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Elina Kakkonen

Regulation of Raft-derived Endocytic Pathways

Studies on Echovirus 1 and Baculovirus

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ABSTRACT

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Yhteenveto: Echovirus 1:n ja bakuloviruksen soluun sisäänmenon reitit ja säätely

To efficiently enter cells, viruses utilize different endocytic pathways. In this thesis, the regulators and entry routes utilized by a human pathogen, echovirus 1 (EV1), as well as an insect pathogen and potential gene therapy vector, baculovirus *Autographa californica* multiple nucleopolyhedrovirus, were studied. EV1 was shown to enter cells with fluid-phase markers in tubulovesicular structures that matured into multivesicular bodies. The pathway was regulated by factors that have been associated generally with macropinocytosis, namely phospholipase C, phosphatidylinositol 3-kinase, Rac1 and p21-activated kinase-1. C-terminal binding protein1/brefeldin A-ADP ribosylated substrate (CtBP1/BARS) was shown to be a novel regulator of epidermal growth factor-induced macropinocytosis, and CtBP1/BARS also had an essential role in the entry of EV1 and α2β1 integrin, the receptor of EV1. EV1 and α2β1 integrin originated from lipid raft-derived plasma membrane areas, and the internalization was dependent on cholesterol both at the plasma membrane and during the later stages of entry. The multivesicular structures formed after α2β1 integrin clustering (α2-MVBs) were sensitive to cholesterol-aggregating drugs, but not to Triton X-100 treatment, indicating that they were enriched in cholesterol and possibly raft lipids. Cholesterol-aggregating drugs also inhibited EV1 uncoating and had a great impact in the cytoplasmic α2-MVB structures.

Baculovirus internalization into mammalian cells was sensitive to a cholesterol-aggregating drug, and the internalization induced membrane ruffling. The virus entered cells together with fluid-phase markers in large, noncoated vesicles. Regulators of the clathrin-mediated pathway or macropinocytosis were not involved in baculovirus entry. Instead, the pathway was regulated by RhoA and Arf6, and the virus triggered the uptake of phagocytic marker *E.coli* into non-phagocytic human cells. These results suggest that baculovirus entry occurs via a mechanism that is reminiscent of phagocytosis.

Keywords: Baculovirus; cholesterol; CtBP1/BARS; echovirus 1; endocytosis; lipid rafts; regulators.

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I Karjalainen M.*, Kakkonen E.*, Upla P., Paloranta H., Kankaanpää P., Liberali P., Renkema G.H., Hyypiä T., Heino J. & Marjomäki V. 2008. A raft-derived, Pak1-regulated entry participates in α2β1 integrin-dependent sorting to caveosomes. Molecular Biology of the Cell, 19:2857-2869.

II Liberali P., Kakkonen E., Turacchio G., Valente C., Spaar A., Perinetti G., Böckmann R.A., Corda D., Colanzi A., Marjomäki V. & Luini A. 2008. The closure of Pak1-dependent macropinosomes requires the phosphorylation of CtBP1/BARS. The Embo Journal, 27:970-981.

III Kakkonen E., Rintanen N., Kirsi M., Upla P., Karjalainen M. & Marjomäki V. Perturbation of cholesterol in α2β1-integrin induced multivesicular bodies inhibits echovirus 1 uncoating and infection. Manuscript.

IV Laakkonen J.P., Mäkelä A.R., Kakkonen E., Turkki P., Kukkonen S., Peränen J., Ylä-Herttuala S., Airenne K.J., Oker-Blom C., Vihinen-Ranta M. & Marjomäki V. Clathrin-independent entry of baculovirus triggers uptake of *E.coli*. Submitted manuscript.

* Equal contribution

RESPONSIBILITIES OF ELINA KAKKONEN IN THE ARTICLES OF THIS THESIS

Article I: I conducted the experiments together with Mikko Karjalainen. I was responsible for the studies of EV1 infection and α2β1 integrin internalization in experiments with dynamin, GPI-AP, CTxB and Pak1. I performed the siRNA-assays together with Mikko Karjalainen. I conducted the transferrin/dextran internalization assays of transfected Pak1 constructs. I was responsible for the confocal microscopy studies together with Mikko Karjalainen. I participated in writing of the article together with Mikko Karjalainen and Varpu Marjomäki.

Article II: I am responsible for the microinjections for EV1 and SV40 experiments. I performed the EV1 infection and internalization studies and the infection studies of SV40. I am responsible for the confocal microscopy and electron microscopy studies of EV1 and α2β1 integrin. I participated in writing of the article together with Prisca Liberali and Varpu Marjomäki.

Article III: I conducted the EV1 infection, α2β1 integrin internalization and confocal microscopy studies together with Nina Rintanen and Maija Kirsi. I performed the electron microscopy experiments. I conducted the EV1 uncoating experiments together with Paula Upla. I wrote the article together with Varpu Marjomäki and processed all figures.

Article IV: I conducted the experiments together with Johanna Laakkonen and Anna Mäkelä. I was responsible for the transfection experiments of Rab34 and Pak1 constructs and the colocalization studies of IL-2 and baculovirus. I performed the siRNA-assays and conducted the colocalization studies of baculovirus and clathrin/flotillin together with Johanna Laakkonen. I participated in experiments of baculovirus colocalization with GPI-EGFP. I conducted the electron microscopy studies of HRP and baculovirus. Johanna Laakkonen and Varpu Marjomäki wrote the article and I participated in finalizing it.

ABBREVIATIONS

293	human embryonic kidney cell line
A431	human epidermoid carcinoma cell line
*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
Acyl-CoA	acyl coenzyme A
Ad	adenovirus
ADP	adenosine diphosphate
AP	adaptor protein
Arf6	ADP-ribosylation factor 6
Arp2/3	actin-related protein 2/3 complex
BV	budded virus
CA	constitutively active
CCP	clathrin coated pit
CCV	clathrin coated vesicle
Cdc42	cell division cycle 42 protein (GTPase)
CR3	complement receptor 3
CtBP1/BARS	C-terminal binding protein1/brefeldin A-ADP ribosylated substrate
CTxB	cholera toxin B subunit
CV-1	African green monkey kidney cell line
DN	dominant-negative
ECM	extracellular matrix
E.coli	*Escherichia coli*
EE	early endosome
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
EIPA	5-(N-ethyl-N-isopropyl)-amiloride
ER	endoplasmic reticulum
EV1	echovirus 1
FcR	receptor for Fc portion of immunoglobulins
FITC	fluorescence isothiocyanate
FYVE	Fab1p-YOPB-Vps27p-EEA1 domain
GAP	guanosine triphosphatase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GED	guanosine triphosphatase effector domain
GEEC	glycosylphosphatidylinositol-anchored protein-enriched early endosomal compartment
GEF	guanine nucleotide exchange factor
GPI-AP	glycosylphosphatidylinositol-anchored protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
GV	granulovirus

HeLa	human cervical adenocarcinoma cell line
HepG2	human hepatocarcinoma cell line
HEV	human enterovirus
HRP	horseradish peroxidase
HSV-1	herpes simplex virus-1
IL-2	interleukin-2
IRES	internal ribosome entry site
LAMP	lysosome-associated membrane protein
LBPA	acid/bis(monoacylglycerol) phosphate
LDL	low density lipoprotein
LE	late endosome
LPA	lysophosphatidic acid
MIDAS	metal-ion dependent adhesion site
MNPV	multiple nucleopolyhedrovirus
MVB	multivesicular body
NAD^+	nicotinamide adenine dinucleotide
NPV	nucleopolyhedrovirus
ODV	occlusion derived virus
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
Pak1	p21-activated kinase-1
PH	pleckstrin homology domain
p.i.	post infection
PI3K	phosphatidylinositol 3-kinase
PI(3)P	phosphatidylinositol 3-phosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PLC	phospholipase C
PM	plasma membrane
PtdIns	phosphatidylinositol
Rab	Ras-like proteins from rat brain (GTPase)
Rac1	Ras-related C3 botulinum toxin substrate 1 (GTPase)
RhoA	Ras homolog gene family member A (GTPase)
RILP	Rab-interacting lysosomal protein
SAOS	human osteosarcoma cell line
SDS	sodium dodecyl sulfate
SH3	rouse sarcoma oncogene homology 3 domain
siRNA	small interfering RNA
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SV40	simian virus 40
TGN	*trans*-Golgi network
TRITC	tetramethylrhodamine isothiocyanate
VP	viral protein
WT	wild type

1 INTRODUCTION

The endocytic traffic inside cells is one of the most important events that cells use to survive. Many vital functions are mediated by endocytic mechanisms, including the regulation of cell-surface receptor expression, uptake of extracellular nutrients and antigen presentation. Having such a critical role in a cell's viability, it is not surprising that a large variety of viruses have evolved to hijack the same endocytic pathways for cell entry: after internalization, viruses get a free ride deeper into the cell and finally to the appropriate location for viral replication. Since these pathways are regulated by the same factors whether viruses are involved or not, finding out the regulators in viral entry may reveal interesting facts about the cellular pathways used.

