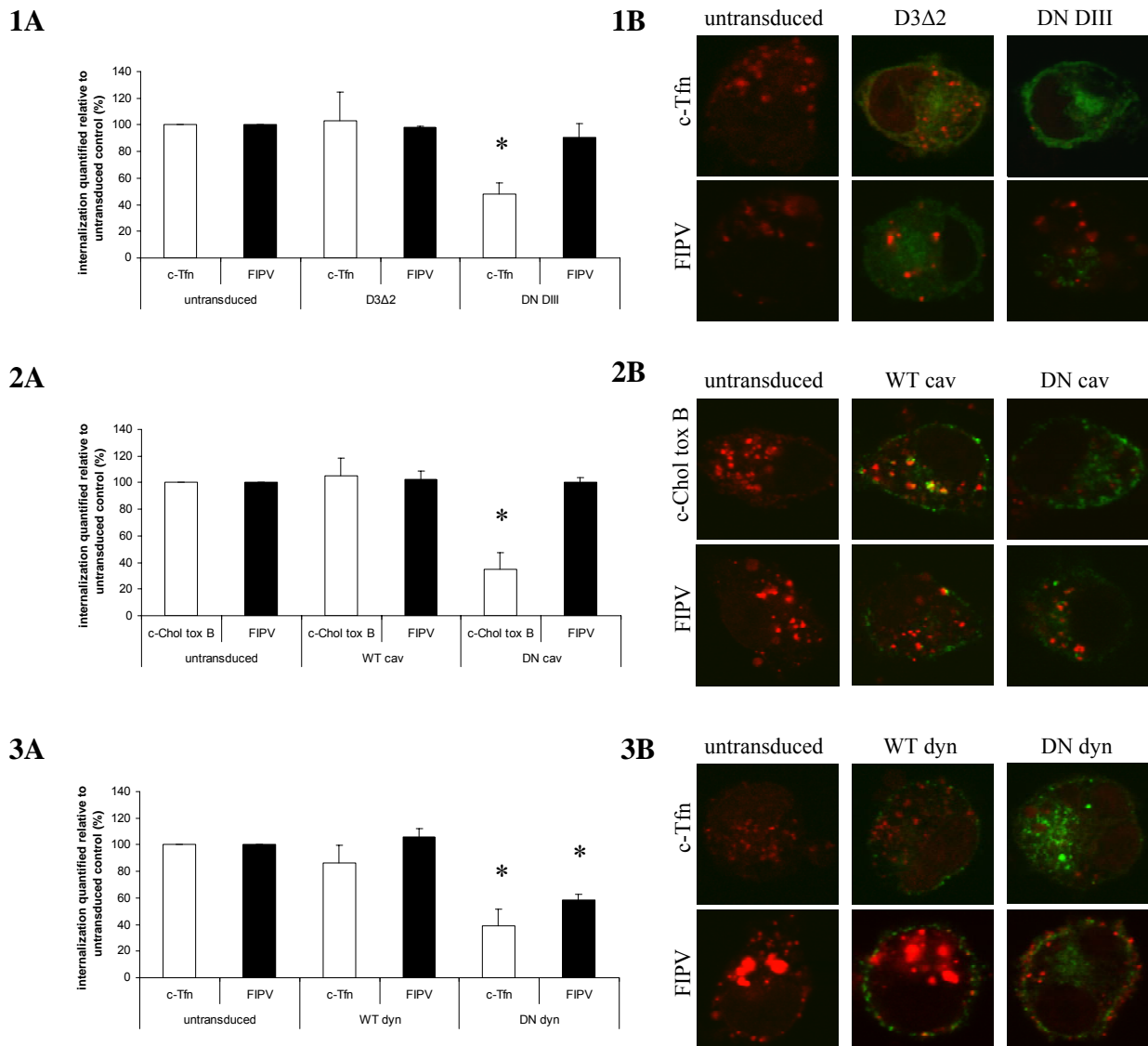


Figure 3: Internalization of control ligands and biotinylated FIPV in transduced monocytes. After transduction, cells were incubated with control ligands or FIPV for 1h at 37°C. Finally, cells were fixed, permeabilized and stained. The results depicted in panel 1 were obtained in untransduced cells and in cells transduced with constructs D3Δ2 and DIII, for non-functional and functional eps15. The results depicted in panel 2 were obtained in untransduced cells and in cells transduced with wild type (WT) and dominant-negative (DN) constructs for caveolin-1 ('cav'). The results depicted in panel 3 were obtained in untransduced cells and in cells transduced with WT and DN constructs for dynamin 2(aa) ('dyn'). Panels A show the graphs in which the quantified internalization for both control ligands (transferrin 'c-Tfn' and Cholera toxin B 'c-Chol tox B') and FIPV is represented by percentages relative to internalization in untransduced cells. Panels B represent confocal images of monocytes. Single optical sections through the cell are depicted. The green signal indicates expression of the transferred gene and the red signal represents control ligands or FIPV inside the cell. * marks values that are significantly different from the control.

Finally, no co-localization between clathrin molecules and FIPV proteins was detected between 0 and 45 minutes after the start of virus uptake (Fig. 4). Taken together, the experiments clearly show that clathrin is not involved in the internalization of FIPV in monocytes.

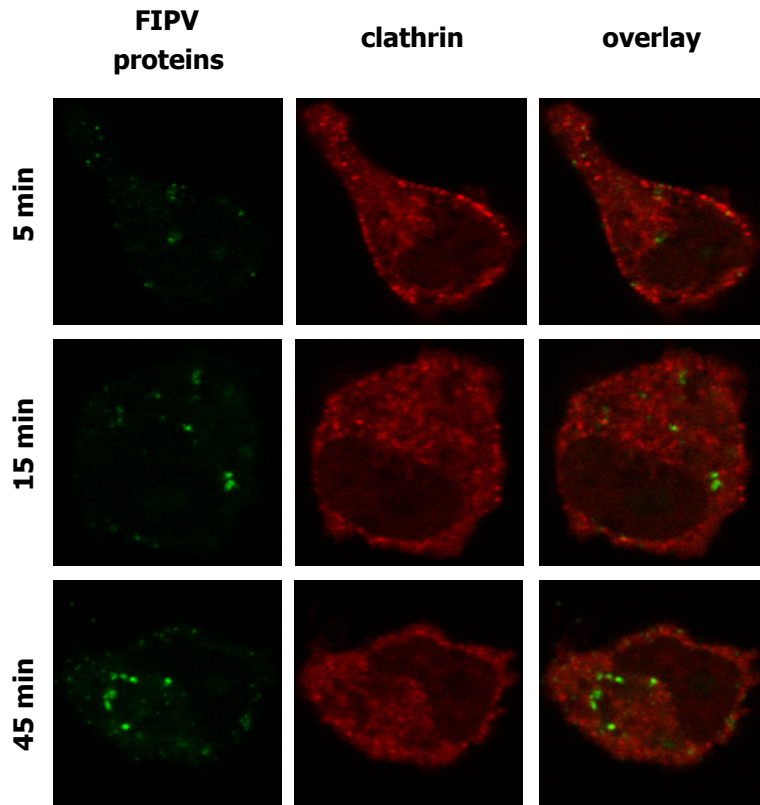


Figure 4: Visualization of FIPV and clathrin during internalization. Confocal images of single sections through cells are shown. Green signals represent FIPV stained with anti-FIPV-FITC and red signals represent clathrin stained by anti-clathrin heavy-chain IgM antibodies, biotin-labelled goat anti-mouse IgM antibodies and streptavidin-Texas red.

FIPV entry is mediated by a caveolae-independent pathway

Another well characterized pathway is caveolae-mediated endocytosis. Like lipid rafts, caveolae are rich in cholesterol and sphingolipids (Brown & London, 1998; Simons & Toomre, 2000). Therefore, sterol-binding drugs, like nystatin and methyl- β -cyclodextrin, disrupt the caveolar function (Rothberg *et al.*, 1992). Genistein blocks caveolae-mediated internalization through inhibition of protein tyrosine kinases. The effect of these drugs on FIPV internalization was investigated. Albumin is internalized through caveolae and was used as a control ligand (Schnitzer *et al.*, 1994). Nystatin and methyl- β -cyclodextrin reduced the uptake of albumin to 54.9 ± 14.5 % and 22.2 ± 4.4 %, respectively, of the control. The uptake of FIPV was reduced slightly to 85.0 ± 1.4 % and 87.4 ± 6.1 %, respectively, of the

control (Fig. 2). Genistein reduced internalization of albumin significantly to 32.6 ± 5.3 % of the control. However, internalization of FIPV was unaffected (107.1 ± 10.7 % of the control) (Fig. 2). Thus, despite the slight effect of sterol-binding drugs, caveolae are probably not involved in FIPV entry as it is not affected by genistein.

Another experiment was performed to confirm that caveolae do not play a role in the internalization of FIPV. The most important protein compounds of the caveolae are caveolins. The presence of DN caveolin-1 directly inhibits the endocytic process through caveolae (Pelkmans *et al.*, 2001; Pelkmans & Helenius, 2002). Monocytes were transduced with either WT or DN caveolin-1. To confirm the effectiveness, the uptake of Cholera toxin B was studied in transduced cells (Montesano, *et al.*, 1982; Parton *et al.*, 1994): in cells with WT caveolin-1, the uptake was 105.1 ± 3.2 % relative to the uptake in untransduced control cells and in cells with DN caveolin-1, the uptake was 34.8 ± 7.8 % relative to the uptake in untransduced control cells. For FIPV, no reduction was observed: in cells with WT caveolin-1, FIPV uptake was 102.1 ± 3.4 % and in cells with DN caveolin-1, FIPV uptake remained at 100.1 ± 7.1 % relative to the untransduced control (Fig. 3, 2A-B). These results indicate that caveolin-1 is not involved in FIPV entry.

Further, most FIPV protein clusters (93.1 ± 4.1 %) did not co-localize with caveolin-1 between 0 and 45 minutes after the start of virus uptake (Fig. 5). Only occasionally, particles were found in the proximity of caveolin-1. A co-localization study between caveolin-1 and an irrelevant protein (transferrin) showed that the observed level of co-localization does not exceed the expected level for coincidental co-localization (data not shown).

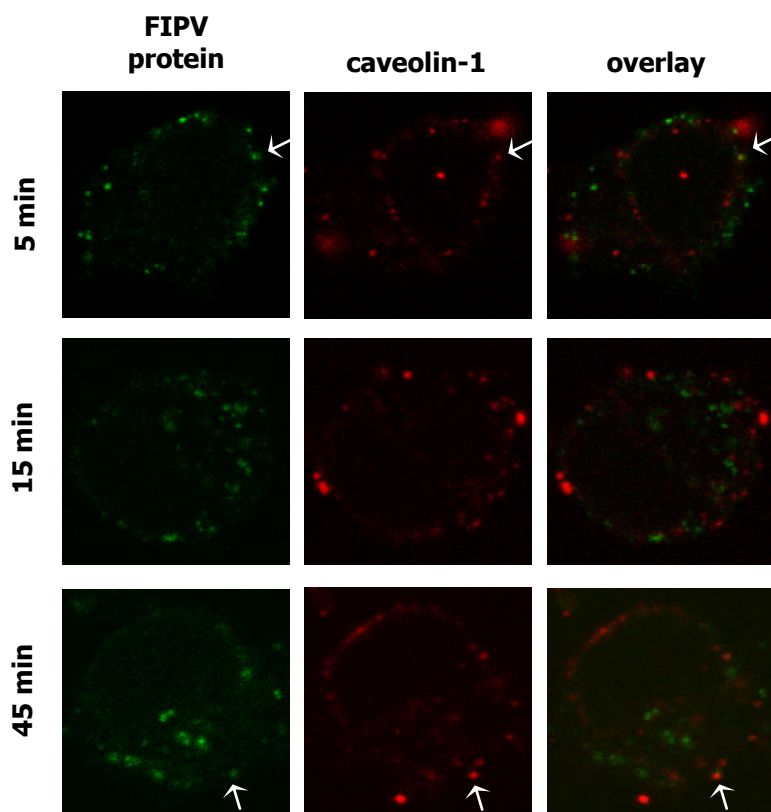


Figure 5: Visualization of FIPV and caveolin-1 during internalization. Confocal images of single sections through cells are shown. Green signals represent FIPV stained with anti-FIPV-FITC and red signals represent caveolin-1 stained by rabbit polyclonal anti-caveolin-1 antibodies and goat anti-rabbit-Texas red. The arrows indicate the few sites of co-localization. In the first picture (5min) FIPV and caveolin-1 completely co-localize, in the last picture (45min) they only partly co-localize.

Thus, both clathrin- and caveolae-mediated pathways are not involved in FIPV entry in monocytes. The sterol-binding inhibitors, nystatin and methyl- β -cyclodextrin, slightly influenced the uptake of FIPV in monocytes.

FIPV entry depends on dynamin

Dynamin is a GTPase that is involved in many internalization pathways: phagocytosis, clathrin-mediated internalization, caveolae-mediated internalization and some clathrin- and caveolae-independent pathways (Gold *et al.*, 1999; Hinshaw, 2000; Mayor & Pagano, 2007). Dynamin is thought to pinch off formed vesicles from the plasma membrane (Hinshaw, 2000). Cells were treated with dynamin inhibitory peptide (dip) and analysed for internalization of the control ligand transferrin and FIPV (Grabs *et al.*, 1997). Dip (40 μ M) reduced transferrin uptake significantly to 27.4 ± 8.4 % relative to the uptake in untreated cells. For FIPV, a strong, significant reduction was observed to 40.1 ± 9.6 % and 20.3 ± 1.1 % of the uptake in control cells for 40 and 80 μ M, respectively, of dynamin inhibitor (Fig.2).

To confirm these results, the uptake of FIPV was studied in cells transduced with WT and DN dynamin 2(aa) (Cao *et al.*, 1998; 2000). The effectiveness of transduction was studied by monitoring the uptake of transferrin in transduced cells: in cells with WT dynamin 2(aa), the uptake was 86.5 ± 13.4 % relative to the uptake in untransduced control cells and in cells with DN dynamin 2(aa), the uptake was 39.0 ± 12.2 % relative to the uptake in untransduced control cells. Internalization of FIPV was reduced significantly in cells expressing DN dynamin 2(aa), compared with cells expressing WT dynamin 2(aa): in cells with WT dynamin 2(aa), FIPV uptake was 105.6 ± 6.3 % and in cells with DN dynamin 2(aa), FIPV uptake was 58.7 ± 3.9 % relative to the uptake in untransduced control cells (Fig. 3, 3A-B). Clearly, dynamin plays an important role in the internalization of FIPV in monocytes.

Rho GTPases are not involved in FIPV entry

Several independent internalization pathways have been characterized by their dependency on Rho GTPases (Mayor & Pagano, 2007). Rho GTPases are a subfamily of the Ras superfamily of small GTPases. They play an important role in regulating the actin cytoskeleton and in a broad range of aspects of endocytic traffic (Hall, 1998; Ellis & Mellor, 2000). Rho GTPases are involved in phagocytosis and macropinocytosis and also in clathrin- and caveolae-mediated internalization pathways (Ellis & Mellor, 2000; Grimmer *et al.*, 2002). To determine whether they are involved in the internalization of FIPV in monocytes, cells were treated with *Clostridium difficile* toxin B, a general Rho GTPase inhibitor (Just *et al.*, 1995). The activity of the inhibitor was confirmed by its effect on the internalization of fluorescent beads: a significant reduction to 10.0 ± 2.2 % of the uptake in untreated cells (Fig. 2). The internalization of FIPV was not significantly affected: internalization remained at the level of 89.2 ± 11.1 % of the control. These results were confirmed by less general Rho GTPase inhibitors like Rac1 inhibitor (Calbiochem), secramine A [inhibits Cdc42 activation (Pelish *et al.*, 2006)] and Y-27632 (selective inhibitor of the Rho associated protein kinase ROCK; Sigma-Aldrich) (data not shown).

The effect of entry inhibitors on FIPV infection

After establishing the effect of inhibitors on the uptake of virus, the effect on infection was studied. Therefore, cells were inoculated and incubated with FIPV in absence and in presence of entry inhibitors. Bound, non-internalized virions were removed from the cell surface by trypsin wash. Viral replication was stopped after 1 cycle of replication, i.e. at 12 h post inoculation. Figure 6 shows that the inhibitors that did not significantly influence FIPV entry, had no effect on infection rates. The actual influence of the inhibitors on percentages

of infected cells were from 2.1 to 1.9 % for wortmannin, 1.9 to 1.7 % for latrunculin B, 2.1 to 2.1 % for chlorpromazine, 1.9 to 2.3 % for amantadine, 1.9 to 1.9 % for genistein and 1.9 to 2.4 % for toxin B. However, inhibition of FIPV internalization by dynamin inhibitory peptide is reflected in significantly reduced infection (45.7 ± 10.3 % and 23.0 ± 9.5 % of infection of control cells for 40 and 80 μM , respectively, of dynamin inhibitor or reductions from 0.48 to 0.22 % and 0.32 to 0.065 % infected cells). Nystatin treatment during entry led to a reduction to 61.7 ± 5.2 % of infection of control cells or absolutely from 1.9 to 1.2 % infected cells.