Early entry is the first encounter between viruses and host cells. The focus of this thesis was to elucidate the regulators of endocytic pathways used by EV1 and baculovirus. Although the clathrin- and caveolin mediated pathways are probably the most exploited ones among the currently known viruses, neither of the viruses studied here was shown to utilize these routes. Instead, the regulators of EV1 pathway were those that are generally associated with macropinocytosis. Furthermore, CtBP1/BARS was shown to be a novel regulator of macropinocytosis and also to be involved in EV1 entry. Interestingly, internalization and infection of the nonenveloped EV1 were shown to be dependent on a component of lipid rafts, cholesterol, which is more often associated with enveloped virus entry, assembly and budding. The entry of baculovirus originated similarly from cholesterol-enriched plasma membrane areas, but it was regulated by factors that have been shown to play key roles in phagocytosis.

The studies concerning endocytic pathways in viral entry not only give information about the molecular basis of host cell susceptibility, but they are also needed for understanding the regulatory events during the virus–cell encounter. Understanding the events during viral entry will provide opportunities to block these intricate interactions, or, in the case of gene therapy, to develop suitable and efficient virus-derived gene therapy vectors.

2 REVIEW OF THE LITERATURE

2.1 Endocytic pathways in mammalian cells

Recent studies in the field of endocytosis have increased our knowledge about the mechanisms by which cells internalize particles and macromolecules. Although we can still divide the mechanisms into the two large categories ('phagocytosis' meaning uptake of large particles, and 'pinocytosis' meaning uptake of fluids and solutes), classifications under these categories get more and more challenging. In this thesis, pinocytosis has been divided into caveolae- and raft-mediated endocytosis, clathrin-mediated endocytosis and macropinocytosis. However, it is likely that this categorization will encounter some modifications in the future. For example, raft-mediated pathways are a group of endocytic routes that cannot be categorized by classical endosomal markers and which differ slightly from each other and from the previously known pathways (Lamaze et al. 2001, Sabharanjak et al. 2002, Damm et al. 2005, Kirkham & Parton 2005, Glebov et al. 2006). In the future, also these pathways might be classified more specifically into their own groups.

Regardless of the possible problems in classification, endocytic pathways have a crucial role e.g. in maintaining cellular homeostasis, development, nutrient uptake, degradation, antigen presentation and signal transduction. Endocytosis is a highly regulated process wherein membrane-bound or soluble ligands and transmembrane proteins are trapped in plasma membrane-derived vesicles that pinch off from the membrane. Following formation, vesicles are often targeted to early endosomes, from which the cargo can be transported to late endosomes and further to lysosomes for degradation. Within recycling endosomes, the cargo can be transported from early endosomes back to the plasma membrane. Different pathways may also include other intracellular structures that participate in cargo delivery. The endocytic machinery is often shared with the pathways, thereby forming a complex endocytic network inside the cell (Figure 1). Endocytosis is also utilized by a wide range of viruses and bacteria (Table 1) as an elegant way to invade cells.

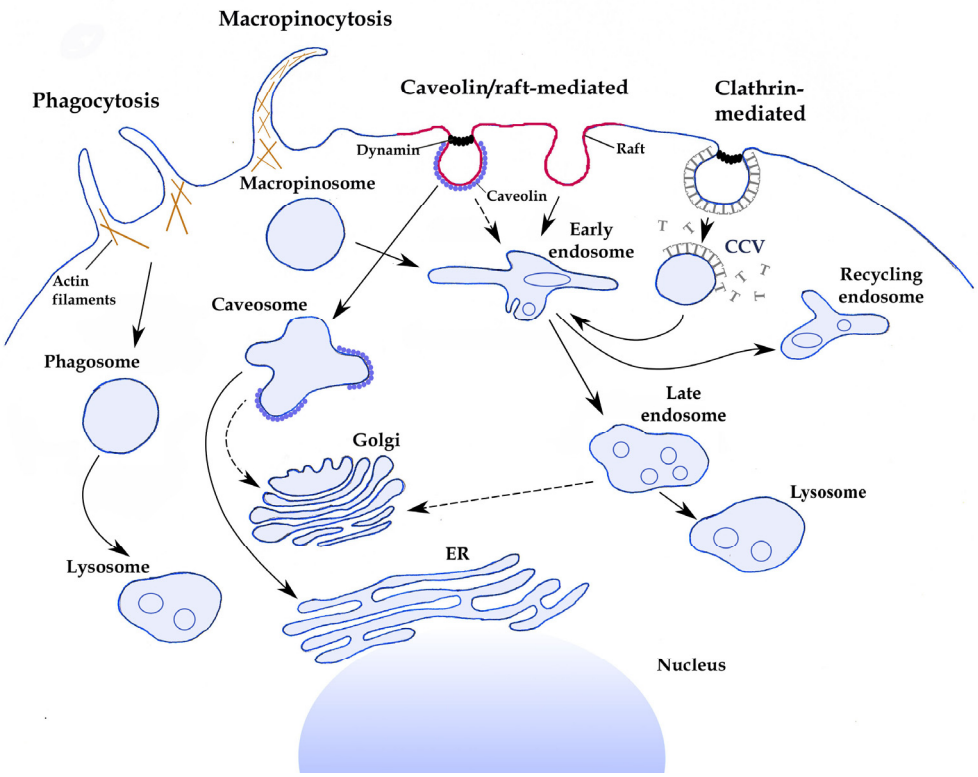

FIGURE 1 Endocytic pathways in mammalian cells. The intracellular machinery is often shared with different pathways. CCV, clathrin coated vesicle; ER, endoplasmic reticulum. Modified from Marsh & Helenius (2006) and Mayor & Pagano (2007).

2.1.1 Phagocytosis

Phagocytosis is a mechanism of innate immune defence, where specialized, 'professional' cells such macrophages, monocytes and neutrophils use it as a mechanism to get rid of large microbial pathogens (e.g. bacteria) and large debris (e.g. apoptotic cells) (Greenberg & Grinstein 2002, Underhill & Ozinsky 2002). In primitive organisms, phagocytosis is predominately a mechanism to supply nutrients, but in higher organisms it has a crucial role in triggering inflammation and defence mechanisms, and also in embryonic development and tissue remodelling (Aderem & Underhill 1999, Aderem 2003). In addition to these 'professional phagocytes', phagocytosis is also applied with similar mechanisms in 'non-professional phagocytes' such as fibroblasts and epithelial cells, although to a lesser degree due to their lack of efficient phagocytic receptors (Rabinovitch 1995). Cells bind the pathogens via phagocytic receptors after which the particle is rapidly internalized into specific membrane structures, phagocytic cups that mature into phagosomes. The phagosome further matures into a phagolysosome, where the internalized particle can be degraded (Beron et al. 1995).

Receptor engulfment leads to an extremely complex series of events, during which kinase activation and alterations in phospholipid metabolism are

required. Protein kinase C (PKC) participates in formation of actin filaments at least with five isomers (Allen & Aderem 1995), and it is activated by phospholipase C (PLC). Phosphatidylinositol 3-kinase (PI3K) is required for membrane pseudopod extension (Cox et al. 1999), a process which is also mediated by vesicle-associated membrane protein VAMP-3 (Bajno et al. 2000). Several myosin motors are recruited to the phagosome, where they appear with different kinetics and are needed in particle engulfment and phagosome closure (Diakonova et al. 2002, Olazabal et al. 2002). Dynamin's role in phagocytosis is rather controversial. Gold and colleagues (1999) have reported dynamin to be essential for the formation of phagosomes in macrophages, but the importance of dynamin in phagocytosis has also been questioned (Boleti et al. 1999, Tse et al. 2003).