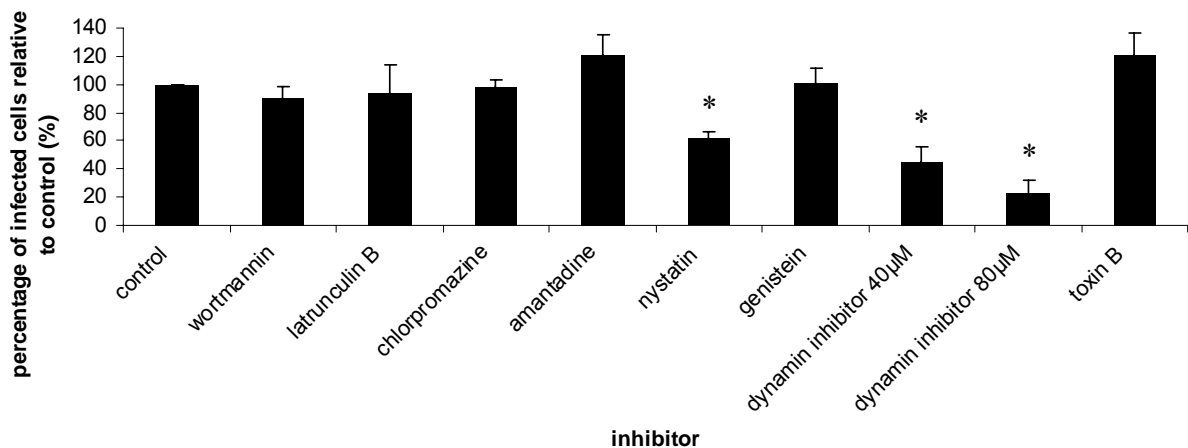


Figure 6: The effect of various entry inhibitors on infection of monocytes with FIPV. Cells were pre-treated with the inhibitors for 30 minutes at 37°C. FIPV was added to the cells in the absence ('control') or presence of inhibitor and subsequently incubated with the cells for 1h at 37°C. After trypsin wash, cells were further incubated for 11h. Then, cells were fixed and stained and infection was quantified. Percentages shown are percentages of infected cells in the presence of a certain inhibitor, relative to the percentage of infected 'control' cells. Data represent means \pm standard deviations of triplicate assays. * marks values that are significantly different from the control.

DISCUSSION

Previously, we showed that FIPV enters its target cell via endocytosis and not via direct fusion with the plasma membrane (Van Hamme *et al.*, 2007). In the present study, the mechanism of endocytosis involved in FIPV entry was determined. Multiple strategies were combined: internalization pathways were blocked by use of chemical inhibitors and expression of DN mutants, and co-localization assays were performed.

FIPV is not phagocytosed nor macropinocytosed as the inhibitors wortmannin and latrunculin B did not affect the entry and Rho GTPases were not involved in the internalization. Independency from clathrin was proven based on the facts that inhibition with chlorpromazine and amantadine was ineffective and that expression of DN eps15 had no effect on FIPV internalization. FIPV and clathrin did not co-localize either. Further, it was shown that caveolae are not involved in FIPV internalization as genistein and expression of DN caveolin-1 had no effect on FIPV internalization, and FIPV and caveolin-1 did not co-localize inside the cell. In conclusion, FIPV enters monocytes through a clathrin- and caveolae-independent pathway. This pathway is further characterized by its dependency on dynamin and independency from Rho GTPases.

Further, the sterol-binding drugs nystatin and methyl- β -cyclodextrin slightly affected FIPV internalization. Cholesterol depletion affects internalization pathways associated with lipid rafts. The association with rafts can be crucial for initiating and proceeding signalling cascades necessary for internalization. However, requirement for cholesterol is not necessarily related to lipid rafts. For example, cholesterol depletion disturbs clathrin-mediated internalization, which is not associated with lipid rafts (Subtil *et al.*, 1999; Nichols & Lippincott-Schwartz, 2001). As the observed decrease in internalization of FIPV is much smaller than the decrease in internalization of the control ligand and as the observed reduction due to methyl- β -cyclodextrin is smaller than those described in literature (ranging from 30 to >90 % for different pathways) (Rodal *et al.*, 1999; Subtil *et al.*, 1999; Grimmer *et al.*, 2002; Sanchez-San Martin *et al.*, 2004; Barrias *et al.*, 2007), it seems likely that the pathway is not dependent on cholesterol. Possibly, a fraction of the virus binding receptor is present in lipid rafts. Disturbance of rafts could then cause a decrease in receptor availability. Reduced virus binding subsequently leads to a drop in the number of internalized virions. Further research is needed to confirm the effect of sterol-binding drugs and to enlighten the underlying cause.

To our knowledge, no identical physiological internalization pathway has been described. However, the characteristics of the internalization of the β -chain of interleukin 2 receptors (IL-2R- β) are very similar to those of FIPV internalization (Lamaze *et al.*, 2001). Like FIPV, IL-2R- β is internalized via a clathrin- and caveolae-independent pathway and is dependent on dynamin and sensitive to cholesterol depletion. The sensitivity to cholesterol depletion is caused by raft-association of the receptor. A clear difference with the uptake of FIPV, is the dependency on Rho GTPases. The internalization of the common cytokine receptor γ (γ c) is categorized in the same group as the IL-2R- β -pathway (Kirkham & Parton, 2005; Sauvonnnet *et al.*, 2005). The γ c receptor is a subunit shared by several cytokine receptors, namely the

IL-2, -4, -7, 9, -15, and -21 receptors (Schluns & Lefrancois, 2003). When expressed by itself, the γ c receptor is rapidly and efficiently endocytosed (Morelon & Dautry-Varsat, 1998). Clathrin- and caveolae-independent pathways have also been linked to the entry of many viruses (Marsh & Helenius, 1989; Sieczkarski & Whittaker, 2002a; Sanchez-San Martin *et al.*, 2004). Like FIPV, SARS virus is internalized through a clathrin- and caveolae-independent pathway that is sensitive to treatment with methyl- β -cyclodextrin (Wang *et al.*, 2008). The involvement of dynamin and Rho GTPases has however not been studied. Rotavirus cell entry resembles FIPV entry as it is also internalized via a clathrin- and caveolae-independent pathway that depends on dynamin, but the pathway is highly cholesterol depletion sensitive. The role of Rho GTPases in this endocytic process has not been studied either. In addition, there are similarities between the entry pathways used by influenza virus and FIPV. Influenza virus enters HeLa cells via a clathrin- and caveolae-independent pathway that is not associated with lipid rafts. It is not known whether this pathway depends on dynamin (Sieczkarski & Whittaker, 2002b). Influenza virus entry in Mv-1 lung cells has been shown to depend on dynamin but further characterization has not been performed (Roy *et al.*, 2000). As the route of entry depends on the host cell type, it is impossible to predict whether influenza virus entry in HeLa cells will also depend on dynamin. Thus, for both FIPV and influenza virus, the internalization pathways need further characterization to compare them. HCoV-229E belongs to the same phylogenetic group as FIPV and is the only member of the group of which the internalization process has been characterized. It was shown that HCoV-229E binds to its receptor, human aminopeptidase N (APN), in rafts and enters human fibroblasts through caveolae (Nomura *et al.*, 2004). Human APN has been reported to be a component of rafts in various cell types (Danielsen, 1995; Santos *et al.*, 2000; Riemann *et al.*, 2001; Nomura *et al.*, 2004). For TGEV, that is endocytosed after binding to porcine APN, it has been suggested that clathrin might be involved in the internalization (Hansen *et al.*, 1998). For type II FIPV, feline APN is the receptor for entry in cell lines. Whether this is also a receptor in primary target cells is not known. Even if APN would be the internalizing receptor, no conclusions can be made based on the entry mechanisms of HCoV and TGEV as these coronaviruses already use two different mechanisms of internalization through APN.

Despite the low number of infected cells, which is an inevitable obstacle for FIPV research in target cells, infection assays in the presence of entry inhibitors qualitatively confirmed the results from the internalization assays and show that virions infect their host cell via the described pathway. Inhibition of dynamin function and nystatin treatment reduced infection. The reduction in infection caused by nystatin is larger than expected based on the reduction

of internalization. Possibly, cholesterol binding by nystatin affects post-entry events, e.g. intracellular transport, despite the trypsin wash.

In this study, the mechanism of internalization for FIPV 79-1146 in monocytes was determined. The pathway is clathrin- and caveolae-independent, strongly depends on dynamin and is slightly cholesterol depletion sensitive. The identified pathway proceeds similarly, though not exactly like IL-2R- β endocytosis and SARS- and rotavirus entry. Gaining insights in the initial virus-cell interactions is valuable in the search for an effective treatment or prevention of FIP. Obviously, the pathway should be further characterized and its components should be identified in order to obtain well-defined targets for antiviral therapy.

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REFERENCES

- Araki, N., Johnson, M. T. & Swanson, J. A. (1996).** A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* **135**, 1249-1260.
- Barrias, E. S., Dutra, J. M. F., De Souza, W. & Carvalho, T. M. U. (2007).** Participation of macrophage membrane rafts in *Trypanosoma cruzi* invasion process. *Biochem Biophys Res Commun* **363**, 828-834.
- Benmerah, A., Lamaze, C., Bègue, B., Schmid, S. L., Dautry-Varsat, A. & Cerf-Bensussan, N. (1998).** AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J Cell Biol* **140**, 1055-1062.
- Brodsky, F. M., Chen, C., Knuehl, C., Towler, M. C. & Wakeham, D. E. (2001).** Biological basket weaving: Formation and function of clathrin-coated vesicles. *Annu Rev Cell Dev Biol* **17**, 517-568.
- Brown, D. A., & London, E. (1998).** Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* **14**, 111-136.
- Cao, H., F. Garcia, & McNiven, M. (1998).** Differential distribution of dynamin isoforms in mammalian cells. *Mol Biol Cell* **9**, 2595-2609.
- Cao, H., H. Thompson, E. Krueger, & McNiven, M. (2000).** Disruption of golgi structure and function in mammalian cells expressing a mutant dynamin. *J Cell Sci* **113**, 1993-2002.
- Choi, K. S., H. Aizaki, & Lai, M. M. C. (2005).** Murine coronavirus requires lipid rafts for virus entry and cell-cell fusion but not for virus release. *J Virol* **79**, 9862-9871.
- Conner, S. D., & Schmid, L. S. (2003).** Regulated portals of entry into the cell. *Nature* **422**, 37-44.
- Danielsen, E. M. (1995).** Involvement of detergent-insoluble complexes in the intracellular transport of intestinal brush border enzymes. *Biochemistry* **34**, 1596-1605.
- Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2005).** Replication of feline coronaviruses in peripheral blood monocytes. *Arch Virol* **150**, 2483-2500.

Eifart, P., Ludwig, K., Böttcher, C., de Haan, C. A. M., Rottier, P. J. M., Korte, T. & Herrmann, A. (2007). The role of endocytosis and low pH in cell entry of the murine hepatitis virus MHV-A59. *J Virol* **81**, 10758-10768.

Ellis, S. & Mellor, H. (2000). Regulation of endocytic traffic by Rho family GTPases. *Trends Cell Biol* **10**, 85-88.

Gold, E. S., Underhill, D. M., Morrissette, N. S., Guo, J., McNiven, M. A. & Aderem, A. (1999). Dynamin 2 is required for phagocytosis in macrophages. *J Exp Med* **12**, 1849-1856.

Grabs, D., Slepnev, V. I., Songyang, Z., David, C., Lynch, M., Cantley, L. C. & De Camilli, P. (1997). The SH3 domain of amphiphysin binds the proline-rich domain of dynamin at a single site that defines a new SH3 binding consensus sequence. *J Biol Chem* **20**, 13419-13425.

Grimmer, S., van Deurs, B. & Sandvig, K. (2002). Membrane ruffling and macropinocytosis in A431 cells require cholesterol. *J Cell Sci* **115**, 2953-2962.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.

Hall, A., & C. D. Nobes. (2000). Rho GTPases: Molecular switches that control the organization and dynamics of the actin cytoskeleton. *Phil Trans R Soc Lond B* **355**, 965-970.

Hansen, G. H., Delmas, B., Besnardeau, L., Vogel, L. K., Laude, H., Sjöström, H. & Norèn, O. (1998). The coronavirus transmissible gastroenteritis virus causes infection after receptor-mediated endocytosis and acid-dependent fusion with an intracellular compartment. *J Virol* **72**, 527-534.

Harding, C., Heuser, J. & Stahl, P. (1983). Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* **97**, 329-339.

Hinshaw, J. E. (2000). Dynamin and its role in membrane fission. *Annu Rev Cell Dev Biol* **16**, 483-519.

Inoue, Y., Tanaka, N., Tanaka, Y., Inoue, S., Morita, K., Zhuang, M., Hattori, T. & Sugamura, K. (2007). Clathrin-dependent entry of severe acute respiratory syndrome coronavirus into target cells expressing ACE2 with the cytoplasmic tail detected. *J Virol* **81**, 8722-8729.

Just, I., Selzer, J., Wilm, M., Von-Eichel-Streiber, C., Mann, M. & Aktories, K. (1995). Glucosylation of rho proteins by *Clostridium difficile* toxin B. *Nature* **375**, 500-503.

Kim, O. J., Lee, D. H. & Lee, C. H. (2006). Close relationship between SARS-coronavirus and group 2 coronaviruses. *J Microbiol* **44(1)**, 83-91.

Kirkham, M., & Parton, R. G. (2005). Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim Biophys Acta* **1746(3)**, 349-363.

Kurzchalia, T., Dupree, P., Parton, R. G., Kellner, R., Virta, H., Lehnert, M. & Simmons, K. (1992). VIP21, a 21kD membrane protein is an integral component of trans-golgi-derived transport vesicles. *J Cell Biol* **118**, 1003-1014.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A. & Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol Cell* **7**, 661-671.

Marsh, M. & Helenius, A. (1989). Virus entry into animal cells. *Adv Virus Res* **36**, 107-151.

Mayor, S. & Pagano, R. E. (2007). Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol* **8(8)**, 603-612.

McKeirnan, A. J., Evermann, J. F., Hargis, A., Miller, L. M. & Ott, R. L. (1981). Isolation of feline coronaviruses from two cats with diverse disease manifestations. *Feline Pract* **11**, 16-20.

Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H. M. & Nauwynck, H. J. (2005). Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. *J Gen Virol* **86**, 2057-2068.

Montesano, R., Roth, J., Robert, A. & Orci, L. (1982). Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. *Nature* **296**, 651-653.

Morelon, E. & Dautry-Varsat, A. (1998). Endocytosis of the common cytokine receptor γ c chain: identification of sequences involved in internalization and degradation. *J Biol Chem* **273**, 22044-22051.

Nichols, B. J. & Lippincott-Schwartz, J. (2001). Endocytosis without clathrin coats. *Trends Cell Biol* **11**, 406-412.

Nomura, R., Kiyota, A., Suzaki, E., Kataoka, K., Ohe, Y., Miyamoto, K., Senda, T. & Fujimoto, T. (2004). Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. *J Virol* **78**, 8701-8708.

Parton, R. G., Joggerst, B. & Simons, K. (1994). Regulated internalization of caveolae. *J Cell Biol* **127**, 1199-1215.

Pelish, H. E., Peterson, J. R., Salvarezza, S. B., Rodriguez-Boulan, E., Chen, J., Starnes, M., Macia, E., Feng, Y., Shair, M. D. & Kirchhausen, T. (2006). Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation *in vitro*. *Nat Chem Biol* **2**, 39-46.

Pelkmans, L. & Helenius, A. (2002). Endocytosis via Caveolae. *Traffic* **3**, 311-320.

Pelkmans, L. & Helenius, A. (2003). Insider information: what viruses tell us about endocytosis. *Curr Opin Cell Biol* **15**, 414-422.

Pelkmans, L., Kartenbeck, J. & Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol* **3**, 473-483.

Phonphok, Y. & Rosenthal, K. S. (1991). Stabilization of clathrin coated vesicles by amantadine, tromantadine and other hydrophobic amines. *FEBS Lett.* **281**, 188-190.

Racoosin, E. L. & Swanson, J. A. (1994). Labeling of endocytic vesicles using fluorescent probes for fluid-phase endocytosis, p.375-380. *In* J. E. Celis (ed.), *Cell Biology: A Laboratory Handbook* (vol.2). Academic Press, New York.

Riemann, D., Hansen, G. H., Niels-Christiansen, L., Thorsen, E., Immerdal, L., Santos, A. N., Kehlen, A., Langner, J. & Danielsen, E. M. (2001). Caveolae/lipid rafts in fibroblast-like synoviocytes: ectopeptidase-rich membrane microdomains. *Biochem J* **354**, 47-55.

Rodal, S. K., Skretting, G., Garred, Ø., Vilhardt, F., van Deurs, B. & Sandvig, K. (1999). Extraction of cholesterol with methyl- β -cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell* **10**, 961-974.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R. & Anderson, R. G. (1992). Caveolin, a protein compound of caveolae membrane coats. *Cell* **68**, 673-682.

Roy, A.M., Parker, J. S., Parrish, C. R. & Whittaker, G. R. (2000). Early stages of influenza virus entry into Mv-1 lung cells: involvement of dynamin. *Virology* **267**, 17-28.

Sanchez-San Martin, C., Lopez, T., Arias, C. F. & Lopez, S. (2004). Characterization of rotavirus cell entry. *J Virol* **78(5)**, 2310-2318.

Santos, A. N., J. Langner, M. Hermann, & D. Riemann, (2000). Aminopeptidase N/CD13 is directly linked to signal transduction pathways in monocytes. *Cell Immunol* **201**, 22-32.

Sauvonnet, N., Dujeancourt, A. & Dautry-Varsat, A. (2005). Cortactin and dynamin are required for the clathrin-independent endocytosis of γ c cytokine receptor. *J Cell Biol* **168**, 155-163.

Savarino, A., Boelaert, J. R., Cassone, A., Majori, G. & Cauda, R. (2003). Effects of chloroquine on viral infections: an old drug against today's diseases? *Infect Dis* **3**, 722-727.

Schluns, K. S. & Lefrancois, L. (2003). Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* **3**, 269-279.

Schnitzer, J. E., Oh, P., Pinney, E. & Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol* **127**, 1217-1232.

Sieczkarski, S. B. & Whittaker, G. R. (2002a). Dissecting virus entry via endocytosis. *J Gen Virol* **83**, 1535-1545.