A wide range of phagocytic receptors have been reported, including scavenger receptors, mannose receptors, Toll-like receptors and integrins. Best characterized are the receptors for opsonins, namely receptors for Fc portions of immunoglobulins (FcRs) and complement receptor 3 (CR3) (Underhill & Ozinsky 2002, Niedergang & Chavrier 2004). Phagocytosis is crucially dependent on the remodelling of the actin cytoskeleton, but the regulators of actin reorganization are determined by the ligand to be ingested. Ligation and clustering of FcRs (type I phagocytosis) triggers actin polymerization and particle internalization induced by the Rho GTPases Rac1 and Cdc42 (Caron & Hall 1998), and also the GTPase ADP-ribosylation factor (Arf)6 is activated (Niedergang et al. 2003). By contrast, CR3-mediated phagocytosis (type II phagocytosis) is induced only after Rho-activation (Caron & Hall 1998). The differences in activation of Rho GTPases and their downstream effectors could explain the lack of inflammation response in type II phagocytosis (Coso et al. 1995). At least one member of the Rab family has been associated with phagosome formation: Rab11 was shown to regulate membrane recruitment from recycling endosomes to phagosomes in FcR-mediated phagocytosis (Cox et al. 2000).

Soon after closure, phagosomes fuse with early endosomes, followed by fusions with late endosomes and finally lysosomes (Desjardins et al. 1994, Desjardins et al. 1997). The molecular mechanisms regulating phagosome maturation are largely unknown, but it is speculated that the same proteins that regulate fusion and fission events in the endocytic pathways are also involved in phagosome maturation. Members of the Rab family (see 2.2.4) (Desjardins et al. 1994) and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins syntaxin 7 and 13 (Collins et al. 2002) are associated with phagocytosis. During the maturation process proteins from early endosomal compartments (such as Rab5), late endosomes (Rab7) and lysosomes (lysosome-associated membrane protein (LAMP)-1) accumulate on the phagosomes (Vieira et al. 2002, Niedergang & Chavrier 2004) due to the multiple fusion/fission events. Class III phosphatidylinositol 3-phosphate (PI3P) has been shown to direct the fusion between phagosome and late endosomes/lysosomes (Vieira et al. 2001).

2.1.2 Macropinocytosis

Cells use macropinocytosis to internalize large amounts of fluid and membrane. As a response to cell stimulation, closure of lamellipodia occurs at the sites of membrane ruffling which leads to formation of large (0.2–5 μm), irregular macropinosomes that are readily labeled with fluid-phase markers (Swanson & Watts 1995, Swanson 2008). The process can be induced by growth factors or phorbol esters (Racoosin & Swanson 1989, Swanson 1989, West et al. 1989). However, macropinocytosis occurs constitutively in macrophages and dendritic cells where it is responsible for antigen presentation on both major histocompatibility complex (MHC) class I and class II molecules (Sallusto et al. 1995, Norbury et al. 1995, Norbury, et al. 1997). Membrane ruffling is strictly dependent on actin polymerization, driven by the Rho family GTPases Rac1 and Cdc42 (Ridley et al. 1992, Garrett et al. 2000). The closure of ruffles to form macropinosomes is mediated by PI3K (Amyere et al. 2000). Macropinocytosis is also regulated by Src family kinases (Veithen et al. 1996, Kasahara et al. 2007), PKC (Grimmer et al. 2002), PLC (Amyere et al. 2000), p21-activated kinase-1 (Pak1; downstream target of Rac1/Cdc42) (Dharmawardhane et al. 2000) and Rab34 GTPase (Sun et al. 2003). In addition, inhibition of amiloride-sensitive Na^+/H^+ exchanger at the plasma membrane inhibits macropinocytosis (West et al. 1989).

Newly formed macropinosomes undergo a cell type–dependent maturation process. In macrophages, they travel into the centre of the cell, decrease in size and became acidified, which is followed by fusion with late endosomes and finally lysosomes (Racoosin & Swanson 1992, 1993). This maturation is regulated by recruitment of sorting nexins (Kerr et al. 2006) that are a group of hydrophilic proteins widely employed in endosomal sorting and signaling (Cullen 2008). However, in nonmacrophage cell lines, macropinosomes form a distinct vesicle population that do not acidify or recruit markers of late endosomes or lysosomes, but recycle often back to the plasma membrane (Hewlett et al. 1994).

The early regulatory events in macropinocytosis share common features with phagocytosis. Both pathways require a dynamic actin cytoskeleton in cup formation and closure, regulated by small GTPases. In macropinocytosis, receptor signaling increases ruffling at the cell surface, and, analogously to phagocytosis, the signaling results in extension of the actin-rich phagocytic cup. In FcR-mediated phagocytosis and in epidermal growth factor receptor (EGFR)-stimulated macropinocytosis, the activated receptors recruit a similar combination of cytoplasmic proteins (Swanson 2008). Also, the essential signaling components required during particle ingestion, especially PI3K and the phosphoinositides generated (see 2.2.3) as well as phospholipases, are similar in macropinocytosis and phagocytosis. Both endocytic pathways also require PKC for efficient receptor endocytosis (Swanson 2008).

2.1.3 Clathrin-mediated endocytosis

A wide range of membrane proteins enter cells via clathrin-mediated endocytosis. These include nonsignaling receptors mediating the uptake of essential cellular nutrients, such as low density lipoprotein (LDL) and iron-laden transferrin (Conner & Schmid 2003), as well as receptors that trigger signal transduction events, such as most of the known growth factor receptors and G-protein-coupled receptors (Sorkin 2004). Clathrin-mediated endocytosis is also crucial in maintaining cellular homeostasis and in controlling intercellular communication during tissue and organ development (Conner & Schmid 2003).

In response to receptor-mediated internalization signals, clathrin is recruited to the cytoplasmic side of the plasma membrane which further leads to clathrin assembly into basket-like, regular polygonal lattices that surround vesicle invaginations called clathrin coated pits (CCPs) (Hirst & Robinson 1998). The clathrin assembling unit is clathrin triskelion, composed of three heavy chains, each with an associated regulatory light chain (Kirchhausen & Harrison 1981, Ungewickell & Branton 1981). The formation of CCPs requires also a variety of accessory and adaptor proteins that stimulate clathrin assembly or mediate the binding of clathrin to the membrane (Benmerah & Lamaze 2007). The major clathrin adaptor protein at the plasma membrane is adaptor protein 2 (AP2), which provides a bridge between cytoplasmic domains of endocytic receptors and the clathrin coat, thus concentrating receptors selectively into coated pits (Lewin & Mellman 1998). With the help of actin polymerization and dynamin activity, CCPs are gradually invaginated into clathrin coated vesicles (CCVs) (Hinshaw & Schmid 1995, Lamaze et al. 1997). Other accessory proteins, such as endophilin, epsin, amphiphysin and intersectin are also involved in formation of CCVs (Conner & Schmid 2003).

After CCV budding into the cell, the clathrin coat is rapidly removed by an uncoating ATPase (Schlossman et al. 1984) and CCVs fuse with early endosomes, considered the primary sorting stations in endocytic pathways. Low pH (6.2) in these compartments dissociates ligands from their receptors, after which constitutively recycling receptors, like transferrin and LDL receptors, are transported back to the plasma membrane via less acidic recycling endosomes. Downregulated receptors are transported further into late endosomes via intermediates with multivesicular appearance (multivesicular bodies, MVB) that fuse with late endosomes (Gruenberg 2001). Whether the membrane delivery in endocytic pathways occurs via transport vesicles between distinct compartments of stable identity (Helenius et al. 1983, Griffiths & Gruenberg 1991) or via vesicle maturation process (Helenius et al. 1983, Murphy 1991, Dunn & Maxfield 1992) has been the subject of much debate. Lately, the maturation model in which early endosomes increase the amount of internal vesicles during the maturation process into MVBs and finally into late endosomes, have gained attention (Russell et al. 2006, Woodman & Futter 2008). In addition, a model reconciling both the maturation and vesicle-transport models has been presented (Rink et al. 2005).