Sieczkarski, S. B. & Whittaker, G. R. (2002b). Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. *J Virol* **76**, 10455-10464.

Simons, K. & Toomre, D. (2000). Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31-39.

Spector, I., Shochet, N. R., Kashman, A. & Groweiss, Y. (1983). Latrunculins—novel marine toxins that disrupt microfilament organization in cultured cells. *Science* **219**, 493-495.

Stove, V., Van de Walle, I., Naessens, E., Coene, E., Stove, C., Plum, J. & Verhasselt, B. (2005). Human immunodeficiency virus Nef induces rapid internalization of the T-cell coreceptor CD8 $\alpha\beta$. *J Virol* **79**, 11422-11433.

Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H. & McGraw, T. E. (1999). Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci* **96**, 6775-6780.

Swanson, J. A. & Watts, C. (1995). Macropinocytosis. *Trends Cell Biol* **5**, 424-428.

Thorp, E. B. & Gallagher, T. M. (2004). Requirements for CEACAMs and cholesterol during murine coronavirus cell entry. *J Virol* **78**, 2682-2692.

Van de Walle, G. R., Favoreel, H. W., Nauwynck, H. J., van Oostveldt, P. & Pensaert, M. B. (2001). Involvement of cellular cytoskeleton components in antibody-induced internalization of viral glycoproteins in pseudorabies virus-infected monocytes. *Virology* **288**, 129-138.

Van Hamme, E., Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2007). Attachment and internalization of feline infectious peritonitis virus in feline blood monocytes and Crandell feline kidney cells. *J Gen Virol* **88**, 2527-2535.

Wang, H., Yang, P., Liu, K., Guo, F., Zhang, Y., Zhang, G. & Jiang, C. (2008). SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. *Cell Res* **18**, 290-301.

Wang, L., Rothberg, K. G. & Anderson, R. G. W. (1993). Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J Cell Biol* **123**, 1107-1117.

Yang, Z.-Y., Huang, Y., Ganesh, L., Leung, K., Kong, W.-P., Schwartz, O., Subbarao, K. & Nabel, G. J. (2004). pH-Dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by the dendritic cell transfer through DC-SIGN. *J Virol* **78**, 5642-5650.

**GENOME RELEASE AFTER ENDOCYTOSIS OF FELINE
INFECTIOUS PERITONITIS VIRUS TYPE II IN PRIMARY
MONOCYTES**

Evelien Van Hamme, Hannah L. Dewerchin, Els Cornelissen & Hans J. Nauwynck

ABSTRACT

Upon entry via endocytosis, feline infectious peritonitis virus (FIPV) particles are 'trapped' in intracellular vesicles. To initiate viral replication, transcription and translation, the virus needs to release its genome into the cytosol. This uncoating process occurs through fusion of the viral envelope with membranes of the intracellular vesicles. In general, such a process can be initiated by low pH, cleavage by proteases, conformational changes by receptor interaction or a combination of these factors. In this study, the process of serotype II FIPV uncoating was visualized in time by specifically staining nucleocapsids that have been released from intracellular vesicles into the cytosol. To perform this staining, the plasma membrane was selectively permeabilized without affecting intracellular membranes. At each time point, the percentage of cells with genome release was determined. Further, the requirement for endosomal low pH for FIPV infection was checked. The results indicate that the percentage of cells with genome release increased over time to a maximum (8.4 % of the cells) at 35 minutes after the start of internalization, and then decreased again. This release did not depend on a low pH as infection could not be blocked by lysosomotropic agents. In conclusion, despite the uptake of FIPV in practically all monocytes, release from vesicles appears to be restricted to a subpopulation. These findings suggest that the presence of uncoating mediators may be a determining factor in cell susceptibility for FIPV type II.

INTRODUCTION

Entry of enveloped viruses via endocytosis results in intact virions 'trapped' in intracellular vesicles. Most internalized vesicles travel through the endosomal/lysosomal pathway and the virus needs to escape before lysosomal degradation is completed. To escape from intracellular vesicles, the viral envelope has to fuse with the membrane of the intracellular vesicle. This process is mediated by viral fusion proteins. The viral envelope is left behind in the membrane of the intracellular vesicle while the nucleocapsid is released in the cytosol so that genome replication, transcription and translation can be initiated.

Based on structural similarities, there are two classes of viral fusion proteins (Lescar *et al.*, 2001). Class I viral proteins contain 4,3 hydrophobic (heptad) repeat (HR) regions and an N-terminal or N-proximal fusion peptide. Class II viral fusion proteins lack HR regions and have an internal fusion peptide. The fusion peptide is the actual mediator of fusion. The initiation of membrane fusion occurs upon induction of conformational changes in the viral fusion protein. These changes bring HR regions in class I fusion proteins in a conformation that inserts the fusion peptide into the cell membrane during the fusion event. As a result, the cellular and viral membrane are brought closely together for the actual fusion event (Eckert & Kim, 2001).

The viral spike protein is the main protein involved in coronavirus entry. This protein was extensively studied for the group 2 coronavirus, mouse hepatitis virus (MHV). The fusion core complex was structurally and functionally characterized and the data indicated that this coronavirus spike protein is a class I virus fusion protein (Bosch *et al.*, 2003). Sequence alignments of HR regions in spike proteins among coronaviruses, including feline coronavirus spikes, show significant similarity, suggesting that they all are class I fusion proteins (Xu *et al.*, 2004). To bring spike proteins into a fusogenic state, conformational changes in the S-protein are required. These conformational changes can be induced in several ways (Smith & Helenius, 2004). Certain viruses require protonation and depend on the pH drop in late endosomes for the initiation of fusion activity. Dependency on low pH in endosomes can be shown by inhibiting virus entry by lysosomotropic weak bases like chloroquine or ammonium chloride. Other viruses undergo a conformational change through the interaction with a certain receptor. A third group requires cleavage by proteases to reveal the fusogenic domains. Possibly there are more, still unknown, triggers for fusion.

There are some data available on uncoating of group 1 coronaviruses. It has been suggested that canine coronavirus entry depends on low pH in endosomal organelles (Savarino *et al.*, 2003). For human coronavirus 229E, both pH drop and proteases, more specifically

cathepsin L, are necessary to mediate genome release into the cytosol (Kawase *et al.*, 2009). For the group 2a coronavirus MHV, binding of viral spikes of the strains MHV-A59 and MHV-4 to their receptor induces a conformational change into a fusogenic state (Matsuyama & Taguchi, 2002; Zelus *et al.*, 2003; Miura *et al.*, 2004). On the other hand, infection by MHV-3 is sensitive to lysosomotropic agents and therefore depends on the endosomal pH drop (Krzystyniak and Dupuy, 1984). Protease activity is not required. However, for strain MHV-2, low pH is not required but cathepsins B and L are (Qiu *et al.*, 2006). For severe acute respiratory syndrome virus, a human coronavirus belonging to group 2b, both low pH and cathepsins mediate viral entry via endocytosis (Huang *et al.*, 2006; Wang *et al.*, 2008). Recently it has been reported that sequential cleavage by trypsin at the S1/S2 boundary and inside S2 might prime the spike protein for direct fusion (Belouzard *et al.*, 2009). Avian infectious bronchitis virus, a group 3 coronavirus, undergoes a conformational change after exposure to low pH, probably inducing membrane fusion after endocytosis (Chu *et al.*, 2006).

Studies on uncoating of feline coronaviruses have resulted in many contradictory statements. Takano *et al.* claimed that the entry of feline infectious peritonitis virus (FIPV) strain 79-1146 depends on acidification of the endosome (2008), while Regan *et al.* claimed that this is not true for FIPV strains 79-1146 and DF2, but only for enteric feline coronavirus (eFCoV) strains like 79-1683 (2008). Further, Regan *et al.* stated that FCoV spikes are cleaved by cathepsins, more specifically by cathepsin B for the FIPV strains and both cathepsins B and L for the eFCoV strains (2008). However, de Haan *et al.* claimed that cathepsins are refractory for FIPV infection (2008). To provide more insights on these contradictory statements, we performed the present study. Therefore, a kinetical study was performed to situate uncoating in the entry and infection process. In addition, the effect of pH drop inhibitors on FIPV infection was investigated.

METHODS

Cells and virus

Feline blood monocytes were isolated from blood collected from feline coronavirus-, feline leukaemia virus- and feline immunodeficiency virus-negative cats and cultured as described before (Dewerchin *et al.*, 2005). FIPV serotype II strain WSU 79-1146 was kindly provided by Dr Egberink (Department of Infectious Diseases and Immunology, Utrecht, The Netherlands) and passaged on CrFK cells (McKeirnan *et al.*, 1981).

Antibodies

The monoclonal antibody (mAb) against FIPV nucleocapsid (N) protein was produced in the laboratory as described previously by Lefebvre *et al.* (2008) after immunization of Balb/c mice with FIPV serotype II strain WSU 79-1146 infected cells. Polyclonal anti-FIPV antibodies labelled with FITC were purchased from Veterinary Medical Research and Development (VMRD).

Lysosomotropic agents

The lysosomotropic weak base ammonium chloride and bafilomycin A1, a specific inhibitor of vacuolar type H⁺-ATPase (Sigma-Aldrich) were used to prevent acidification of endosomal vesicles. Ammonium chloride was used at a concentration of 5 mM and bafilomycin A1 at a concentration of 10nM.

Kinetics of uncoating

At 68 h post seeding, cells were chilled on ice and incubated with virus at 4 °C. After 2 h, when the maximal number of virions was bound to the cells as determined previously (Van Hamme *et al.*, 2007), cells were shifted to 37 °C. At different time points (after 15, 25, 35, 45, 55 and 65 minutes), cells were fixed with formaldehyde (1 %). After washing with phosphate buffered saline (PBS), cells were put at 4 °C and washed with ice-cold buffer that consisted of 110 mM KOAc, 20 mM Hepes at pH 7.2 and 2mM MgCl in ultra pure H₂O (KHM buffer). Then, the plasma membrane was permeabilized with 30 µg digitonin ml⁻¹ KHM buffer for 4 minutes without affecting intracellular membranes. After washing with PBS, released nucleocapsid proteins were stained with anti-nucleocapsid mAbs for 1 h at 37 °C. Cells were washed and permeabilized with 0.1 % Triton X-100. Finally, virus particles were stained with FITC-labelled anti-FIPV antibodies (Molecular probes).

Infection inhibition assay

At 56 h post-seeding, cells were washed with RPMI 1640 and pre-incubated for 1 h at 37 °C with pH drop inhibitors. After pre-treatment, cells were inoculated with FIPV in the presence of the inhibitors used for pre-treatment. Cells were infected at an moi of 5. Then, after 1 h the inoculum was replaced by medium supplemented with the inhibitors used for pre-treatment. The cells were incubated for another 11 h at 37 °C. Finally, cells were washed, fixed and permeabilized. Permeabilization was followed by 1 h of incubation at 37 °C with anti-FIPV polyclonal antibodies labelled with FITC and 10 minutes with Hoechst 33342 (Molecular Probes). Coverslips were mounted onto microscope slides and analyzed by

confocal microscopy. Cells with cytoplasmic expression of viral proteins were scored as infected cells. All cells on the coverslips were evaluated.

Microscopy and statistics

All assays were analyzed by a DM IRB inverted microscope (Leica Microsystems GmbH). Triplicate assays were performed and compared using the Mann-Whitney U test from the SPSS software package (version 12.0, SPSS). P values ≤ 0.05 were considered significantly different.

RESULTS

Kinetics of uncoating

Kinetics of uncoating were determined by evaluating the presence of 'free' nucleocapsids in the cytosol at different time points after internalization. To visualize these nucleocapsids that are released from intracellular vesicles into the cytosol, nucleocapsid protein (N) was stained after selectively permeabilizing the plasma membrane (with digitonin), leaving the intracellular vesicular membranes intact. At each time point, the percentage of cells with released genomes, was determined. The first cells with released genomes in the cytosol, appeared immediately after internalization as shown in the kinetics presented in Figure 1. The maximum percentage of cells with uncoating of endocytosed particles was observed at 35 minutes after the start of virus uptake. Then, in 8.4 ± 3.0 % of the cells, nucleocapsids were observed in the cytosol. From 35 minutes onwards, that percentage of cells with uncoating decreased. This might be due to the release of nucleocapsid protein from the RNA genome for translation, transcription and replication. Possibly, single N-proteins are not visible under the microscope or they become incorporated in replication/transcription complexes and are therefore no longer accessible for antibodies.

Effect of lysosomotropic agents on FIPV infection of monocytes

Figure 2 shows the influence of treatment of monocytes with ammonium chloride and bafilomycin A1 on FIPV infection. None of these pH drop inhibitors had a significant effect on FIPV infection. It seems that release from FIP virions out of endosomal/lysosomal vesicles does not depend on a drop in pH.

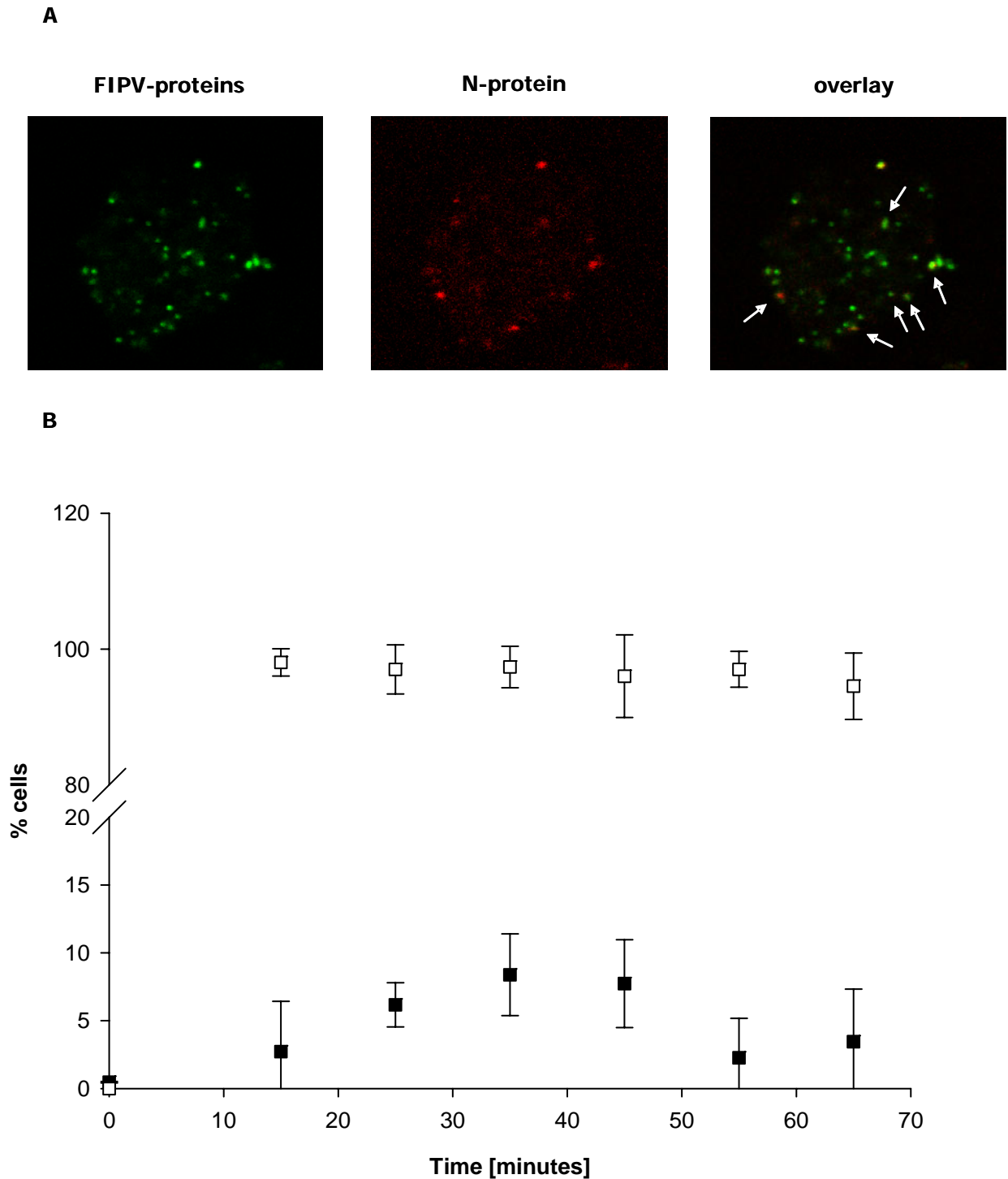


Figure 1: (A) Visualization of genome release in monocytes. Virus particles are stained with anti-FIPV-FITC and released N-protein by a mAb followed by Texas Red labelled goat anti-mouse antibodies. The arrows indicate sites of genome release. **(B) Kinetics of internalization (open squares) and kinetics of uncoating of the FIP virions after endocytosis (filled squares).** The percentage of monocytes with internalized virions and the percentage of cells with uncoated particles released in the cytosol, are represented in function of time after the start of virus uptake. Data represent means \pm standard deviations of triplicate assays.