Sorting in early endosomes can result from the nature of the cargo: membrane-bound cargo is recycled back to the plasma membrane whereas cargo in fluid-phase is targeted further to late endosomes (Mayor et al. 1993). Furthermore, lipid composition (Mukherjee et al. 1999, Watanabe et al. 1999b) and specific sorting signals in receptors (Tanowitz & von Zastrow 2003) have been suggested to play a role in the sorting events. Late endosomes are highly complex and dynamic multivesicular structures, from which the cargo is transported to the final destination, lysosomes, where it is degraded by lysosomal enzymes. The endocytic structures in the clathrin-mediated pathway can share common proteins, but they still have their unique protein compositions, fusion events mainly mediated by the SNARE proteins together with the Rab family of GTPases (see 2.2.4), and possibly proteins of the annexin family (Gruenberg & Maxfield 1995, Gruenberg 2001).

2.1.4 Caveolae/raft-mediated endocytosis

2.1.4.1 Caveolae-mediated endocytosis

The caveolar pathway is, together with the clathrin-mediated pathway, one of the best characterized dynamin-dependent endocytic routes so far. It has been linked to many cellular functions, including signal transduction (Lisanti et al. 1994), transcytosis (Schnitzer et al. 1994) and calcium entry and signaling (Fujimoto 1993, Isshiki & Anderson, 2003). Morphologically, caveolae are smooth, flask-shaped, 50-80 nm invaginations of the plasma membrane that are rich in cholesterol and sphingolipids. The main protein component, caveolin-1 (caveolin-3 in muscle cells, Way & Parton 1995), is an integral, cholesterol-binding protein that has been shown to be essential for the formation of caveolae (Rothberg et al. 1992, Drab et al. 2001). Caveolins undergo two stages of oligomerization, during which caveolin monomers are first assembled into oligomers which then interact with each other and form the characteristic caveolae-shaped structures (Monier et al. 1995, Sargiacomo et al. 1995). The cells lacking caveolin-1 have no membrane invaginations classified as caveolae, but, on the other hand, caveolae formation can be induced in such cells by expressing caveolin-1 (Fra et al. 1995, Engelman et al. 1997). Caveolin-1 is not exclusively expressed on the plasma membrane, since it can reside also in the Golgi complex (where also the assembly of caveolar domains takes place, Tagawa et al. 2005), in recycling endosomes (Gagescu et al. 2000) and in the endoplasmic reticulum (ER) (Smart et al. 1994).

The plasma membrane caveolae are enriched in cholesterol and sphingolipids, thereby forming a subclass of specific microdomains, lipid rafts (see 2.1.4.2). Caveolar structures are highly immobile at the plasma membrane, anchored by actin cytoskeleton (Parton et al. 1994). This rigidity can be overrun by inducing a signaling cascade, possibly by cross-linking caveolae-located transmembrane tyrosine kinase receptors, or by cross-linking other caveolar components such as glycosylphosphatidylinositol-anchored proteins (GPI-APs)

(Pelkmans & Helenius 2002). The composition of the signaling complexes and the activity of signaling molecules are regulated by caveolins that act as a scaffold onto which specific signaling molecules (e.g. Src family tyrosine kinases) can bind (Li et al. 1996, Okamoto et al. 1998). Tyrosine phosphorylation of caveolin-1 eventually induces the internalization of caveolae (Aoki et al. 1999, Nomura & Fujimoto 1999). The uptake of cargo by the caveolar-mediated pathway leads to fusion with caveolin-1 positive, pH neutral organelles termed caveosomes (Pelkmans et al. 2004) from which the cargo can be further sorted to other cellular locations (Le & Nabi 2003). Caveolae themselves can fuse with early endosomes in a Rab5-dependent manner (Pelkmans et al. 2004) and enter the classical degradative pathway that leads to multivesicular bodies or lysosomes (Botos et al. 2007).

An important feature of caveolin-1 is its ability to bind cholesterol with high affinity (Murata et al. 1995). Caveolin-1 has been shown to play a role in the efflux of plasma membrane derived cholesterol to high density lipoprotein acceptors (Fielding & Fielding 1995), and also in the transport of intracellular, LDL-derived cholesterol back to the plasma membrane (Fielding & Fielding 1996). Furthermore, caveolin-1 is suggested to be involved in the transport of newly synthesized cholesterol from the ER to the caveolae on the plasma membrane (Smart et al. 1996, Uittenbogaard et al. 1998).

2.1.4.2 Endocytosis via lipid rafts

Cell membranes can incorporate microdomains enriched in certain specialized lipids and associated proteins. The most actively studied lipid domains are lipid rafts, dynamic islets of sphingolipids and cholesterol (Simons & Ikonen 1997). These domains are thought to form clusters in exoplasmic leaflets, where sphingolipids and cholesterol pack tightly excluding lipids with unsaturated hydrocarbon tails (Simons & Ikonen 1997, Brown & London 1998). Highly ordered lipid rafts with saturated hydrocarbons form distinct liquid-ordered phases in the lipid bilayer and they are laterally mobile in the surrounding plane of more liquid-disordered phase of unsaturated glycerolipids (Simons & Ikonen 1997, Simons & Toomre 2000, Wang & Silvius 2001). The insolubility of many lipid raft components in some detergents, such as the nonionic detergent Triton X-100 (Brown & Rose 1992), is due to the tight packing of the raft lipids and it is critically dependent on the presence of cholesterol. Lipid raft domains are suggested to be typically small and short-lived under steady-state conditions, and require clustering by ligand binding to induce raft stabilization and function (reviewed in Kusumi et al. 2004).

Lipid rafts are complex structures which vary both in size and protein content (Edidin 2001). The dynamic association/dissociation of raft proteins allows rafts to concentrate specific signaling components while excluding others. Lipid rafts have an essential role in protein sorting and signaling by forming platforms to which proteins can attach (Simons & Toomre 2000). Many raft-associated proteins have been defined, including GPI-APs (Brown & London 1998, Sargiacomo et al. 1993), cholesterol-associated and palmitoylated

proteins (Rietveld et al. 1999), and dually acylated proteins (e.g. the Src family tyrosine kinases) (Resh 1999). It is not known how the proteins interact with lipid rafts, but it is likely that they induce raft clustering, which ultimately leads to formation of a signaling complex composed of raft proteins and raft-interacting proteins (Simons & Toomre 2000). Rafts are most abundant at the plasma membrane, but their distribution between apical and basolateral membranes depend on cell type (Simons & Ikonen 1997). In addition to residing at the plasma membrane, rafts can also be found intracellularly in exocytic and endocytic compartments. These include at least recycling endosomes (Gagescu et al. 2000), late endosomes (Sobo et al. 2007), lysosomes (Simons & Gruenberg 2000) and exosomes (Wubbolts et al. 2003). However, the concept of lipid rafts is rather controversial. Debate has arisen e.g. from the size and formation of lipid rafts (Munro 2003, Mayor & Rao 2004, Shaw 2006, Kenworthy 2008).

Raft-dependent endocytosis encompasses a diverse set of clathrin-independent, cholesterol-sensitive endocytic pathways. Although these pathways share some common mechanisms, they vary e.g. in their dependence on expression of caveolin-1 and dynamin-2 (Le & Nabi 2003, Kirkham & Parton 2005). A clathrin- caveolin- and dynamin-independent, raft-mediated internalization pathway has been demonstrated for cholera toxin (Kirkham et al. 2005) and simian virus 40 (SV40) (Damm et al. 2005). Of note, cholera toxin (Henley et al. 1998) and SV40 (Pelkmans et al. 2001) can also internalize cells via dynamin- and caveolin -dependent, raft-associated mechanisms that differ in their dependence on Arf6 GTPase. In fact, Rho family GTPases regulate various raft-dependent endocytic routes. GPI-anchored proteins have been shown to enter cells via a Cdc42-regulated, dynamin-, clathrin- and caveolin-independent pathway (Sabharanjak et al. 2002). Also, uptake of lipid raft-associated interleukin-2 (IL-2) receptors occurs in RhoA-regulated, clathrin- and caveolin-independent but dynamin-dependent manner (Lamaze et al. 2001). Recently found, raft-located flotillin-1 defines its own, clathrin-, caveolin- and dynamin-independent pathway of cell entry (Glebov et al. 2006, Rajendran et al. 2007), whereas the autocrine motility factor uses a clathrin- and caveolin-independent but dynamin-dependent raft pathway (Kojic et al. 2007).