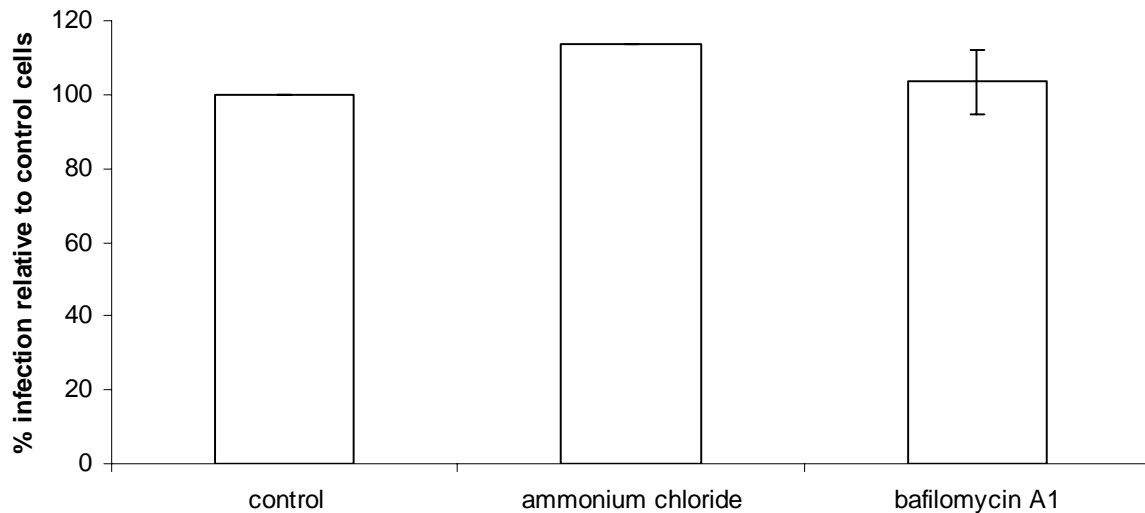


Figure 2: The influence of lysosomotropic agents on FIPV infection. For monocytes treated with ammonium chloride or bafilomycin A1, the relative percentage of infection was determined relative to the percentage of infection that was observed in control cells. Data for ammonium chloride represent means for duplicate assays, while data for bafilomycin A1 represent means \pm standard deviations of triplicate assays.

DISCUSSION

In this study, the process of uncoating of endocytosed FIP virions was monitored by determining the kinetics of uncoating. To selectively visualize uncoated viral genomes in the cytosol, the plasma membrane was permeabilized with digitonin that leaves intracellular membranes intact. Digitonin permeabilizes membranes by complexing with membrane cholesterol, producing holes in the membrane (Schulz, 1990). The selectivity of this process is due to the relatively high cholesterol content of the plasma membrane (Adam *et al.*, 1992). The digitonin-based membrane permeabilization method has been previously applied in macrophages by Diaz *et al.* (1989) and LeDoan *et al.* (1999).

Uncoating apparently starts immediately after internalization and at 35 minutes, the maximum number of cells in which nucleocapsid is detected in the cytosol, is reached. This number decreases after 35 minutes. These kinetics can be interpreted in two ways. Possibly the population of cells that is capable of mediating uncoating is restricted to approximately 8.4 %, or alternatively, released genomes only remain visible for a short period of time. In that case the number of cells actually mediating release, is the total of the number of cells with uncoating visualized at each time point. In the future, this will be studied by visualizing genome release in Crandell feline kidney cells, to determine how long uncoated

nucleocapsids can be detected in the cytosol. In these cells, release is expected to occur in all cells as they are all susceptible to infection. Because we know that experiments with cells from cell lines can give indications about processes in target cells but need to be confirmed in the target cell itself, release in monocytes should be studied in an alternative assay to check the results obtained in CrFK cells.

Using our assay, it seems that only a subpopulation of monocytes released the FIPV genome in the cytosol. This suggests that the capacity of a cell to mediate FIPV uncoating, might be a factor that determines if a cell is susceptible to infection with FIPV. This would explain (partially) why infection in monocytes is restricted to approximately 1 % of the cells, while virus uptake occurs in the majority of cells (Dewerchin *et al.*, 2005; Van Hamme *et al.*, 2007). It is evident that there might be other limiting factors that characterize the subpopulation of monocytes susceptible for FIPV infection.

In literature, there are conflicting data regarding the dependency of FIPV genome release on the pH drop in endosomal vesicles (Regan *et al.*, 2008; Takano *et al.*, 2008). Our results suggest that low pH is not required to initiate uncoating of FIPV 79-1146 in monocytes. This could have been expected as genome release seems to be a selective process while acidification of endosomes is likely to occur in all cells. Regan *et al.* also suggested that entry of serotype II FIPV occurs independently from a low pH (2008). Their results were obtained solely in feline cell lines but they did use a variety of type II FIPV strains. The concentrations of inhibitor used in this study lie in the range of concentrations used in the study performed by Regan. In contrast, Takano *et al.* claim that genome release of FIPV in monocytes does depend on low pH (2008). Their study was performed in monocytes. The effect of the inhibitors was evaluated by comparing virus titres after three days of cultivation instead of counting infected cells after one cycle of replication as done by Regan and us. Incubation of monocytes with lysosomotropic agents for several days at relatively high concentrations (500 nM of bafilomycin A1) might have side effects that could influence the result of the assay.

Another possible trigger for uncoating is cleavage by proteases. There are four main categories of proteases, named serine proteases, cysteine proteases, aspartyl proteases and metalloproteases (Barrett, 1994). Preliminary experiments suggest that a serine and/or cysteine protease might be necessary for FIPV infection of monocytes (data not shown). In the future, the involvement of each class of proteases in FIPV infection will be studied by using specific inhibitors: 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), pepstatin A and

phosphoramidon. Then, the specific involvement of these proteases in the process of genome release, will be studied. Because we want to identify proteases necessary for uncoating, it needs to be checked if the observed protease activity is not due to the virus-encoded proteins necessary for processing of the polyproteins pp1a/pp1ab in the early steps of virus replication (Hegyí & Ziebuhr, 2002). When the exact class(es) of proteases involved in the process of genome release is determined, inhibitors can be applied to specify which protease(group) is involved. Identification of a specific protease may increase the chance of finding an inhibitor that is able to reduce infection. Involvement of protease activity can be explained by a necessity for cleavage of the spike to reveal a fusion peptide (Bosch *et al.*, 2008). After identification of a certain protease, it should be confirmed and checked if and how the viral spike is cleaved by this protease. Further, it would be interesting to verify whether the expression pattern of the protease in the population of monocytes may correlate with the apparent restriction on genome release to a limited number of cells. If not, this implies that another factor is crucial for efficient uncoating, for example the presence of another receptor that induces a conformational switch into a fusogenic protein. It is clear that the available data and knowledge need to be extended, before a model for feline coronavirus uncoating can be proposed.

This study reveals that uncoating of endocytosed FIP virions may not occur in all cells. Possibly, the cellular capacity to mediate uncoating could be an important determinant for cell susceptibility. A low pH seems not necessary to enable uncoating. The involvement of proteases will be studied in the future.

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REFERENCES

- Adam, S. A., Sterne-Marr, R. & Gerace, L. (1992).** Nuclear protein import using digitonin-permeabilized cells. *Methods Enzymol* **219**, 97-110.
- Barrett, A. J. (1994).** Classification of peptidases. *Meth Enzymol* **244**, 1-15.
- Belouzard, S., Chu, V. C. & Whittaker, G. R. (2009).** Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *PNAS* **106**, 5871-5876.
- Bosch, B. J., Bartelink, W., R. & Rottier, P. J. M. (2008).** Cathepsin L functionally cleaves the severe acute respiratory syndrome coronavirus class I fusion protein upstream of rather than adjacent to the fusion peptide. *J Virol* **82**, 8887-8890.
- Bosch, B. J., van der Zee, R., de Haan, C. A. M. & Rottier, P. J. M. (2003).** The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J Virol* **77**, 8801-8811.
- Chu, V. C., McElroy, L. J., Chu, V., Bauman, B. E. & Whittaker, G. R. (2006).** The avian coronavirus infectious bronchitis virus undergoes direct low-pH-dependent fusion activation during entry in host cells. *J Virol* **80**, 3180-3188.
- de Haan, C. A. M., Haijema, B. J., Schellen, P., Wichgers Schreur, P., te Lintelo, E., Vennema, H. & Rottier, P. J. M. (2008).** Cleavage of group 1 coronavirus spike proteins: how furin cleavage is traded off against heparan sulfate binding upon cell culture adaptation. *J Virol* **82**, 6078-6083.
- Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2005).** Replication of feline coronaviruses in peripheral blood monocytes. *Arch Virol* **150**, 2483-2500.
- Diaz, R., Wileman, T. E., Anderson, S. J. & Stahl, P. (1989).** The use of permeabilized cells to study the ion requirements of receptor-ligand dissociation in endosomes. *Biochem J* **260**, 127-134.
- Eckert, D. M. & Kim, P. S. (2001).** Mechanisms of viral membrane fusion and its inhibition. *Annu Rev Biochem* **70**, 777-810.
- Hegy, A. & Ziebuhr, J. (2002).** Conservation of substrate specificities among coronavirus main proteases. *J Gen Virol* **83**, 595-599.

Huang, I. C., Bosch, B. J., Li, W., Farzan, M., Rottier, P. M. & Choe, H. (2006). SARS-CoV, but not HCoV-NL63, utilizes cathepsins to infect cells: viral entry. *Adv Exp Med Biol* **581**, 335-338.

Kawase, M., Shirato, K., Matsuyama, S. & Taguchi, F. (2009). Protease-mediated entry via the endosome of human coronavirus 229E. *J Virol* **83**, 712-721.

Krzystyniak, K. & Dupuy, J. M. (1984). Entry of mouse hepatitis virus 3 into cells. *J Gen Virol* **65**, 227-231.

LeDoan, T., Eto, F., Tenu, J., Letourneux, Y. & Agrawal, S. (1999). Cell binding, uptake and cytosolic partition of HIV anti-*gag* phosphodiester oligonucleotides 3'-linked to cholesterol derivatives in macrophages. *Bioorg Med Chem* **7**, 2263-2269.

Lefebvre, D. J., Costers, S., Van Doorselaere, J., Misinzo, G., Delputte, P. L. & Nauwynck, H. J. (2008). Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies. *J Gen Virol* **89**, 177-187.

Lescar, J., Roussel, A., Wien, M. W., Navaza, J., Fuller, S. D., Wengler, G. & Rey, F. A. (2001). The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. *Cell* **105**, 137-148.

Matsuyama, S. & Taguchi, F. (2002). Receptor-induced conformational changes of murine coronavirus spike protein. *J Virol* **76**, 11819-11826.

Miura, H. S., Nakagaki, K. & Taguchi, F. (2004). N-terminal domain of the murine coronavirus receptor CEACAM1 is responsible for fusogenic activation and conformational changes of the spike protein. *J Virol* **78**, 216-223.

Qiu, Z., Hingley, S. T., Simmons, G., Yu, C., Das Sarma, J., Bates, P. & Weiss, S. R. (2006). Endosomal proteolysis by cathepsins is necessary for murine coronavirus mouse hepatitis virus type 2 spike-mediated entry. *J Virol* **80**, 5768-5776.

Regan, A. D., Shraybman, R., Cohen, R. D. & Whittaker, G. R. (2008). Differential role of low pH and cathepsin-mediated cleavage of the viral spike protein during entry of serotype II coronaviruses. *Vet Microbiol* **132**, 235-248.

Savarino, A., Boelaert, J., Cassone, A., Majori, G. & Cauda, R. (2003). Effects of chloroquine on viral infections: an old drug against today's diseases? *Infect Dis* **3**, 722-727.

Schultz, I. (1990). Permeabilizing cells: some methods and applications for the study of intracellular processes. *Methods Enzymol* **192**, 280-300.

Smith, A. E. & Helenius, A. (2004). How viruses enter animal cells. *Science* **304**, 237-242.

Takano, T., Katada, Y., Moritoh, S., Ogasawara, M., Satoh, K., Satoh, R., Tanabe, M. & Hohdatsu, T. (2008). Analysis of the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection: aminopeptidase N is not important and a process of acidification of the endosome is necessary. *J Gen Virol* **89**, 1025-1029.

Van Hamme, E., Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2007). Attachment and internalization of feline infectious peritonitis virus in feline blood monocytes and Crandell feline kidney cells. *J Gen Virol* **88**, 2527-2535.

Wang, H., Yang, P., Liu, K., Guo, F., Zhang, Y., Zhang, G. & Jiang, C. (2008). SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. *Cell Res* **18**, 290-301.

Xu, Y., Liu, Y., Lou, Z., Qin, L., Li, X., Bai, Z., Pang, H., Tien, P., Gao, G. F. & Rao, Z. (2004). Structural basis for coronavirus-mediated membrane fusion. *J Biol Chem* **29**, 30514-30522.

Zelus, B. D., Schickli, J. H., Blau, D. M., Weiss, S. R. & Holmes, K. V. (2003). Conformational changes in the spike glycoprotein of murine coronavirus are induced at 37 °C either by soluble murine CEACAM1 receptors or by pH 8. *J Virol* **77**, 830-840.

GENERAL DISCUSSION

Feline infectious peritonitis virus (FIPV) causes a progressive vasculitis in cats that cannot be treated. The current knowledge on the pathogenesis of FIPV is an unsound basis for the development of effective antivirals. In this thesis, the entry process of FIPV was studied to add to the current knowledge and to evaluate the presence of possible viral and/or host targets for antiviral therapy.

Characteristics of FIPV serotype I and II entry in monocytes and Crandell feline kidney cells

Feline infectious peritonitis virus causes a monocyte-derived viremia (Weiss & Scott, 1981). Therefore, virus entry was studied in feline primary monocytes. Because it is laborious to obtain primary feline monocytes, Crandell feline kidney (CrFK) cells were also included in the study to evaluate if they could be used as a model cell line to study FIPV entry. Unfortunately, FIPV interacted differently with CrFK cells than with monocytes at the level of virus entry. Both attachment and internalization of FIPV were slower and less efficient in CrFK cells than in monocytes (Chapter 3). This confirms what was reported before by Nash & Buchmeier (1997), namely that one should be careful when generalizing data obtained in different cell types because entry can depend on the host cell type. Our studies made it clear that for FIPV, all data that were previously obtained with cell lines, for example the use of receptors for FIPV, should be verified in primary monocytes. Therefore, primary feline monocytes were included in all following experimental work.

FIPV strains are divided in two serotypes: serotype I (FIPV I) and the less prevalent serotype II (FIPV II) (Pedersen *et al.*, 1984; Hohdatsu *et al.*, 1994). In Chapter 3, kinetics of virus binding were determined for one strain of each serotype. It was clear that virus binding to monocytes differed between the serotype I and II strain. This could suggest that serotype I and II strains use different receptors in monocytes, as has been shown by others in cell lines. In those cell lines, type II strains use feline aminopeptidase N (fAPN) as a receptor, while type I strains do not (Hohdatsu *et al.*, 1998; Dye *et al.*, 2007). Further, the kinetics of the serotype II strain showed that virus binding was different between monocytes of two cats. This could be due to differences in expression levels of receptor protein(s) involved in FIPV entry. More virus strains and more cats will need to be included to come to general conclusions.

In general, viruses can enter a cell via two pathways: direct fusion with the plasma membrane or receptor-mediated endocytosis. The pathway that is used, can vary depending on the virus strain or the cell type (Nash & Buchmeier, 1997). Some viruses even use both

pathways in one single cell type (Schaeffer *et al.*, 2004). Based on observations for murine coronavirus, FIPV was supposed to enter cells via direct fusion at the plasma membrane (Krzystyniak & Dupuy, 1984). Nevertheless, the kinetic experiments performed in Chapter 3, showed that both serotypes are internalized via endocytosis and not via direct fusion at the plasma membrane. Direct fusion could be excluded because biotinylated envelopes were present in the cytosol and not left behind in the plasma membrane. Other group 1 coronaviruses, like human coronavirus 229E (HCoV-229E), transmissible gastroenteritis virus (TGEV) and canine coronavirus (CCoV), also enter cells via endocytosis (Hansen *et al.*, 1998; Savarino *et al.*, 2003; Nomura *et al.*, 2004). The kinetics of internalization in Chapter 3 show that internalization of FIPV in monocytes via endocytosis was very efficient for both serotypes. Comparing these results with data on virus entry of TGEV in Madin-Darby canine kidney (MDCK) cells and HCoV-229E in human fibroblasts (Hansen *et al.*, 1998; Nomura *et al.*, 2004), shows that they all occur with comparable efficiency.

It was observed that at 15 minutes after the start of FIPV internalization, envelopes started to accumulate in endosomes. Possibly, these are sites where fusion with endosomal membranes occurs to release the nucleocapsid into the cytosol. Data for TGEV indicate that accumulation in endosomes starts at 10 minutes after virus uptake and genome release is situated around 1 hour after the onset of uptake (Hansen *et al.*, 1998).

In Chapter 3, the efficiency of FIPV internalization is not only noticeable in the quantity and velocity of particle uptake, it is also reflected by the fact that a large majority of the cells takes up virus particles. However, this is in contrast to the low percentage of infection that was previously seen in monocytes (Dewerchin *et al.*, 2005). Combining these observations, we hypothesize that there is a cellular mechanism to resist viral replication in monocytes after virus internalization. This mechanism would restrict infection at the level of genome release, transcription or translation.

Virus-receptor interactions during the entry of FIPV II in monocytes

As mentioned above, cell lines and more specifically CrFK cells interact differently with FIPV during entry compared to monocytes. The two potential receptors described for type II FIPV, fAPN and dendritic cell (DC)-specific intercellular adhesion molecule (ICAM) grabbing non-integrin (DC-SIGN), were studied mainly in cell lines (Tresnan *et al.*, 1996; Hohdatsu *et al.*, 1998; Dye *et al.*, 2007; Regan & Whittaker, 2008). Therefore, the role and interplay between these two proteins during the entry process of FIPV, were evaluated in monocytes and compared with results obtained in cell lines, under the same experimental conditions (Chapter 4).

In our assays, virus binding to CrFK cells was completely blocked by APN-specific antibodies and there was complete co-localization between FIPV II and APN. These data confirmed once more that fAPN is the exclusive virus binding receptor in CrFK cells. Virus bound to fAPN is most likely internalized by APN itself (as FIPV and fAPN also co-localized inside the cell). Entry through fAPN leads to infection of CrFK cells as in our study, infection could also be completely blocked with APN-specific antibodies.

However, in monocytes, the entry process seems to occur differently. APN-blocking antibodies only reduced virus binding by half. Therefore, it can be suggested that FIPV binding partly depends on fAPN and partly on another unknown receptor. The internalization assays in Chapter 4 showed that the fraction of virions that is internalized by the unknown receptor, was similar to the fraction of virions that was internalized when all receptors were available. Thus it is clear that both receptors contribute to the efficiency of internalization observed in Chapter 3. Furthermore, infection could not be blocked completely by shutting down one pathway, so entry via each of both receptors can lead to infection of monocytes. The involvement of a second receptor, besides APN, has also been described for the porcine coronavirus TGEV. TGEV uses porcine APN for entry into cells (Delmas *et al.*, 1992; 1993), but Weingartl & Derbyshire described an additional receptor of approximately 200 kDa, that was not further identified (1994). Possibly the feline variant of that protein is involved in FIPV entry.

Regan & Whittaker reported DC-SIGN as a mediator of feline coronavirus entry into host cells (2008). Therefore, the role of DC-SIGN and potential interplay with APN was studied in the entry process of FIPV. Because feline DC-SIGN (fDC-SIGN) is not completely characterized yet, a human DC-SIGN (hDC-SIGN) construct was used to see if FIPV interacted with hDC-SIGN-expressing cells. Expression of hDC-SIGN in Chinese hamster ovary (CHO) cells enabled the cells to bind and internalize virus, but infection could not be established. It is known that DC-SIGN can target the endosomal/lysosomal pathway (Engering *et al.*, 2002), so probably virus particles are degraded because they lack a factor necessary for genome release. These experiments demonstrated the capacities of hDC-SIGN to bind and internalize FIPV.

Knowing this, the presence of FIPV-fDC-SIGN interactions was also studied in monocytes, and this was done by assessing the effect of blocking fDC-SIGN on virus binding, internalization and infection. In monocytes, no role could be attributed to fDC-SIGN in binding and internalization of FIPV as none of these processes were influenced by incubation

with mannan, a competitor for binding to DC-SIGN. Also when fAPN was blocked, blocking binding to fDC-SIGN did not cause a supplemental inhibition of FIPV binding. Therefore, we can conclude that fDC-SIGN in monocytes is not important for virus binding and internalization and hence, fDC-SIGN cannot be the unknown receptor X that binds and internalizes FIPV. Moreover, these results suggest that FIPV has a relatively low affinity towards fDC-SIGN compared to fAPN and the unknown receptor, and maybe even towards hDC-SIGN. Besides in binding and internalization, we also checked the role of fDC-SIGN in infection by evaluating if mannan could reduce infection. No effect on infection was observed when fDC-SIGN was blocked with mannan. However, when fAPN was blocked, fDC-SIGN did seem to have a role in the infection process. Because fDC-SIGN does not contribute to binding and internalization, the role in infection will most likely be in a step following internalization. Further studies are needed to further elucidate the role of fDC-SIGN in infection.

Having listed the results from this study, a comparison can be made with the results obtained by Regan & Whittaker (2008). They induced susceptibility to infection in unsusceptible cells (mouse 3T3 cells) after transfection with hDC-SIGN. In our study, virus internalization could be induced in transfected CHO cells, but infection did not take place. A possible explanation is that CHO cells lack a factor necessary to release the FIPV genome from intracellular vesicles, while this factor is present in the mouse cells used by Regan & Whittaker. Further, Regan & Whittaker found that transfection of hDC-SIGN in CrFK cells enhanced infection of these cells. This could be explained by an increase of virus binding and internalization. Infection of monocytes could also be blocked with mannan, according to Regan & Whittaker. In our hands, no inhibition of infection was observed after treating monocytes with mannan. There is no obvious explanation for the different outcomes between these studies, but maybe the FIP viruses have a different passaging history. And since it is known that multiple passaging can induce adaptations in viruses, these adaptations might have had an effect on virus-receptor interactions.

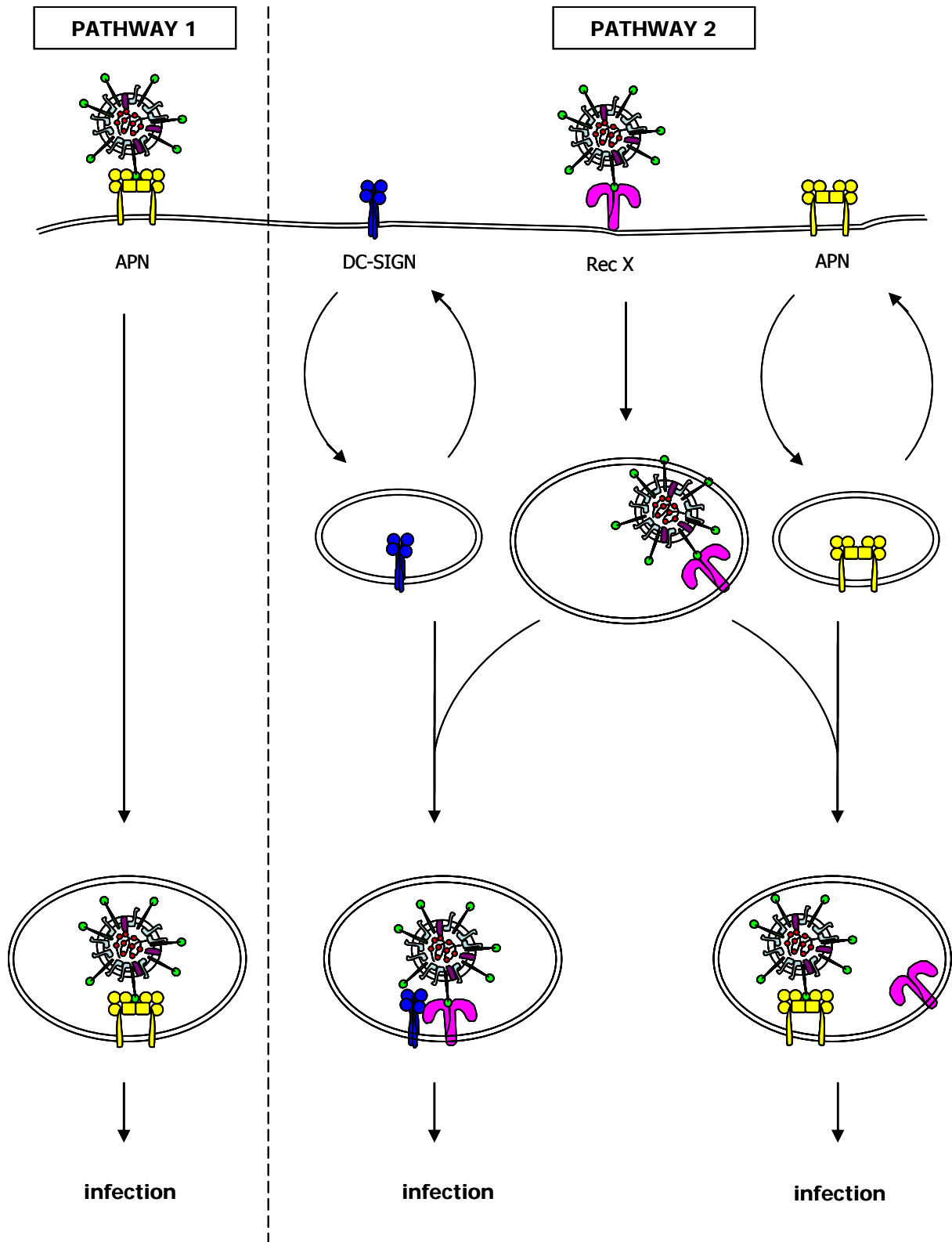


Figure 1: Schematic representation of hypothetical receptor use by FIPV type II upon entry in monocytes. The virus can enter the monocytes via two pathways. 'Rec X' stands for a yet unidentified receptor that can be used by FIPV to gain entry into the cell.

All the data we obtained on interactions between FIPV II and fAPN, the unknown receptor 'rec X', and fDC-SIGN led to the hypothetical model on receptor use in monocytes, depicted in Figure 1. Infection can be mediated by fAPN after virus binding to and internalization via this protein. This is pathway 1 in Figure 1. The unknown rec X can bind and internalize FIPV, but by itself, it can probably not (efficiently) induce infection. Possibly, rec X mediates infection through association with fDC-SIGN or fAPN after internalization to mediate genome release. This is pathway 2 in Figure 1. The association with fDC-SIGN or fAPN can result from vesicle fusion between the internalized FIPV-containing vesicle and a recycling fDC-SIGN-containing or fAPN-containing vesicle. Based on the experimental data, it cannot be excluded that fDC-SIGN or fAPN associate with rec X in the plasma membrane after binding of FIPV to rec X and that fDC-SIGN or fAPN co-internalize with rec X.

Going even one step further, the possibility cannot be excluded that rec X might also associate with fAPN in the plasma membrane in pathway 1. This way, rec X could determine the mechanism of internalization in both pathways.

A potentially interesting observation on the side, was that visualization of a certain epitope on fAPN, recognized by mAb R-G-4, resulted in different staining patterns between cells from feline cell lines and monocytes. In all cells from the CrFK cell line, this fAPN epitope could be visualized in the plasma membrane. In monocytes, this epitope was only present in the plasma membrane of a minority of monocytes, while the full fAPN protein was expressed in the plasma membrane of all monocytes (as shown by staining another epitope of the protein). Possibly, different glycosylation patterns occur in subpopulations of monocytes, that mask the epitope. Alternatively, the epitope might be masked due to di- or multimerization, or interactions with other proteins. It would be very interesting to characterize monocytic fAPN and define the exact differences with fAPN from feline cell lines. Especially since most receptor studies have been performed with constructs expressing the APN gene isolated from such a feline cell line. This would allow to compare interaction sites and binding affinities between FIPV and fAPN from monocytes on the one hand and from feline cell lines on the other hand. For example, it would be interesting to see if affinity for monocytic APN is higher than that for APN from feline cell lines. This can be assumed based on the kinetics obtained in Chapter 3, which indicate that binding of FIPV II to CrFK cells is less efficient than binding to monocytes. Evidently, the presence of the 'extra' receptor X might also contribute to the higher efficiency, by providing a secondary route to deliver virus particles to intracellular vesicles.

To date, the receptor for serotype I FIPV in monocytes is not known. It has been proposed that fAPN is not important for serotype I FIPV (Hohdatsu *et al.*, 1998; Dye *et al.*, 2007). Therefore we would like to suggest that the unknown receptor X, with or without DC-SIGN, might be important for type I FIPV infection. Type I FIPV would then use receptor X for entry, while type II FIPV could use both receptor X as well as fAPN. This hypothesis is in accordance with the observation that type I FIPV cannot bind to CrFK cells which presumably do not express receptor X, as type II FIPV only binds to fAPN on CrFK cells.

Upon the recombination event between serotype I FIPV and CCoV from which serotype II arose (Herrewegh *et al.*, 1998), type II FIPV might have acquired affinity for APN from CCoV (that uses canine APN as a receptor), next to the 'existing' affinity for the type I receptor X. In the kinetics of virus binding (Chapter 3), there were significant differences between type I and type II FIPV, which could be explained if fAPN is not a receptor for type I FIPV while it is for type II FIPV (Chapter 4 - model Fig. 1). A future experiment will evaluate if type II FIPV binding, gives similar results as seen for type I FIPV binding when APN is blocked on monocytes from the same cat. If affinity for APN is a supplemental feature for type II, compared to type I that can only bind receptor X, then blocking APN in the kinetics of type II would result in the kinetics obtained for type I. This could be possible for the second cat included in the study, where binding of serotype II FIPV reaches slightly higher levels in a shorter period of time than binding of serotype I FIPV. However for the first cat, there was less type II FIPV binding than type I FIPV binding, which does not fit the hypothesis. The low level of type II FIPV binding might be due to a lack of or 'different' expression of APN on the monocytes of that cat, what would imply that pathway 1 (in Figure 1) is not used intensely in monocytes of that cat. The small amount of type II virus that did bound, would have bound to receptor X and follow pathway 2 (in Figure 1). The reason that this level of FIPV II binding is lower than the binding observed in the kinetics for type I, could be that type I FIPV has a higher affinity for receptor X than type II FIPV. If this is true, one could hypothesize that the price for type II to gain affinity for fAPN upon the recombination event, was a loss in affinity for receptor X. In the future, it will be studied if pathway 1, where only APN is used, is indeed less available in monocytes of this cat. This could be studied by evaluating if blocking infection of type II FIPV with APN-specific antibodies has less effect on infection of monocytes in this cat compared to the second cat.

In addition, it will also be studied in the near future if type I FIPV infection depends on DC-SIGN.

Taken together, this study obviously provided data on the entry of FIPV II which could be very useful for studying entry of FIPV I.

The mechanism of internalization of FIPV II in monocytes

In Chapter 3, FIPV II was shown to use the endocytosis machinery of the cell to gain entry into monocytes. In Chapter 5, this pathway was further characterized by identifying which cellular proteins are necessary for FIPV internalization. Several strategies were applied: blocking entry with inhibitors, transduction with dominant-negative mutants of internalization proteins and co-localization studies looking at FIPV and internalization proteins. The combination of different strategies guaranteed a reliable result. Including co-localization assays prevented us to draw wrong conclusions due to shifting to another pathway when a certain pathway is blocked.

The experiments showed that the entry of FIPV is independent of phagocytosis and macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis. Blocking dynamin function by an inhibitor and expression of a dynamin dominant-negative mutant strongly reduced virus uptake. Inhibition of Rho GTPases did not. In conclusion, the pathway used by FIPV II to enter monocytes is clathrin- and caveolae-independent and depends on dynamin.

Sterol binding drugs (nystatin and methyl- β -cyclodextrin) slightly reduced FIPV entry in our assay. Reduction by sterol binding drugs could imply the involvement of lipid rafts in the entry process. However, the reduction obtained in virus entry by these drugs was rather small and probably too small to conclude that the entry process is associated with lipid rafts and depends on them to initiate and sustain the necessary signalling cascades.

Perhaps, one of the receptor(s) or components that needs to be recruited for the internalization process is located in lipid rafts. Disturbing rafts might hereby reduce the availability of that receptor/protein and slightly influence the efficiency of internalization, possibly by reducing virus binding.

Another possibility is that the pathway requires cholesterol, like for example clathrin-mediated internalization requires cholesterol independent from lipid rafts (Subtil *et al.*, 1999; Nichols & Lippincott-Schwartz, 2001).

In accordance with the model from Chapter 4, there might be two internalization pathways. If this is true, it would be most likely that both pathways are independent from clathrin and

caveolae. The rather low inhibiting effect of cholesterol binding drugs, could be explained if one of the two pathways is linked to rafts. The moderate effect could be due to a pathway shift or by a bigger contribution by the raft-independent pathway in the internalization process. In pathway 1, fAPN might be present in lipid rafts because its human homologue is localized in rafts (Nomura *et al.*, 2004). For pathway 2, fDC-SIGN or a fraction of fDC-SIGN, might also be localized in rafts. In human monocytes, hDC-SIGN is not localized in rafts immediately after they are brought in culture, but during culture in the presence of IL-4 and GM-CSF a relocation to rafts is observed (Cambi *et al.*, 2004). Of course, nothing is known about possible raft-association of the unknown receptor X. Even if the receptors are located in rafts, it remains uncertain if the association with rafts is necessary for the internalization process. For example, TGEV enters cells through porcine APN (presumably localized in rafts) via clathrin-mediated internalization and this process does not depend on rafts. Possibly, the small effect of the sterol binding drugs, we observed for FIPV, is just due to the localization of the receptors in rafts. Further, it is also not certain that both internalization pathways depend on dynamin as internalization could not be completely blocked by dynamin inhibitors. However, reductions by dynamin inhibitors are much larger than those with sterol binding drugs. Therefore, it seems most likely that both pathways depend on dynamin. Another internalization pathway that is mediated by hAPN, the internalization pathway of HCoV-229E, depends on dynamin (Nomura *et al.*, 2004). However this does not imply that all pathways via APN recruit dynamin. In general, there is no direct interaction between dynamin and the internalizing receptor.

All monocytes internalize FIPV II virus particles, while only a very limited number gets infected. Therefore it might be questioned if the internalization pathways studied, lead to infection. After treatment of monocytes with entry inhibitors, infection of monocytes was reduced to similar levels as virus entry. This suggests that the internalization pathways studied, indeed lead to infection. However, for the sterol binding drugs, the reduction of infection was larger than expected based on the reduction of entry. Therefore, other post-internalization processes are probably also hampered by the inhibitor, like for example intracellular transport. Another possibility is that the small effect of the sterol binding drugs was due to involvement of rafts in one of the two entry pathways or for one of the two receptors. The larger reducing effect on infection would imply that the only pathway that is involved in infection is the one linked to rafts. However, this would contradict earlier results from Chapter 4 that indicate that both pathways are important for infection.

As far as we know, the entry pathway of FIPV II, namely the clathrin- and caveolae-independent pathway that depends on dynamin and is slightly cholesterol depletion sensitive, has no physiological equivalent. However, there are pathways that resemble the entry pathway of FIPV. The β -strain of interleukin 2 receptors (IL-2R) is internalized via a clathrin- and caveolae-independent pathway. The pathway depends on dynamin and is sensitive to cholesterol depletion due to raft-association of the receptor. The pathway depends on Rho GTPases (Lamaze *et al.*, 2001). The internalization of the common cytokine receptor γ (γ c) follows the same route as the IL-2R (Kirkham & Parton, 2005; Sauvonnnet *et al.*, 2005).