The intracellular destination of the raft-derived molecules can vary depending on both the particle itself and the cell type. For example, in epithelial cells GPI-anchored proteins can be selectively recruited to compartments termed GPI-anchored protein-enriched early endosomal compartments (GEECs), and are then delivered via tubular intermediates into recycling endosomes and back to the plasma membrane (Sabharanjak et al. 2002). However, in fibroblasts GPI-anchored proteins can be delivered to early endosomes and further to late endosomes (Fivaz et al. 2002). In the case of IL-2, the intracellular fate of the receptor is either to recycle back to the plasma membrane or to be degraded in lysosomes, depending on the different chains in the multimeric receptor (Hemar et al. 1995).

Raft-dependent endocytosis is a highly complicated process, where similar cargo can utilize different pathways, and where the destination of cargo can

vary. These pathways resemble often the 'clathrin-independent' pathways (reviewed in Kirkham & Parton 2005 and Mayor & Pagano 2007).

TABLE 1 Examples of pathogens utilizing endocytic pathways.

Endocytic pathway	Cargo	Reference
Caveolae/raft-mediated	SV40	Pelkmans et al. 2001
	Polyoma virus	Richterova et al. 2001
	EV1	Marjomäki et al. 2002
	EV11	Stuart et al. 2002
	Ebola virus	Bavari et al. 2002
	Marburg virus	Bavari et al. 2002
	HPV31	Smith et al. 2007
Clathrin-mediated	SFV	Helenius et al. 1980
	Influenza A virus	Matlin et al. 1981
	Ad2	Varga et al. 1991
	HRV14	DeTulleo & Kirchhausen 1998
	Sindbis virus	DeTulleo & Kirchhausen 1998
	HPEV1	Joki-Korpela et al. 2001
	VSV	Sun et al. 2005
	CVB3	Chung et al. 2005
	HCV	Blanchard et al. 2006
Macropinocytosis	HIV-1	Marechal et al. 2001
	Ad2	Meier et al. 2002
	Ad3	Amstutz et al. 2008
	Vaccinia virus	Mercer & Helenius 2008
	Salmonella	Francis et al. 1993
	Chlamydia	Ojcius et al. 1998
	H. influenzae	Ketterer et al. 1999
	M. tuberculosis	Garcia-Perez et al. 2003
Phagocytosis	HSV-1	Clement et al. 2006
	Mimivirus	Ghigo et al. 2008
	L. pneumophila	Horwitz 1983
	Listeria	Tilney & Portnoy 1989

Abbreviations: Ad, adenovirus; CVB3, coxsackievirus B3; EV, echovirus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; HPEV1, human parechovirus 1; HPV31, human papillomavirus type 31; HRV14, human rhinovirus 14; HSV-1, herpes simplex virus-1; SFV, semliki forest virus; SV40, simian virus 40; VSV, vesicular stomatitis virus.

2.2 Overview of regulators in endocytic pathways

2.2.1 Proteins acting in fission machineries at the plasma membrane

2.2.1.1 Dynamin

The large, 100 kDa GTPase dynamin has been considered to be the major regulator in membrane trafficking, since it is required for caveolae- and clathrin-mediated endocytosis, some lipid raft-mediated pathways and it is also implicated in phagocytosis. Dynamin is a mammalian homology to the *Drosophila shibire* gene product, whose temperature-sensitive mutations cause a pleiotropic defect in endocytosis (Kosaka & Ikeda 1983, van der Bliek & Meyerowitz 1991). In mammalian cells, three closely related tissue-specific dynamin isoforms are expressed, of which dynamin-1 is expressed exclusively in neurons (Nakata et al. 1991), dynamin-2 is ubiquitously expressed (Cook et al. 1994) and dynamin-3 is expressed in brain, lung and testis (Cook et al. 1996). In addition to acting in the plasma membrane fission machinery, dynamin may also be involved in membrane traffic at the *trans*-Golgi network (TGN) (Jones et al. 1998).

Dynamin's role has been studied most extensively in the clathrin-mediated pathway. In mammalian cells, overexpression of dominant-negative dynamin blocks endocytic clathrin coated vesicle formation (Damke et al. 1994). Dynamin is recruited to coated pits via interactions between its proline- and arginine-rich domain PRD and Src homology 3 (SH3)-domain containing proteins (Shpetner et al. 1996, Okamoto et al. 1997). Dynamin is evenly distributed on clathrin lattices in its guanosine diphosphate (GDP)-bound form, followed by guanosine triphosphate (GTP) binding that triggers dynamin's redistribution and self-assembly into rings and stacks around the neck of constricted coated pits (Hinshaw & Schmid 1995). The following vesicle budding is dependent on GTP hydrolysis, which is controlled by an intramolecular GTPase activating protein (GAP), encoded within dynamin's GTPase effector domain (GED) (Muhlberg et al. 1997). GTP hydrolysis drives dynamin disassembly and conformational changes, and GDP-bound dynamin is recycled (Warnock et al. 1996). The GTPase activity of dynamin is stimulated by membrane-associated phosphatidylinositol 4,5-biphosphate [$PI(4,5)P_2$] that interacts with dynamin's pleckstrin homology (PH) domain (Klein et al. 1998).

The functions of dynamin in the clathrin-mediated pathway may be dependent on its interactions with a wide range of SH3-domain containing proteins, including membrane-active molecules (e.g. amphiphysin and endophilin; Wigge et al. 1997, Ringstad et al. 1999), proteins from the sorting nexin -family (Lundmark & Carlsson 2003), actin-binding proteins (e.g. cortactin; McNiven et al. 2000b), and scaffolding molecules (e.g. Grb2; Ando et al. 1994). These interactions are, however, still poorly understood. Several models have been proposed to clarify the exact mechanism(s) of dynamin in controlling membrane dynamics in fission machineries. Most of these models

suggest that dynamin is a mechanoenzyme that binds to and hydrolyses GTP, resulting in a net motive force utilized to constrict and sever membrane tubules that leads to formation of transport intermediates (Sweitzer & Hinshaw 1998, Stowell et al. 1999, McNiven et al. 2000a). Other models relate to dynamin's GTPase activity, which regulates a rate-limiting step in endocytosis by recruiting downstream effectors (Sever et al. 2000a, Marks et al. 2001). Also dynamin's ability to regulate the actin cytoskeleton has been suggested to play a role in the fission (Lee & De Camilli 2002, Orth et al. 2002).

2.2.1.2 CtBP1/BARS

CtBP1/BARS is a 50 kDa cellular protein that belongs to a dual-function protein family involved in membrane trafficking and gene transcription. CtBP1 protein was originally discovered as a protein binding to the C-terminus of adenovirus early region protein E1A (thus CtBP1; C-terminal-binding protein) and negatively regulating oncogenic transformation (Boyd et al. 1993, Schaeper et al. 1995). Later, it was also identified as the ADP ribosylation substrate of a fungal toxin, brefeldin A (thus BARS; brefeldin A-ADP ribosylated substrate) (De Matteis et al. 1994, Spano et al. 1999), which disrupts the Golgi complex into a tubular network (Fujiwara et al. 1988). As a transcription corepressor, CtBP1/BARS is involved in numerous transcriptional regulatory programs controlling e.g. development, tumorigenesis, apoptosis and epithelial differentiation (Chinnadurai 2002, Grooteclaes et al. 2003). In cytoplasmic membrane fission, CtBP1/BARS is involved in mitotic Golgi partitioning and in fission of the tubules formed (Hidalgo Carcedo et al. 2004). In addition, CtBP1/BARS controls the fission machinery that participates in formation of post-Golgi transport carriers (Bonazzi et al. 2005) and COP1-coated vesicles (Yang et al. 2005).

CtBP1/BARS is speculated to drive membrane fission events differently and separately from dynamin. Recently, CtBP1/BARS was found to have a role in dynamin-independent fluid-phase endocytosis, since dextran internalization was strongly inhibited in different cell lines treated with siRNAs against CtBP1/BARS. Overexpression of a dominant-negative mutant of CtBP1/BARS caused a similar effect, whereas dynamin inhibition had no effect (Bonazzi et al. 2005). In addition, CtBP1/BARS and dynamin regulate different pathways derived from the TGN: dynamin regulates apical targeted pathways and CtBP1/BARS the basolateral targeted pathways (Bonazzi et al. 2005).