Clathrin- and caveolae-independent pathways are also used by several viruses, including a coronavirus (Marsh & Helenius, 1989; Sieczkarski & Whittaker, 2002; Sanchez-San Martin *et al.*, 2004). The internalization pathway of SARS-CoV in HEK293E cells transfected with ACE2, and Vero E6 cells, is also independent from clathrin and caveolae and associated with lipid rafts (Wang *et al.*, 2008). Further characterization, however, has not been performed to date. Rotavirus entry resembles FIPV entry the most, except for the higher susceptibility for cholesterol depletion. The role of Rho GTPases in this process has not been studied (Sanchez-San Martin *et al.*, 2004).

Not many entry pathways of group 1 coronaviruses are characterized profoundly. The characterized pathways are diverse and different from the pathway described for FIPV. HCoV-229E enters human fibroblasts via caveolae-mediated internalization (Nomura *et al.*, 2004), while TGEV enters cells that stably express APN presumably via clathrin-mediated internalization (Hansen *et al.*, 1998). It is clear that their common characteristics, including the use of APN as a receptor, do not lead to a shared internalization mechanism.

Genome release after internalization via endocytosis of FIPV II

After internalization via endocytosis, the virus is 'trapped' in intracellular vesicles and the genome has to be released into the cytosol for viral replication to occur. In Chapter 6, this uncoating process was visualized. Uncoating could be observed in some cells as soon as internalization started. The maximal percentage of cells with released genomes in the cytosol, was observed 35 minutes after the start of internalization. These data correlate very good with the observed accumulation of FIPV envelopes in endosomes at 15 minutes after the start of internalization in Chapter 3. Moreover we observed that the release of the FIPV genome from these vesicles appears to be restricted to a subpopulation of cells (Chapter 6). This suggests a restriction on the uncoating process which may explain (in part) the discrepancy between the number of cells with internalized particles and the number of

infected cells that was observed in Chapter 3. Still, further research is necessary to verify if there is an additional restriction besides the one on uncoating.

Possible triggers for genome release are low pH, cleavage by proteases and receptor-induced conformational changes. For FIPV type II infection of monocytes, the requirement for low pH has been the subject of contradictory literature (Regan *et al.*, 2008; Takano *et al.*, 2008). Our results from infection inhibition experiments in monocytes with lysosomotropic agents, suggest that low pH is not important to mediate genome release. This is in agreement with the results that Regan *et al.* (2008) obtained in several cell lines for several FIPV strains. In contrast, Takano *et al.* (2008) suggest that low pH is important for FIPV infection in monocytes. They used a different set-up by using relatively high concentrations of inhibitors (500 nM of bafilomycin A1 while we used 10 nM) for a longer incubation time (3 days compared to 6-12 hours). This might explain the different results.

The second possible trigger for genome release is protease activity. Proteases are divided in four main categories: cysteine proteases, serine proteases, aspartyl proteases and metalloproteases. The precise role of proteases in FIPV infection will be studied in the future by treating monocytes with several protease inhibitors. Preliminary experiments suggest that cysteine and/or serine proteases may be involved in FIPV II infection. In the future, it will be determined if (a) cysteine or serine protease(s) is/are important for genome release in particular, and if so, which (subclass of) protease(s). This is of great interest as both furin, a serine protease, and cathepsin B, a cysteine protease, have been suggested to play a role in feline coronavirus entry for at least some strains (Regan *et al.*, 2008; de Haan *et al.*, 2008). The involvement and role of the other proteases (aspartyl proteases and metalloproteases) will also be studied. Possibly, more than one protease is involved in the uncoating, as has been described for HCoV-229E (Kawase *et al.*, 2009).

Another potential trigger is a receptor-induced conformational change. The involvement of conformational changes to initiate fusion for uncoating, was not evaluated in our study and therefore cannot be excluded. The hypothesis in Chapter 4 namely that in pathway 2 fDC-SIGN binding might be necessary to induce uncoating remains possible. However, fDC-SIGN does probably not initiate uncoating itself by inducing a conformational change, but rather targets compartments where other release factors like proteases are present.

Hypothetical model for the entry of FIPV II in feline monocytes

The work in this thesis has led to a hypothetical model of the entry of FIPV II in feline blood monocytes, the *in vivo* target cells. In Figure 2, the different steps during entry are schematically represented. The first pathway of internalization (pathway 1) results from binding to APN in the plasma membrane. Possibly APN resides in lipid rafts, but probably this association is not required for internalization. After binding, dynamin is most likely recruited and a vesicle containing the virus-receptor complex is internalized via a clathrin- and caveolae-independent pathway. To release the viral genome from the vesicle into the cytosol, cysteine and/or serine protease activity might initiate the exposure of a fusion peptide that mediates membrane fusion between the viral envelope and the membrane of the intracellular vesicle. Another, yet unknown receptor can also bind and internalize FIPV (pathway 2). It is not known if this receptor is present in lipid rafts. The internalization also occurs via a clathrin- and caveolae-independent pathway, that probably also depends on dynamin. After internalization, the internalized vesicle can fuse with a DC-SIGN- or an APN-containing vesicle to mediate genome release (unless the association between the unknown receptor and DC-SIGN or APN already took place at the plasma membrane). How genome release is mediated, is not known. The trigger might be a DC-SIGN-induced conformational change or most likely, DC-SIGN might target the virus-receptor complex to an intracellular compartment with the appropriate physiological conditions, like the presence of cysteine and/or serine proteases, to induce fusion.

The relevance of this hypothetical model and the obtained data might be questioned based on the relatively small number of animals included in the study. It would be interesting to compare similar data for other cats. However, the data presented are clearly more representative for the *in vivo* situation than previous data obtained in cell lines.

Knowing that there are big differences in susceptibilities of cats, it can be questioned if the proposed model can be generalized. This could be verified in the future but it seems more likely that differences in susceptibility are caused by differences in the immune response upon infection than by different receptor use or expression.

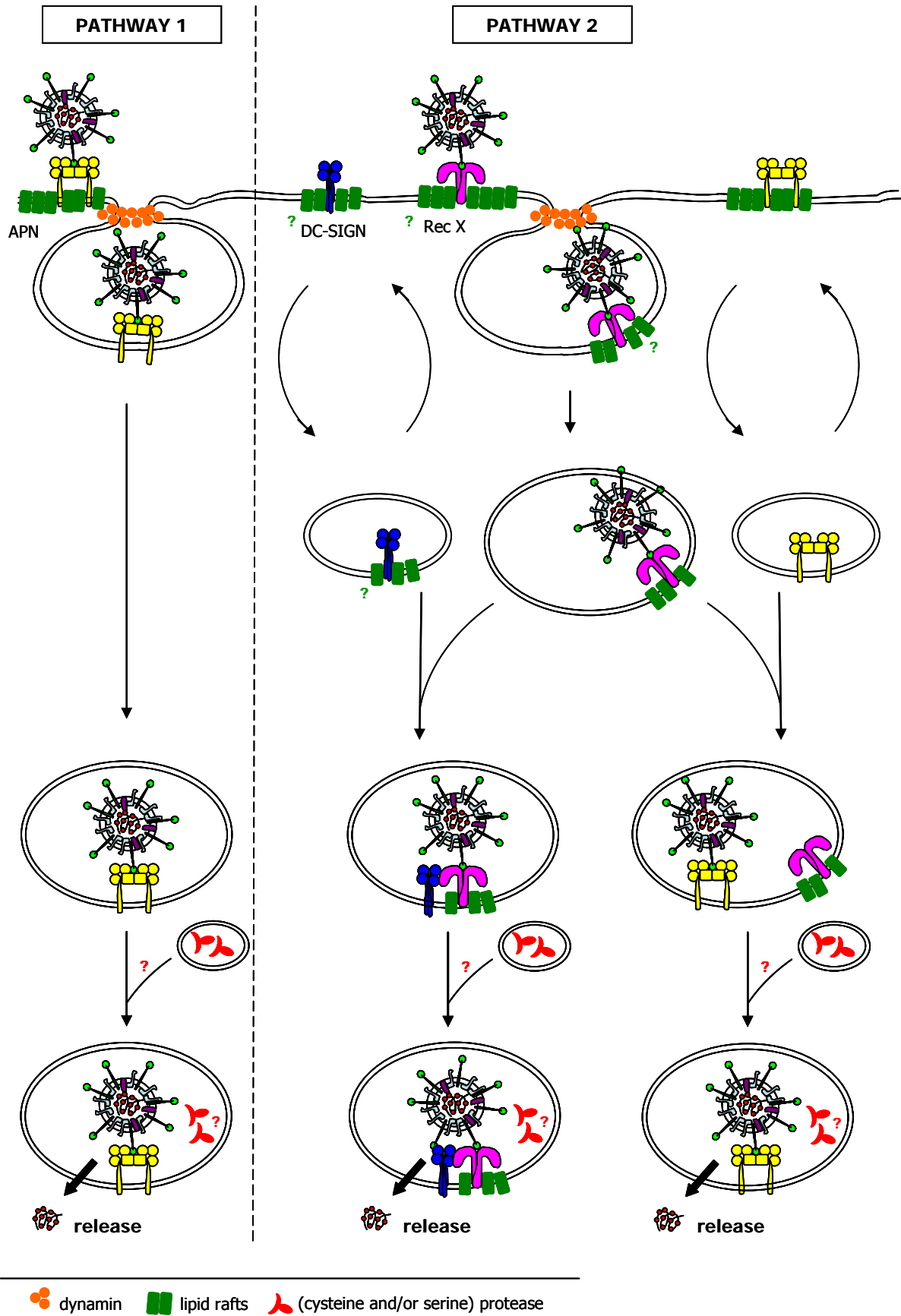


Figure 2: Hypothetical model for the entry of FIPV type II in feline blood monocytes based on the data obtained in this thesis.

The main conclusions that can be drawn from this thesis are:

- Crandell feline kidney cells can not be used as a model cell line to study FIPV entry.
- Serotype I and II FIPV strains might use different receptors on monocytes as they do in CrFK cells.
- FIPV serotype I and II internalization in monocytes is extremely efficient and occurs exclusively via endocytosis.
- fAPN is a virus binding and internalizing receptor for serotype II FIPV on monocytes, while DC-SIGN is not.
- fAPN is not the only receptor for serotype II FIPV on monocytes.
- FIPV serotype II internalization in monocytes is clathrin- and caveolae-independent, depends on dynamin and is slightly cholesterol depletion sensitive.
- Attachment and internalization of FIPV serotype II occurs in most cells, while genome release appears to be restricted to a fraction of the cells which might explain why most cells are not susceptible to FIPV infection.

Fundamental research, like described in this thesis, is extremely important in the search for targets that can be used in the development of new antiviral compounds. Especially in the battle against incurable diseases like feline infectious peritonitis. The studies described in this thesis already provide some ideas on how to fight this disease. For example, FIPV infection of monocytes can be prevented by blocking dynamin. However, blocking dynamin function in an organism will probably have numerous adverse effects.

A lot of questions remain to be studied in the future. First of all, several experiments can be performed to evaluate the relevance of the hypothetical models depicted in Figures 1 and 2. Further, signalling during internalization can be studied in order to find specific regulators for the independent internalization pathway that could be used as targets for treatment of FIP. And finally, it would be of interest to identify the unknown FIPV binding and internalizing receptor.

What is known now about the entry of type II FIPV, also puts us in an ideal position to start analysing the entry of type I FIPV. Knowing more about the entry of type I FIPV is of major interest because most natural infections are serotype I infections. Therefore, if a treatment is developed that targets FIPV entry, it should also be able to reduce type I FIPV entry.

REFERENCES

- Cambi, A., de Lange, F., van Maarseveen, N. M., Nijhuis, M., Joosten, B., van Dijk, E. M., de Bakker, B. I., Fransen, J. A., Bovee-Geurts, P. H., van Leeuwen, F. N., Van Hulst, N. F. & Figdor, C. G. (2004).** Microdomains of the C-type lectin DC-SIGN are portals of entry into dendritic cells. *J Cell Biol* **164**, 145-155.
- de Haan, C. A. M., Haijema, B. J., Schellen, P., Wichgers Schreur, P., te Lintelo, E., Vennema, H. & Rottier, P. J. M. (2008).** Cleavage of group 1 coronavirus spike proteins: how furin cleavage is traded off against heparan sulfate binding upon cell culture adaptation. *J Virol* **82**, 6078-6083.
- Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L. K., Sjöström, H., Norén, O. & Laude, H. (1992).** Amino-peptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature* **357**, 417-419.
- Delmas, B., Gelfi, J., Sjöström, H., Norén, O. & Laude, H. (1993).** Further characterization of amino-peptidase N as a receptor for coronaviruses. *Adv Exp Med Biol* **342**, 293-298.
- Dewerchin, H. L., Cornelissen, E. & Nauwynck, H. J. (2005).** Replication of feline coronaviruses in peripheral blood monocytes. *Arch Virol* **150**, 2483-2500.
- Dye, C., Temperton, N. & Siddell, S. G. (2007).** Type I feline coronavirus spike glycoprotein fails to recognize amino-peptidase N as a functional receptor on feline cell lines. *J Gen Virol* **88**, 1753-1760.
- Engering, A., Gijtenbeek, T. B. H., van Vliet, S. J., Wijers, M., van Liempt, E., Demareux, N., Lanzavecchia, A., Fransen, J., Figdor, C. G., Piguet, V. & van Kooyk, Y. (2002).** The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol* **168**, 2118-2126.
- Hansen, G. H., Delmas, B., Besnardeau, L., Vogel, L. K., Sjöström, H., Laude, H. & Norén, O. (1998).** The coronavirus transmissible gastroenteritis virus causes infection after receptor-mediated endocytosis and acid-dependent fusion with an intracellular compartment. *J Virol* **72**, 527-534.
- Herrewegh, A. A., Smeenk, I., Horzinek, M. C., Rottier, P. J. M. & de Groot, R. J. (1998).** Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. *J Virol* **72**, 4508-4514.

Hohdatsu, T., Izumiya, Y. & Yokoyama, Y. (1998). Differences in virus receptor for type I and type II feline infectious peritonitis virus. *Arch Virol* **143**(5), 839-850.

Hohdatsu, T., Tokunaga, J. & Koyama, H. (1994). The role of IgG subclass of mouse monoclonal antibodies in antibody-dependent enhancement of feline infectious peritonitis virus infection of feline macrophages. *Arch Virol* **139**, 273-285.

Kawase, M., Shirato, K., Matsuyama, S. & Taguchi, F. (2009). Protease-mediated entry via the endosome of human coronavirus 229E. *J Virol* **83**, 712-721.

Krzystyniak, K. & Dupuy, J. M. (1984). Entry of mouse hepatitis virus 3 into cells. *J Gen Virol* **65**, 227-231.

Kwon, D. S., Gregorio, G., Bitton, N., Hendrickson, W. A. & Littman, D. R. (2002). DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. *Immunity* **16**, 135-144.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A. & Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol Cell* **7**, 661-671.

Marsh, M. & Helenius, A. (1989). Virus entry into animal cells. *Adv Virus Res* **36**, 107-151.

Nash, T. C. & Buchmeier, M. J. (1997). Entry of mouse hepatitis virus into cells by endosomal and nonendosomal pathways. *Virology* **233**, 1-8.

Nichols, B. J. & Lippincott-Schwartz, J. (2001). Endocytosis without clathrin coats. *Trends Cell Biol* **11**, 406-412.

Nomura, R., Kiyota, A., Suzaki, E., Kataoka, K., Ohe, Y., Miyamoto, K., Senda, T. & Fujimoto, T. (2004). Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. *J Virol* **78**, 8701-8708.

Pedersen, N. C., Black, J. W., Boyle, J. F., Evermann, J. F., McKeirnan, A. J. & Ott, R. L. (1984). Pathogenic differences between various feline coronavirus isolates. Coronaviruses; molecular biology and pathogenesis. *Adv Exp Med Biol* **173**, 365-380.

Regan, A. D., Shraybman, R., Cohen, R. D. & Whittaker, G. R. (2008). Differential role of low pH and cathepsin-mediated cleavage of the viral spike protein during entry of serotype II coronaviruses. *Vet Microbiol* **132**, 235-248.

Regan, A. D. & Whittaker, G. R. (2008). Utilization of DC-SIGN for entry of feline coronaviruses into host cells. *J Virol* **82**, 11992-11996.

Sanchez-San Martin, C., Lopez, T., Arias, C. F. & Lopez, S. (2004). Characterization of rotavirus cell entry. *J Virol* **78(5)**, 2310-2318.

Sauvonnet, N., Dujeancourt, A. & Dautry-Varsat, A. (2005). Cortactin and dynamin are required for the clathrin-independent endocytosis of γ c cytokine receptor. *J Cell Biol* **168**, 155-163.

Savarino, A., Boelaert, J. R., Cassone, A., Majori, G. & Cauda, R. (2003). Effects of chloroquine on viral infections: an old drug against today's diseases? *Lancet Infect Dis* **3**, 722-727.

Schaeffer, E., Soros, V. B. & Greene, W. C. (2004). Compensatory link between fusion and endocytosis of human immunodeficiency virus type I in human CD4 T lymphocytes. *J Virol* **78**, 1375-1383.

Sieczkarski, S. B. & Whittaker, G. R. (2002). Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. *J Virol* **76**, 10455-10464.

Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H. & McGraw, T. E. (1999). Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci* **96**, 6775-6780.

Takano, T., Katada, Y., Moritoh, S., Ogasawara, M., Satoh, K., Satoh, R., Tanabe, M. & Hohdatsu, T. (2008). Analysis of the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection: aminopeptidase N is not important and a process of acidification of the endosome is necessary. *J Gen Virol* **89**, 1025-1029.

Tresnan, D. B., Levis, R. & Holmes, K. V. (1996). Feline aminopeptidase N serves as a receptor for feline, canine, porcine and human coronaviruses in serogroup I. *J Virol* **70**, 8669-8674.

Wang, H., Yang, P., Liu, K., Guo, F., Zhang, Y., Zhang, G. & Jiang, C. (2008). SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. *Cell Res* **18**, 290-301.

Weingartl, H. M. & Derbyshire, J. B. (1994). Evidence for a putative second receptor for porcine transmissible gastroenteritis virus on the villous enterocytes of newborn pigs. *J Virol* **68**, 7253-7259.

Weiss, R. C. & Scott, F. W. (1981). Pathogenesis of feline infectious peritonitis: nature and development of viremia. *Am J Vet Res* **42**, 382-390.

Chapter 8

SUMMARY - SAMENVATTING

SUMMARY

Feline coronaviruses occur in two virulence variants of which the feline infectious peritonitis virus (FIPV) is the most threatening. It causes a monocyte-derived viremia that leads to a progressive vasculitis and is mostly fatal for the infected cat. There is no effective treatment available. To address this matter, an improved knowledge of the pathogenesis of FIPV is needed. The process of viral entry is an attractive target for the development of new therapeutic agents. However, little is known about the entry of FIPV. It is even questioned if the virus enters cells via direct fusion with the plasma membrane or after receptor-mediated internalization.

In this thesis, the entry process of FIPV is studied to add upon the knowledge in FIPV pathogenesis and to evaluate the presence of possible targets for antiviral therapy.

In **chapter 1**, an introduction is given about FIPV and virus entry in general. In the first section of chapter 1, the taxonomy and pathogenesis of FIPV are described. Further, insights are provided in the structure of a FIP virion and the production of new virions upon infection of a cell. In the second section of chapter 1, the process of viral entry is discussed. The different receptors that can be used by group 1 coronaviruses are listed and their use by the different coronaviruses is described. An overview of possible internalization pathways for viruses is given. And finally, different coronaviruses are linked to their internalization pathways.

In **chapter 2**, the outline of the aims is given.

In **chapter 3**, a quantitative analysis of the different steps in viral entry, i.e. attachment and internalization, was performed. Kinetics of attachment and internalization were determined by evaluating the number of attached virions and the percentage of internalized virions at different time points after inoculation. Kinetics were determined in both the *in vivo* target cells, feline primary monocytes and in Crandell feline kidney (CrFK) cells, cells from a continuous cell line. This was done to evaluate if this cell line could be used as a model cell line for FIPV entry studies. Unfortunately, attachment and internalization were less efficient in CrFK cells, so entry experiments in CrFK cells cannot predict interactions of FIPV with monocytes during virus entry.

Two virus strains were included in the study, namely the serotype I strain Black and the serotype II strain 79-1146, to evaluate differences between both serotypes. Virus binding to

monocytes differed between the serotype I and II strain. This might suggest that both serotypes use different receptors in monocytes, like was shown previously in cell lines. Further, for the serotype II strain, virus binding was different between monocytes of two cats. This could be due to differences in expression levels of receptor protein(s) involved in FIPV entry. It is clear that these hypotheses should be verified by evaluating virus binding for more virus strains and more cats.

Virus internalization was very efficient in monocytes. Besides the fact that the majority of bound virions was internalized within 1 minute, internalization also occurred in the majority of cells. This was in contrast to the restricted number of cells that is susceptible to infection. It was also shown that this internalization process proceeded via receptor-mediated endocytosis and not via direct fusion at the plasma membrane.

In **chapter 4**, the role of feline aminopeptidase N (fAPN) and dendritic cell-specific intercellular adhesion molecule grabbing non-integrin (DC-SIGN), as receptors for FIPV serotype II strain 79-1146, has been studied in both monocytes and cells from continuous cell lines. First, the effect of blocking the potential receptor(s) was evaluated and secondly, the level of co-localization of FIPV and the receptors was determined.

It was found that blocking fAPN inhibited binding and infection in CrFKs completely and that FIPV co-localized with fAPN. In contrast, on monocytes, binding and infection were only reduced by 53 % and 44 %, respectively, and only 60 % of bound FIPV co-localized with fAPN. Thus, APN is capable of binding and internalizing FIPV in monocytes and APN-mediated entry leads to infection in monocytes. However, this is not the exclusive receptor for FIPV on monocytes, like it appears to be in CrFK cells.

To evaluate the FIPV binding and internalizing capacities of human DC-SIGN (hDC-SIGN), CHO cells were transduced with hDC-SIGN. Transduction rendered the cells susceptible to binding and internalization of FIPV but not to infection. In monocytes, blocking fDC-SIGN did not influence binding and infection and there was no co-localization. It is clear that fDC-SIGN is not the second receptor next to fAPN on monocytes. However, blocking fDC-SIGN caused a further inhibition of infection from 56 % up to 18 % infection in APN-blocked monocytes. It seems that DC-SIGN is not directly required for attachment or internalization in monocytes, but might be necessary for infection via an alternative pathway independent of APN.

In conclusion, it was clear that virus-receptor interactions on CrFK cells are not representative for the interactions on *in vivo* target cells. The results obtained in this chapter also led to a model for receptor interactions upon FIPV type II entry in monocytes.

In **chapter 5**, the mechanism of internalization of FIPV serotype II strain 79-1146 in monocytes was studied. In chapter 3, it was shown that FIPV enters cells via endocytosis and not via direct fusion at the plasma membrane. This study aimed to specify which endocytic pathway was used by FIPV in monocytes. There are two main categories of endocytic pathways: phagocytosis and pinocytosis. Pinocytosis can be divided in macropinocytosis, clathrin-mediated internalization, caveolae-mediated internalization and several clathrin- and caveolae-independent internalization pathways. To identify the pathway that was used by FIPV, three strategies were applied: internalization pathways were blocked with inhibitors or by expressing dominant-negative internalization proteins in monocytes, and double stainings were performed to analyse co-localization between FIPV and internalization proteins. Phagocytosis, macropinocytosis and clathrin-mediated internalization were clearly not involved in FIPV entry. Caveolae-mediated internalization was not involved either but sterol-binding drugs, applied to block this pathway non specifically, had a slight but significant reducing effect on FIPV uptake. Further, blocking the function of dynamin by an inhibitory peptide and expression of dominant-negative dynamin significantly reduced both the internalization and infection with FIPV in monocytes. Rho-GTPases were not involved in internalization. It was concluded that the entry of FIPV II in monocytes proceeded via a clathrin- and caveolae-independent pathway that depends on dynamin and is slightly cholesterol-depletion sensitive.

In **chapter 6**, the step after internalization, the release of the genome of FIPV type II strain 79-1146 from endosomes into the cytosol, was studied. This process was analysed by visualizing released nucleocapsids in the cytosol at different time points after the start of internalization. At 35 minutes after this starting point, the maximum number of cells with released genomes was observed. Compared to the percentage of cells with internalization, the percentage of cells with genome release was low. It appears that there is a restriction to infection at this level. This might explain why only a small fraction of the cells is susceptible to infection.

The release of nucleocapsids from endosomes occurs via fusion between viral envelopes and endosomal membranes. This process can be triggered by a number of factors such as the pH drop in late endosomes, protease activity, a receptor-induced conformational change or other unknown factors. By using inhibitors, it was shown that genome release of FIPV is not induced by low pH. Preliminary data suggest a role for serine and/or cysteine protease(s). In the future, the involvement of proteases will be further studied to identify the exact protease(s) that are involved in FIPV entry.

As a **general conclusion**, it can be stated that the process of FIPV entry in monocytes does not resemble FIPV entry in CrFK cells. However, serotype I and serotype II strains probably use different receptors in both cell types.

FIPV serotype II entry in monocytes occurs in a large majority of cells and proceeds exclusively via endocytosis. Based on the results obtained in chapters 3 to 6, a model for FIPV type II entry in monocytes was proposed. This is shown in Figure 1. The first pathway of internalization (pathway 1) results from binding to APN in the plasma membrane. Possibly APN resides in or is transported to lipid rafts. After binding, most likely, dynamin is recruited and a vesicle containing the virus-receptor complex is internalized via a clathrin- and caveolae-independent pathway. To release the viral genome from the vesicle into the cytosol, cysteine and/or serine protease activity might be necessary. Another, yet unknown receptor can also bind and internalize FIPV (pathway 2). It is not known if this receptor (rec X) is present in lipid rafts. The internalization also occurs via a clathrin- and caveolae-independent pathway, that probably also depends on dynamin. After internalization, the internalized vesicle might fuse with a DC-SIGN- or an APN-containing vesicle (this association between rec X and DC-SIGN or APN may also occur at the plasma membrane). How genome release is mediated, is not known but possibly cysteine and/or serine proteases induce fusion.

It seems like only a fraction of the cells that internalize virus, release free nucleocapsids from endosomes. This might explain - at least in part - the discrepancy between the fraction of cells that internalize virus and the fraction that is susceptible to infection.

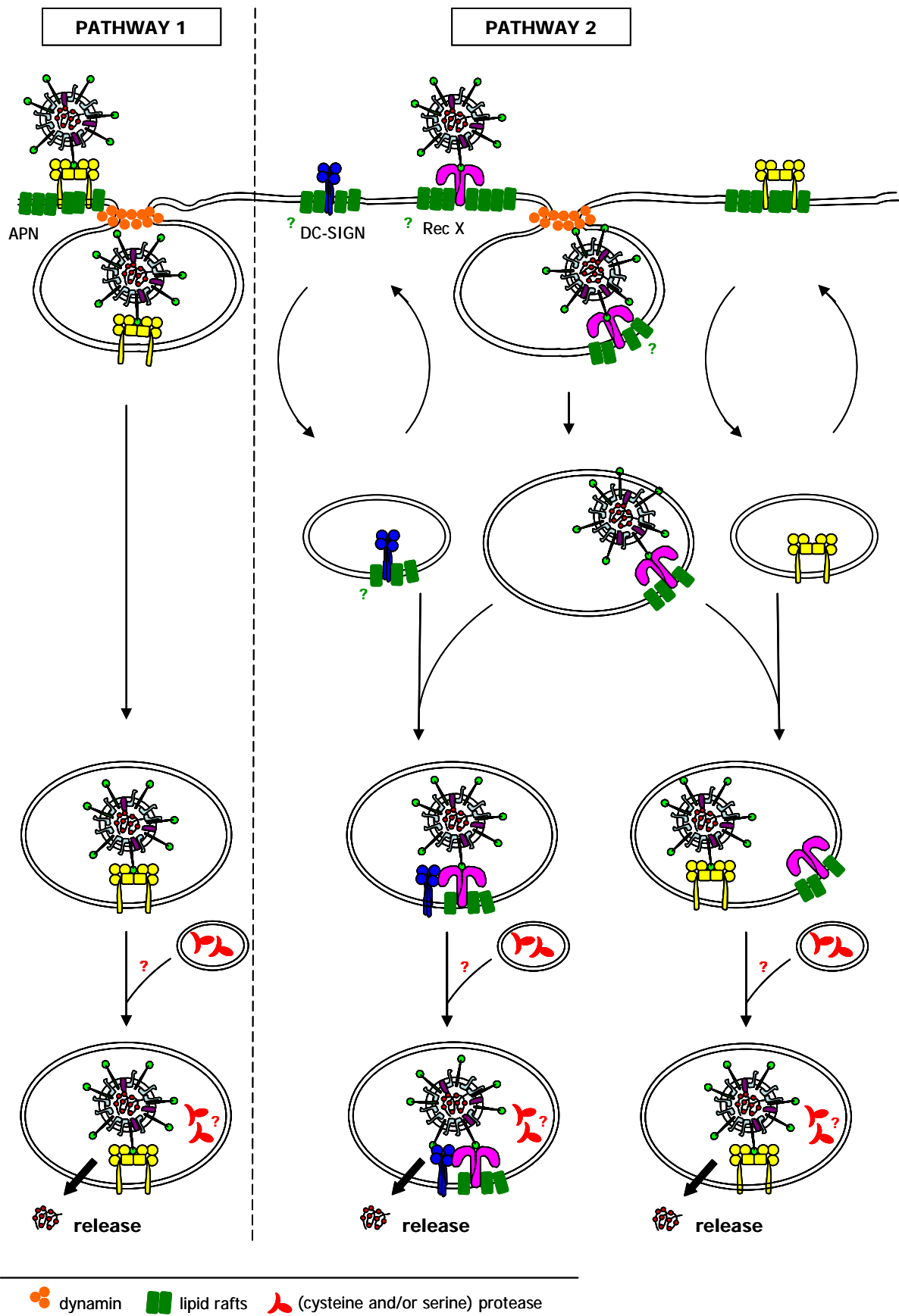


Figure 1: Hypothetical model for the entry of FIPV type II in feline blood monocytes based on the data obtained in this thesis.

The main conclusions that can be drawn from this thesis are:

- Crandell feline kidney cells can not be used as a model cell line to study FIPV entry.
- Serotype I and II FIPV strains might use different receptors on monocytes as they do in CrFK cells.
- FIPV serotype I and II internalization in monocytes is extremely efficient and occurs exclusively via endocytosis.
- fAPN is a virus binding and internalizing receptor for serotype II FIPV on monocytes, while DC-SIGN is not.
- fAPN is not the only receptor for serotype II FIPV on monocytes.
- FIPV serotype II internalization in monocytes is clathrin- and caveolae-independent, depends on dynamin and is slightly cholesterol depletion sensitive.
- Attachment and internalization of FIPV serotype II occurs in most cells, while genome release appears to be restricted to a fraction of the cells which might explain why most cells are not susceptible to FIPV infection.

SAMENVATTING

Binnen de feline coronavirussen bestaan er twee virulente varianten. Het feline infectieuze peritonitis virus (FIPV) is de meest bedreigende. Het virus veroorzaakt een monocyt-geassocieerde viremie die leidt tot een progressieve vasculitis. De infectie kent meestal een fatale afloop en er is geen effectieve behandeling beschikbaar. Om hierin verandering te brengen, is er een verbeterde kennis van de pathogenese van FIPV nodig. Vele nieuwe antivirale middelen zijn gericht tegen virusopname. Voor FIPV, is er echter weinig gekend over dit proces. Het is zelfs niet duidelijk of FIPV zijn doelwitcel, de monocyt, binnendringt via directe membraanfusie of via receptor-gemedieerde endocytose.

In deze thesis, is de opname van FIPV bestudeerd met als doel een deeltje van de pathogenese op te helderen en zo een stap dichterbij een effectieve behandeling.

In **hoofdstuk 1**, worden het FIP virus en het proces van virus opname beschreven. Het eerste deel van hoofdstuk 1 handelt over de taxonomie en pathogenese van FIPV. Verder worden de structuur van het virion en de productie van virale partikels bij infectie van een cel, beschreven. Het tweede deel van hoofdstuk 1 handelt over virus opname. De verschillende receptoren van coronavirussen in groep 1 worden opgesomd en de binding aan deze receptoren wordt besproken. Daarnaast worden alle mogelijke opnamewegen beschreven en worden verschillende coronavirussen gelinkt aan de opnameweg die hen in hun doelwitcel brengt.

In **hoofdstuk 2** wordt de doelstelling van deze thesis toegelicht.

In **hoofdstuk 3**, werd een kwantitatieve analyse gemaakt van de verschillende stappen in het proces van virusopname, namelijk binding en internalisatie. De kinetieken van binding en internalisatie werden bepaald door het aantal viruspartikels dat gebonden is aan een cel en het percentage van partikels die opgenomen zijn per cel, te kwantificeren op verschillende tijdstippen na inoculatie met virus. Deze kinetieken werden bepaald in de *in vivo* doelwitcel van het virus, feline bloedmonocyten, en in cellen van een continue cellijn, 'Crandell feline kidney' (CrFK) cellen. Deze cellijn werd ingesloten in de studie om te evalueren of ze gebruikt kon worden als model cellijn voor de studie van opname van FIPV in monocyten. Binding en internalisatie bleken helaas minder efficiënt te verlopen in de CrFK cellijn dan in monocyten, dus kunnen opname experimenten met deze cellen niet voorspellen hoe het virus met monocyten zal interageren.

Er werden twee virusstammen gebruikt in deze studie, een serotype I stam Black en een serotype II stam 79-1146, om zo eventuele verschillen tussen serotypes te bekijken. Virus binding was verschillend voor beide stammen, dit zou kunnen betekenen dat beide serotypes verschillende receptoren gebruiken in monocytten, zoals ze doen in CrFK cellen. Voor binding van het serotype II virus, werden ook verschillen opgemerkt tussen monocytten afkomstig van twee verschillende katten. Dit zou het gevolg kunnen zijn van verschillende expressie van een receptor die betrokken is bij virus opname. Deze hypothesen moeten nog verder onderzocht worden door meer stammen en meer katten in te sluiten in de studie.

Virus internalisatie was een zeer efficiënt proces in monocytten. Niet alleen werd de meerderheid van de gebonden partikels binnen 1 minuut geïnternaliseerd, dit proces voltrok zich ook in de meerderheid van de cellen. Dit staat in contrast met het beperkte aantal cellen dat gevoelig is voor infectie. Er werd ook aangetoond dat internalisatie gebeurde via receptor-gemedieerde endocytose en niet via directe fusie met de plasma membraan.

In **hoofdstuk 4**, werd de rol van felien aminopeptidase N (fAPN) en 'dendritic cell-specific intercellular adhesion molecule grabbing non-integrin' (DC-SIGN), als receptoren voor de serotype II FIPV stam 79-1146, bestudeerd in monocytten en in cellen van continue cellijnen. Eerst werd het effect bepaald van het blokkeren van deze proteïnen, en vervolgens werd de colocalisatie van FIPV en de receptoren bepaald.

Er werd vastgesteld dat het blokkeren van fAPN, binding en infectie in CrFK cellen verhindert en dat het virus volledig colocaliseert met deze receptor. In monocytten werden binding en infectie 'slechts' gereduceerd met respectievelijk 53 % en 44 %. Colocalisatie tussen FIPV en fAPN was beperkt tot 60 % van de viruspartikels. Virus kan dus via fAPN binden en opgenomen worden in monocytten en opname langs deze weg kan tot infectie leiden. Toch is er naast fAPN nog een receptor voor binding en internalisatie, wat niet het geval is in CrFK cellen.