The precise mechanisms of CtBP1/BARS function are mainly unknown. The protein is recruited to the nucleus by DNA transcription factors that contain a PXDLS motif (where X is often leucine or valine) (Schaeper et al. 1995, Turner & Crossley 2001). CtBP1/BARS interacts with methyl transferases, demethylases and histone deacetylases, suggesting that its corepressive activity in gene transcription is mediated by a variety of chromatin remodeling agents (Shi et al. 2003). CtBP1/BARS is a dimer, of which each monomer is divided into large and small domains separated by a flexible hinge region (Figure 2) (Kumar et al. 2002). Based on its structure, CtBP1/BARS displays a significant

homology to nicotinamide adenine dinucleotide [NAD(H)]-dependent dehydrogenases and it binds both to NAD(H) and acyl coenzyme A (acyl-CoA) (Kumar et al. 2002). The NAD(H) binding probably promotes a close dimeric conformation and transcriptional activity by permitting the protein to make critical contacts with essential transcription factors or cofactors (Zhang et al. 2002, Mani-Telang et al. 2007), whereas acyl-CoA induces an open monomeric structure and is required for CtBP1/BARS-induced fission events (Weigert et al. 1999, Nardini et al. 2003). Activated by CtBP1/BARS, acyl-CoAs catalyze lysophosphatidic acid (LPA) acylation into phosphatidic acid (PA) which leads to accumulation of PA in membranes and further to a change in membrane curvature and membrane fission (Weigert et al. 1999). However, the acyl transferase activity of CtBP1/BARS has also been questioned (Gallop et al. 2005).

CtBP1/BARS was shown to be phosphorylated on a specific serine by Pak1 (Barnes et al. 2003), a downstream effector of Rac1 and Cdc42 in macropinocytosis (Dharmawardhane et al. 2000). Pak1-mediated phosphorylation had a regulatory function in nuclear-to-cytoplasmic translocation of CtBP1/BARS, thereby mediating the subcellular localization of CtBP1/BARS. Furthermore, CtBP1/BARS phosphorylation was shown to modulate the nuclear corepressor function of the protein by blocking its dehydrogenase activity (Barnes et al. 2003).

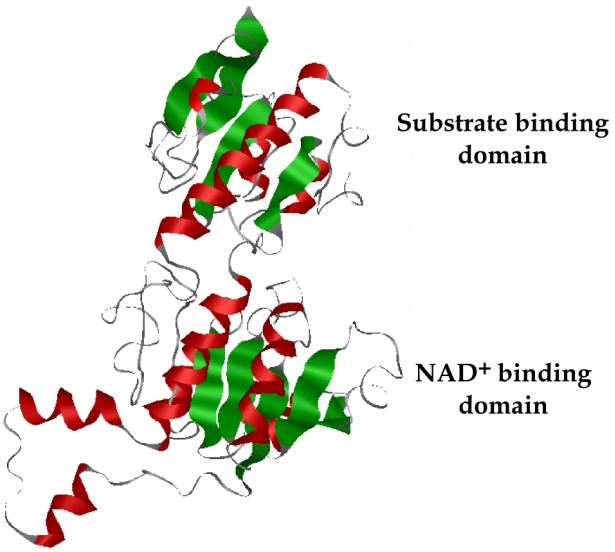

FIGURE 2 Ribbon diagram of the CtBP1/BARS monomer. The monomer is composed of the substrate binding domain and the NAD^+ binding domain, separated by a deep cleft. α-helixes are shown in red and β-sheets in green. The figure was created using Qmol version 4.02. The atomic coordinates were published by Nardini et al. (2003) and the coordinates were obtained from the Protein Data Bank, Brookhaven National Laboratory.

2.2.2 Rho GTPases

Rho GTPases are a subgroup of the large Ras superfamily of GTPases, other subgroups being Ras, Rab, Arf and Ran. The family of mammalian Rho GTPases consists of 20 intracellular signaling molecules that form eight different subfamilies (Figure 3) (Etienne-Manneville & Hall 2002, Heasman & Ridley 2008). Most Rho GTPases cycle between a GDP-bound (inactive) and a GTP-bound (active) state. When bound to GTP, Rho GTPases are stimulated by adhesion molecules, growth factors and cytokines, relaying signals to a large number of downstream effectors. The GTPase cycle is controlled by three types of regulatory proteins: guanine nucleotide exchange factors (GEFs) which stimulate the exchange of GDP allowing its replacement by GTP, GTPase-activating proteins (GAPs) that stimulate GTP hydrolysis, and guanine nucleotide dissociation inhibitors (GDIs) that bind to the GDP-bound form and maintain the GTPases in the inactive state (reviewed in Symons & Settleman 2000). Some Rho GTPases (RhoBTBs 1 and 2, RhoU, RhoH, RhoV and Rnd:s 1, 2 and 3) are predominantly GTP-bound and they are regulated by other mechanisms than GEFs and GAPs, e.g. phosphorylation, level of gene expression or protein-protein interactions (Symons & Settleman 2000, Aspenström et al. 2007).

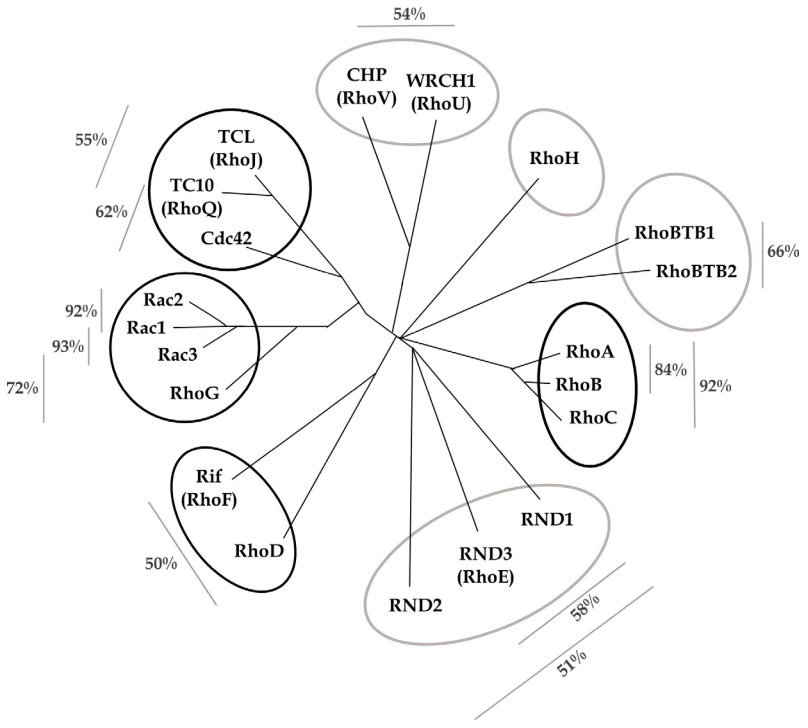

FIGURE 3 The relationship between eight Rho GTPase subfamilies composed of 20 different Rho GTPases. Black circles, classical Rho GTPases; grey circles, atypical Rho GTPases; %, amino acid sequence identity. Modified from Heasman & Ridley (2008).

Rho GTPases control a variety of essential cellular processes, including gene transcription, cell cycle progression, cell polarity, migration and cytokinesis, but their role in regulating the actin cytoskeleton is by far the most extensively studied (Hall 1998, Nobes & Hall 1999, Etienne-Manneville & Hall 2002). Three well known Rho GTPases, Rac1, Cdc42 and RhoA participate in regulation of the actin cytoskeleton by acting in the formation of lamellipodia, filopodia and actin stress fibres, respectively (Nobes & Hall 1995). In addition, other Rho GTPases such as RhoG, RhoD, Rif (RhoF) and TC10 (RhoQ) are also able to induce actin-based protrusions at the plasma membrane (Murphy et al. 1999, Ellis & Mellor 2000, Aspenström et al. 2004). Actin polymerization is stimulated via the actin-related protein (Arp)2/3 complex by Wiskott-Aldrich syndrome proteins that are in turn activated by Rac1 and Cdc42 (Aspenström et al. 1996, Rohatgi et al. 1999). The Arp2/3 complex nucleates actin filaments and stimulates the formation of branched actin filament networks (Millard et al. 2004). Other Rho GTPases activate Diaphanous-related formins that stimulate actin nucleation and extension of linear actin filaments (Wasserman 1998, Watanabe et al. 1999a, Pellegrin & Mellor 2005). Actin reorganization can also be regulated by the actin depolymerizing factor cofilin, which is phosphorylated and deactivated by LIM-kinases (Arber et al. 1998). LIM-kinases are in turn phosphorylated by the Rho-associated coiled-coil -containing kinase ROCK, which is activated by RhoA, RhoB and RhoC (Maekawa et al. 1999, Ridley 2006) or Pak-kinases 1-3 (activated by Rac1, Rac2 and Rac3) (Yang et al. 1998, Edwards et al. 1999, Wu et al. 2003, Ridley 2006).