Om te evalueren of humaan DC-SIGN (hDC-SIGN) FIPV kon binden en internaliseren, werden CHO cellen met hDC-SIGN getransduceerd. De transductie zorgde ervoor dat deze cellen virus konden binden en opnemen, maar de cellen konden niet geïnfecteerd worden. Het blokkeren van fDC-SIGN in monocytten had geen effect op virus binding en infectie. Verder was er ook geen colocalisatie tussen FIPV en fDC-SIGN. fDC-SIGN bleek duidelijk niet de tweede receptor, naast fAPN, op monocytten te zijn. Het blokkeren van fDC-SIGN in cellen waarin fAPN ook geblokkeerd is, veroorzaakte echter wel een bijkomende inhibitie van infectie van 56 naar 18 %. Het lijkt alsof fDC-SIGN niet nodig is voor binding en internalisatie in monocytten, maar wel een rol speelt in infectie onafhankelijk van fAPN.

Uit deze studie bleek dat virus-receptor interacties in CrFK cellen niet representatief zijn voor interacties in *in vivo* doelwit cellen. Verder kon ook een model opgesteld worden van de receptor interacties bij opname van serotype II FIPV in monocytten.

In **hoofdstuk 5**, werd het mechanisme van internalisatie van FIPV serotype II stam 79-1146 in monocytten bepaald. In hoofdstuk 3 werd aangetoond dat FIPV monocytten binnendringt via receptor-gemedieerde endocytose en niet via directe fusie met de plasma membraan. Deze studie had als doel te bepalen langs welke weg internalisatie van FIPV in monocytten gebeurt. Endocytose kan opgedeeld worden in twee klassen: fagocytose en pinocytose. Pinocytose omvat macropinocytose, clathrine-gemedieerde internalisatie, caveolae-gemedieerde internalisatie en verschillende clathrine- en caveolae-onafhankelijke internalisatie wegen. Om de opnameweg van FIPV te bepalen, werden drie strategieën toegepast: internalisatie wegen werden geblokkeerd met inhibitoren of door expressie van dominant-negatieve internalisatie proteïnen in monocytten, en dubbelkleuringen werden uitgevoerd om de colocalisatie tussen virus en internalisatie proteïnen te bekijken. Fagocytose, macropinocytose en clathrine-gemedieerde internalisatie bleken duidelijk niet betrokken bij de opname van FIPV. Caveolae-gemedieerde internalisatie was ook niet betrokken maar de sterol-bindende middelen die gebruikt werden om caveolae-gemedieerde internalisatie specifiek te inhiberen, hadden een klein, maar significant reducerend effect op de opname van FIPV. Het inhiberen van de functie van dynamine, met een dynamine inhiberend peptide en expressie van dominant-negatief dynamine, reduceerde opname en infectie met FIPV significant. Rho-GTPasen bleken niet betrokken bij opname van FIPV. Er werd geconcludeerd dat de opname van FIPV serotype II in monocytten gebeurt via een clathrine- en caveolae-onafhankelijke weg die afhankelijk is van dynamine en licht gevoelig is voor het onttrekken van cholesterol.

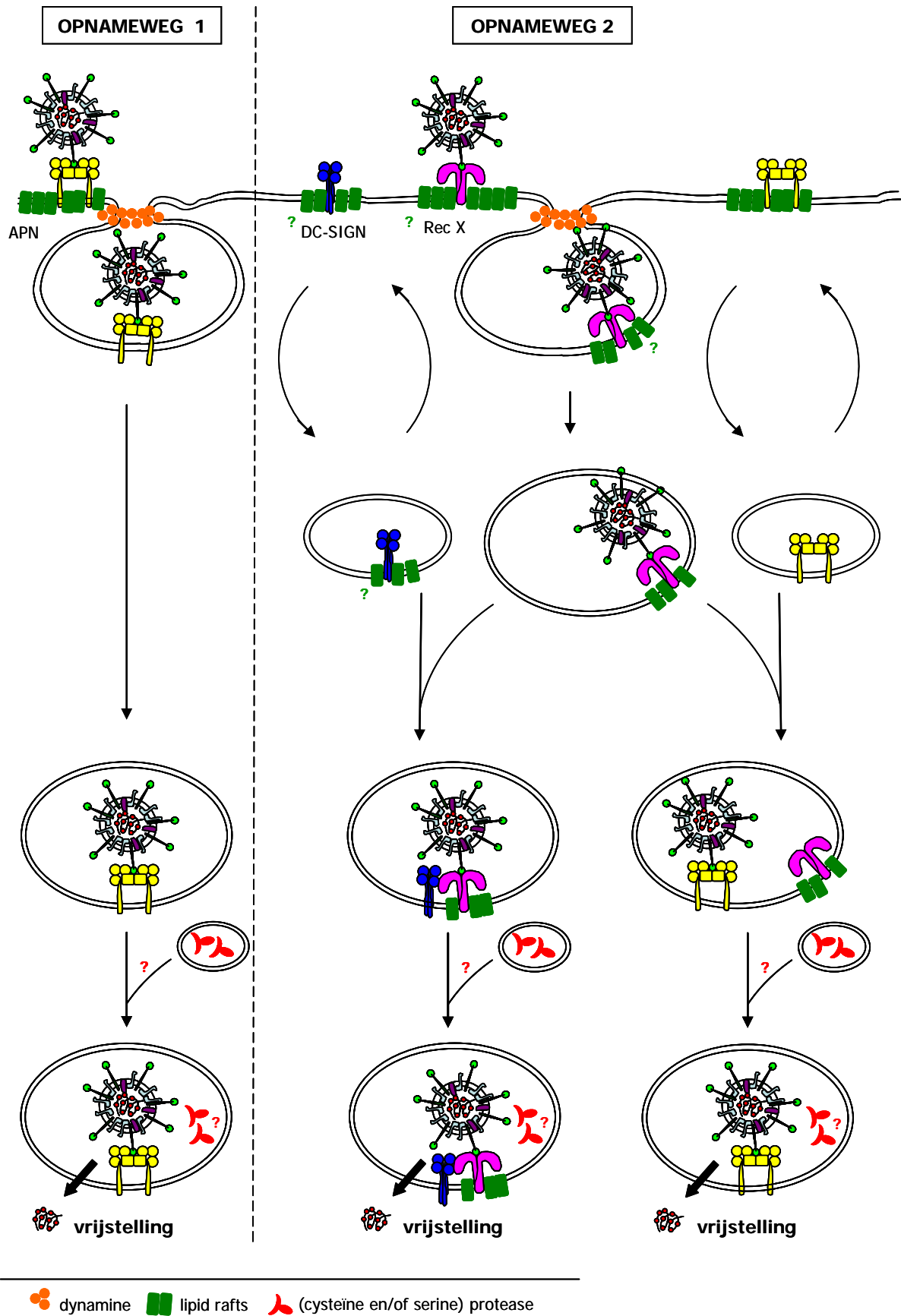
In **hoofdstuk 6**, werd de stap bestudeerd die volgt op internalisatie via endocytose, namelijk de vrijstelling van het genoom in het cytosol. Dit proces werd geanalyseerd door vrije nucleokapsids in het cytosol te visualiseren op verschillende tijdstippen na de start van internalisatie. Op 35 minuten na de start van internalisatie, werd het maximaal percentage cellen met vrijgestelde nucleokapsids gedetecteerd. In vergelijking met het aantal cellen die virus internaliseren, was het aantal cellen met vrijstelling laag. Het lijkt alsof genoomvrijstelling een stap is in de replicatiecyclus die niet in alle cellen kan doorgaan. Dit zou kunnen verklaren dat slechts een laag percentage cellen geïnfecteerd kan worden.

De vrijstelling van nucleokapsieds uit endosomen resulteert uit fusie van de virale envelop met de membraan van endosomen. Deze fusie kan geïnduceerd worden door verschillende factoren zoals de verlaagde pH in late endosomen, protease activiteit, een receptor-geïnduceerde conformatie verandering of andere onbekende factoren. Aan de hand van inhibitoren werd aangetoond dat de verlaagde pH in endosomen niet noodzakelijk is voor de vrijstelling van FIPV. Volgens preliminaire experimenten, zouden serine en/of cysteine proteasen echter wel betrokken zijn bij de genoomvrijstelling van FIPV. In de toekomst zal de rol van proteasen in de genoomvrijstelling van FIPV verder onderzocht worden zodat de proteasen geïdentificeerd kunnen worden die de vereiste membraanfusie induceren.

Als **algemene conclusie**, kan gesteld worden dat de opname van FIPV in monocytten niet vergelijkbaar is met opname in CrFK cellen. Toch gebruiken serotype I en II stammen waarschijnlijk verschillende receptoren in beide celtypes.

FIPV serotype II opname in monocytten vindt plaats in de meeste monocytten en gebeurt via endocytose. Op basis van de resultaten die bekomen zijn in de hoofdstukken 3 tot 6, werd een hypothetisch model voorgesteld voor de opname van serotype II FIPV in monocytten. Dit is weergegeven in figuur 1. De eerste opnameweg (opnameweg 1) wordt gevolgd na binding aan APN in de plasma membraan. Het is mogelijk dat APN gelokaliseerd is in of getransporteerd wordt naar lipid rafts. Na binding, wordt dynamine waarschijnlijk gerecruiteerd naar het internaliserend vesikel dat uiteindelijk opgenomen wordt via clathrine- en caveolae-onafhankelijke internalisatie. Serine en/of cysteine protease activiteit zou nodig kunnen zijn om genoomvrijstelling uit de endosomen te induceren. FIPV zou ook kunnen binden aan en internaliseren via een tweede, nog onbekende receptor (opnameweg 2). Uiteraard is niet bekend of deze receptor (rec X) zich in lipid rafts bevindt. Waarschijnlijk is deze opnameweg ook onafhankelijk van clathrine en caveolae, maar afhankelijk van dynamine. Na internalisatie, zou het vesikel kunnen fusioneren met een DC-SIGN- of APN-bevattend vesikel (tenzij de associatie tussen rec X en DC-SIGN of APN reeds ter hoogte van de plasma membraan plaats vindt). Hoe de daaropvolgende genoomvrijstelling gebeurt, is niet gekend, maar mogelijks zijn serine en/of cysteine proteasen hierbij betrokken.

Het lijkt alsof slechts een fractie van de cellen met virus opname, in staat zijn om nucleokapsieds vrij te stellen uit endosomen. Dit zou - allerm minst gedeeltelijk - het verschil tussen de fractie cellen die virus internaliseren en de fractie die gevoelig is voor infectie, kunnen verklaren.



Figuur 1: Hypothetisch model voor de opname van serotype II FIPV in feliene bloed monocytten op basis van de data bekomen in deze thesis.

De belangrijkste conclusies van deze studie zijn:

- De 'Crandell feline kidney' cellijn kan niet gebruikt worden als model cellijn om de opname van FIPV te bestuderen.
- Er zijn indicaties dat serotype I en II stammen in monocyten verschillende receptoren gebruiken zoals aangetoond in CrFK cellen.
- Internalisatie van serotype I en II FIPV in monocyten is uiterst efficiënt en vindt uitsluitend plaats via endocytose.
- fAPN is een virus bindende en internaliserende receptor voor serotype II FIPV, maar DC-SIGN niet.
- fAPN is niet de enige receptor voor serotype II FIPV op monocyten.
- Internalisatie van serotype II FIPV in monocyten is clathrine- en caveoline-onafhankelijk, afhankelijk van dynamine en licht gevoelig voor het onttrekken van cholesterol.
- Binding en internalisatie van serotype II FIPV komt voor in de meerderheid van de cellen, terwijl genoomvrijstelling beperkt lijkt te zijn tot een fractie van cellen. Dit zou kunnen verklaren waarom de meeste cellen niet gevoelig zijn voor infectie.

CURRICULUM VITAE

Personalia

Evelien Van Hamme werd geboren in Zottegem op 25 juni 1980. In 1998 behaalde zij het getuigschrift van hoger secundair onderwijs aan het Sint-Franciscusinstituut te Brakel in de richting Latijn-wiskunde. In de Provinciale Industriële Hogeschool te Kortrijk volgde zij de opleiding tot Industrieel Ingenieur in de Biochemie. In 2003 studeerde zij daar af met grote onderscheiding. Daarna studeerde ze verder aan de Universiteit Gent en behaalde in 2005 het diploma van Bio-ingenieur in de Cel- en Genbiotechnologie met grote onderscheiding. Datzelfde jaar nog startte zij aan het laboratorium voor Virologie, vakgroep Virologie, Parasitologie en Immunologie van de Faculteit Diergeneeskunde, Universiteit Gent met het onderzoek beschreven in deze thesis. Zij beschikte hiervoor over een doctoraatsbeurs van het Bijzonder Onderzoeksfonds van de Universiteit Gent in de periode van oktober 2005 tot oktober 2009. Het onderzoek handelde over de binding en opname van het feliene infectieuze peritonitis virus in gastheercellen, namelijk bloed monocyten van de kat.

Publicaties

Publicaties in internationale wetenschappelijke tijdschriften

Cornelissen, E., Dewerchin, H. L., Van Hamme, E. and Nauwynck, H. J. (2009). Absence of antibody-dependent, complement-mediated lysis of feline infectious peritonitis virus infected cells. *Virus Res*, **144**, 285-289.

Dewerchin, H. L., Cornelissen, E., Van Hamme, E., Smits, K., Verhasselt, B. and Nauwynck, H. J. (2008). Surface-expressed viral proteins in feline infectious peritonitis virus-infected monocytes are internalized through a clathrin- and caveolae-independent pathway. *J Gen Virol* **89**, 2731-2740.

Van Hamme, E., Dewerchin, H. L., Cornelissen, E., Verhasselt, B. and Nauwynck, H. J. (2008). Clathrin- and caveolae-independent entry of feline infectious peritonitis virus in monocytes depends on dynamin. *J Gen Virol* **89**, 2147-2156.

Van Hamme, E., Dewerchin, H. L., Cornelissen, E. and Nauwynck, H. J. (2007). Attachment and internalization of feline infectious peritonitis virus in feline blood monocytes and Crandell feline kidney cells. *J Gen Virol* **88**, 2527-2532.

Cornelissen, E., Dewerchin, H. L., Van Hamme, E. and Nauwynck, H. J. (2007). Absence of surface expression of feline infectious peritonitis virus (FIPV) antigens on infected cells isolated from cats with FIP. *Vet Mic* **31**, 121-131.

Publicaties in voorbereiding

Van Hamme, E., Dewerchin, H. L., Cornelissen, E. and Nauwynck, H. J. Intriguing interplay between aminopeptidase N and DC-SIGN in feline infectious peritonitis virus entry in blood monocytes. In preparation.

Abstracts

Cornelissen, E., Dewerchin, H. L., Van Hamme, E., Bosseler, L. and Nauwynck, H. J. Absence of antibody-dependent, complement-mediated lysis of feline infectious peritonitis virus infected cells.

- XIth international symposium on Nidoviruses, 22nd-27th June 2008, Oxford, United Kingdom.
- Belgian Society for Microbiology: 14th annual symposium, 12th December 2008, Brussels, Belgium.

Dewerchin, H. L., Cornelissen, E., Van Hamme, E. and Nauwynck, H. J. A new internalization pathway revealed by an immune evasion strategy used by feline infectious peritonitis virus.

- XIth international symposium on Nidoviruses, 22nd-27th June 2008, Oxford, United Kingdom.
- Belgian Society for Microbiology: 14th annual symposium, 12th December 2008, Brussels, Belgium.

Van Hamme, E., Dewerchin, H. L., Cornelissen, E., Verhasselt, B. and Nauwynck, H. J. Clathrin- and caveolae-independent entry of feline infectious peritonitis virus in monocytes depends on dynamin.

- XIth international symposium on Nidoviruses, 22nd-27th June 2008, Oxford, United Kingdom.
- Belgian Society for Microbiology: 14th annual symposium, 12th December 2008, Brussels, Belgium.

Van Hamme, E., Dewerchin, H. L., Cornelissen, E. and Nauwynck, H. J. Attachment and internalization of feline infectious peritonitis virus in monocytes and Crandell feline kidney cells.

- Early steps of the viral life cycle: molecular and cellular insights, 4th-5th October 2007, Paris, France.
- Belgian Society for Microbiology: 13th annual symposium, 23rd November 2007, Brussels, Belgium.

Cornelissen, E., Dewerchin, H. L., Van Hamme, E. and Nauwynck, H. J. Absence of surface expression of feline infectious peritonitis virus (FIPV) antigens on infected cells isolated from cats with FIP. 7th International Congress of Veterinary Virology - ESVV, 24th-27th September 2006, Lisbon, Portugal.

Dewerchin, H. L. & Cornelissen, E., Van Hamme, E. and Nauwynck, H. J. Possible immune evasion strategies of feline infectious peritonitis virus. Belgian Society for Microbiology: 12th annual symposium, 24th November 2006, Brussels, Belgium.

Mondelinge presentaties

Van Hamme, E., Dewerchin, H. L., Cornelissen, E., Verhasselt, B. and Nauwynck, H. J. Clathrin- and caveolae-independent entry of feline infectious peritonitis virus in monocytes depends on dynamin.

- XIth international symposium on Nidoviruses, 22nd-27th June 2008, Oxford, United Kingdom.
- Belgian Society for Microbiology: 14th annual symposium, 12th December 2008, Brussels, Belgium.

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Werken aan een doctoraat is in zekere zin heel individueel, je werkt aan je eigen onderzoek en bepaalt uiteindelijk zelf wat wanneer moet gebeuren. Anderzijds is het voltooien van een doctoraat niet mogelijk zonder een 'serieuze' achterban. Aan die achterban is dit laatste woordje gewijd...

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