Several Rho GTPases localize at the plasma membrane and/or different intracellular membrane structures (Table 2). Their interaction with membranes and precise localization is dependent on post-translational modifications, mostly prenylation and in some cases palmitoylation (Wennerberg & Der 2004). Although RhoA, Rac1 and Cdc42 are the three well known regulators of different plasma membrane derived endocytic pathways (reviewed in Ridley 2001 and Symons & Rusk 2003), and Cdc42 is a regulator of Golgi-to-ER transport (Luna et al. 2002), other Rho GTPases are also likely to participate in vesicle trafficking both in endocytic and exocytic pathways (Ridley 2001). In addition to regulating the membrane protrusions and membrane trafficking via reorganizing the actin cytoskeleton, Rho GTPases may also affect these events through microtubule-associated dynamics (Watanabe et al. 2005) and by regulating phosphoinositide metabolism (Oude Weernink et al. 2004).

TABLE 2 Localization of mammalian Rho GTPases.

Rho GTPase	Localization	Reference
Rac1	PM	Michaelson et al. 2001
Rac2	PM, Golgi, ER, NE	Michaelson et al. 2001 Roberts et al. 2008
Rac3	PM, endomembranes	Mira et al. 2000 Joyce & Cox 2003
RhoA	PM, cytosol	Adamson et al. 1992
RhoB	PM, Golgi, LE	Adamson et al. 1992 Robertson et al. 1995 Michaelson et al. 2001
RhoC	PM, cytosol	Adamson et al. 1992
RhoD	PM, EE	Murphy et al. 1996
RhoH	endomembranes	Roberts et al. 2008
RhoG	PM, endosomes	Prieto-Sanchez & Bustelo 2003
RhoBTB1	vesicular	Aspenström et al. 2004
RhoBTB2	vesicular	Aspenström et al. 2004
Rnd1	PM	Nobes et al. 1998
Rnd2	EE	Tanaka et al. 2002
Rnd3 (RhoE)	PM, Golgi, cytosol	Riento et al. 2003
Rif (RhoF)	PM	Ellis & Mellor 2000
Cdc42	PM, Golgi, ER, NE	Nobes & Hall 1995 Erickson et al. 1996 Michaelson et al. 2001
TC10 (RhoQ)	PM, perinuclear	Murphy et al. 1999
TCL (RhoJ)	PM, EE	de Toledo et al. 2003
Wrch1 (RhoU)	PM, EE	Berzat et al. 2005
Chp (RhoV)	PM, EE	Chenette et al. 2005

Abbreviations: EE, early endosome; ER, endoplasmic reticulum; NE, nuclear envelope; LE, late endosome; PM, plasma membrane. Modified from Ridley (2006).

2.2.3 Lipids

The timing and localization of endocytic membrane trafficking events is controlled by different kinds of lipids that are involved in protein recruitment in the transport machinery, regulation of protein sorting and formation of lipid microdomains (for lipid rafts, see 2.1.4.2). Phosphoinositides, short-lived derivatives of phosphatidylinositol (PtdIns), are enriched in the plasma membrane and in particular intracellular organelles (Figure 5) where they control signaling effects through the binding of their head groups to cytosolic proteins or to cytosolic domains of membrane proteins (Simonsen et al. 2001). Protein domains that bind phosphoinositides include the pleckstrin homology (PH) domain, the Fab1p-YOPB-Vps27p-EEA1 (FYVE) domain, the phox homology (PX) domain and the epsin N-terminal homology domain (ENTH) (Gillooly et al. 2001, Itoh & Takenawa 2002). Mediated by a variety of kinases and phosphatases, PtdIns can undergo rapid phosphorylation/

dephosphorylation cycles at positions 3', 4' and 5' of their inositol ring, thereby forming seven phosphoinositide species (Figure 4) (De Matteis & Godi 2004). Small GTPases, such as Ras, Arf6, RhoA, Rac1 and Cdc42, are primary regulators of the recruitment and/or activation of phosphatidylinositol kinases (Rodriguez-Viciana et al. 1994, Honda et al. 1999, Weernink et al. 2004).

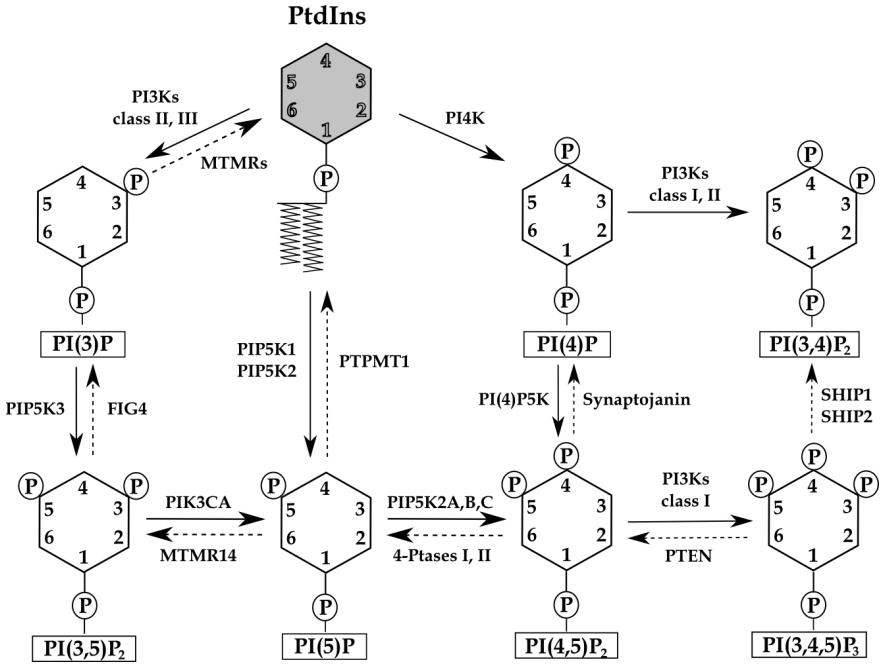

FIGURE 4 Phosphoinositides. Seven different phosphoinositides are formed by phosphorylation of 3′, 4′ or 5′ positions in the inositol ring of phosphatidylinositol (PtdIns). Some of the phosphorylation/ dephosphorylation pathways are illustrated. Kinases are indicated with black arrows and phosphatases with dashed arrows. Modified from Simonsen et al. (2001) and Lecompte et al. (2008).

Phosphatidylinositol 4,5-biphosphate [PI(4,5)P_2] is a key regulator of the formation, scission and uncoating of clathrin coated vesicles (Jost et al. 1998). Several proteins involved in clathrin-mediated endocytosis, such as dynamin (Schmid et al. 1998), epsin (Ford et al. 2002), two subunits of the AP2 complexes (Gaidarov & Keen 1999, Rohde et al. 2002) and AP180 (Ford et al. 2001) interact directly with PI(4,5)P_2 through their different PI-binding domains (De Matteis & Godi 2004, Haucke 2005). Dephosphorylation of PI(4,5)P_2 by synaptojanin may function during the uncoating of coated vesicles (Cremona & De Camilli 2001). Class I phosphatidylinositol 3-kinases convert PI(4,5)P_2 to PI(3,4,5)P_3, which acts by recruiting effectors, such as GAPs and GEFs for small GTPases, to membranes at which these factors regulate signaling pathways (Cantley 2002, Lindmo & Stenmark 2006). PI(4,5)P_2 has been shown to accumulate at the phagosomal cup, followed by a rapid disappearance upon recruitment of PLC and formation of diacylglycerol (Botelho et al. 2000). The products of its metabolism, e.g. PI(3,4,5)P_3, are also likely to have an essential role in pseudopod extension and phagosome closure (Botelho et al. 2000). In macropinocytosis, PI3K interacts with Rac, which then stimulates PI(4)P5-

kinase to produce PI(4,5)P_2, the target of PLC (Carpenter et al. 1997, Simonsen et al. 2001).

Phosphatidylinositol 3-phosphate [PI(3)P] is highly enriched in early endosomes and in the intralumenal vesicles of multivesicular bodies (Figure 5). Proteins containing FYVE zinc finger domains, e.g. early endosomal antigen (EEA)1 (Stenmark et al. 1996, Itoh et al. 2002), hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs) (Komada et al. 1997), and Smad anchor for receptor activation (SARA) (Itoh et al. 2002), are recruited to endosomal membranes by PI3P, where they have diverse functions in membrane trafficking (Gillooly et al. 2001). Several PI3P-binding proteins bind also the small GTPase Rab5, which in turn regulates PI(3)P synthesis (Shin et al. 2005). Furthermore, PI3P may also be involved in ubiquitin-dependent selection of proteins destined to degradation and in the biogenesis of transport intermediates (Gruenberg 2003). PI(3)P is phosphorylated to PI(3,5)P_2, which is important for the sorting of membrane proteins late in the endocytic pathway (Shaw et al. 2003). The internal vesicles of late endosomes are enriched in phospholipid lysobisphosphatidic acid/bis(monoacylglycerol) phosphate (LBPA), which through dephosphorylation by phospholipase A2 (Ito et al. 2002) could facilitate the multivesicular membrane invagination process (Gruenberg 2003). LBPA-membranes may also function in selecting proteins and lipids that are destined for degradation (Gruenberg 2003). In addition to phosphoinositides, other phospholipids, such as phosphatidic acid and phosphatidylserine, may have regulatory roles in intracellular membrane traffic (Haucke & Di Paolo 2007).

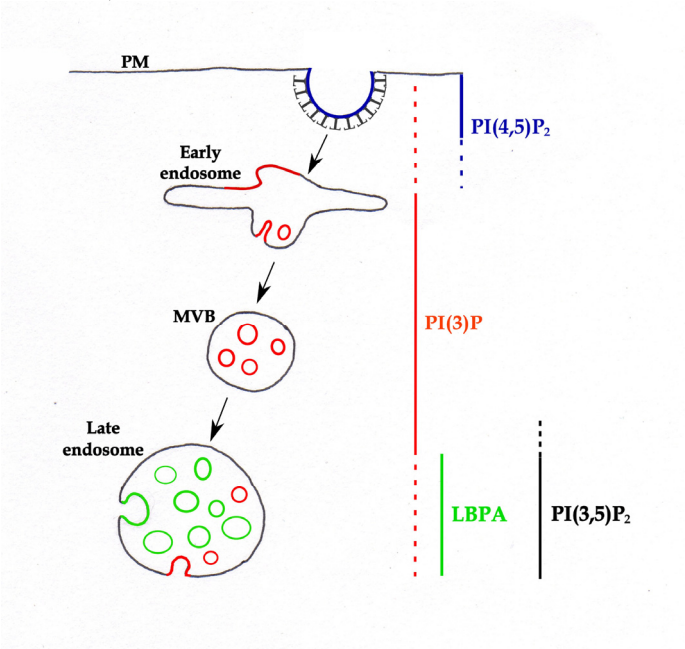

FIGURE 5 Different lipid territories in the endocytic pathway. PI(4,5)P_2 (blue) is predominantly found at the plasma membrane (PM), whereas PI(3)P (red) and PI(3,5)P_2 (black) are abundant in endosomes. Late endosomes are rich in LBPA (green) and also other membrane domains, e.g. cholesterol-rich lipid rafts (not shown). Modified from Gruenberg (2003).

As integral parts of special lipid microdomains (Simons & Ikonen 1997), cholesterol and sphingomyelins take part in most of the endocytic internalization routes currently known. In different intracellular endosomal compartments, cholesterol is present in variable amounts. Early and recycling endosomes as well as the internal membranes of multivesicular bodies contain high amounts of cholesterol, whereas late endosomes and lysosomes are normally poor in cholesterol (Kobayashi et al. 1998, Hao et al. 2002, Möbius et al. 2003). Cells acquire cholesterol by *de novo* synthesis in the ER and as cholesteryl ester in the form of low density lipoprotein, which is endocytosed and hydrolyzed in late endosomes/lysosomes into free cholesterol and fatty acids (Goldstein et al. 1985). Free cholesterol is then released to the plasma membrane and to the ER. The maintenance of cellular cholesterol balance in endosomes is crucial. For instance, cholesterol accumulation in late endocytic organelles has been implicated in a fatal neurological disease, Niemann Pick type C (Vanier & Millat 2003). Interestingly, Rab7 and Rab9 have been found to reduce cholesterol accumulation in late endosomes (Choudhury et al. 2002). Moreover, Rab GTPases, essential regulators of membrane trafficking (see 2.2.4), and their effectors have been shown to be involved in cholesterol recycling back to the plasma membrane (Hölttä-Vuori & Ikonen 2006, Takahashi & Kobayashi 2008).

An important feature of membrane lipids is their ability to generate membrane curvature. Several studies have shown that cholesterol is essential for the generation of high-curvature clathrin-coated buds and synaptic vesicles (Rodal et al. 1999, Subtil et al. 1999, Thiele et al. 2000). Selective accumulation of cholesterol into the budding leaflet of the membrane by two cholesterol-binding proteins, caveolin (Murata et al. 1995) and synaptophysin (Thiele et al. 2000), may decrease membrane rigidity and facilitate vesicle budding (Farsad & De Camilli 2003). Also asymmetries between the membrane leaflets, generated by flippase-mediated transfer of lipids, could enhance the formation of membrane curvatures (Farge et al. 1999). The direction of membrane curvature can be determined by the different geometries of membrane lipids, so that type I lipids ('cone shaped', e.g. lysophosphatidylcholine) create positive curvature and type II lipids ('inverted cone shaped', e.g. phosphatidylethanolamine) induce negative membrane curvature (Burger 2000). Lipids can also affect the membrane curvature indirectly, by interacting with integral membrane proteins that have intrinsic curvature or have curvature on oligomerization (Burger et al. 2000, Ford et al. 2002, Peter et al. 2004, Lee et al. 2005, McMahon & Gallop 2005).

2.2.4 The Rab family

Rab GTPases, members of the Ras superfamily, are essential regulators of vesicular trafficking pathways. More than 60 mammalian Rab GTPases, each localized to distinct subcellular membrane-bound compartments (Figure 6), participate in vesicle formation, motility, tethering and fusion to the acceptor membrane (Stenmark & Olkkonen 2001, Zerial & McBride 2001, Pfeffer &

Aivazian 2004). Some Rab GTPases are cell- or tissue-specific or are localized in a cell type-specific manner, but many are ubiquitous in their expression (Zerial & McBride 2001).

In cytosol, Rab GTPases are first recognized by a soluble chaperone-like Rab escort protein REP (Alexandrov et al. 1994), which transfers Rab GTPases to Rab geranylgeranyl transferase for the addition of one or two geranylgeranyl groups to cysteine residues present in the C-terminus of Rab GTPases (Seabra et al. 1992, Anant et al. 1998). Prenylated Rab proteins bind to GDP-dissociation inhibitor (GDI), which recognizes prenylated Rabs in their inactive, GDP-bound conformations. These complexes have been shown to carry all the information needed for the delivery of Rab GTPases to their appropriate membrane compartments (Soldati et al. 1994, Ullrich et al. 1994). At the donor membrane, GDI-displacement factor (GDF) catalyses the dissociation of Rab–GDI complexes (Dirac-Svejstrup et al. 1997). Converted by GEFs to their active, GTP-bound conformation, Rab proteins can bind and be stabilized by effector proteins located on the organelle, thereby fulfilling their various functions in membrane traffic (reviewed in Zerial & McBride 2001). A diverse set of effectors and their downstream signaling proteins (effector-binding proteins) have been reported (Segev 2001, Zerial & McBride 2001, Grosshans et al. 2006). Rab effectors are involved in various membrane trafficking events, including cargo sequestration during vesicle budding (e.g. Rab9 effector TIP47, Carroll et al. 2001), vesicle movement in microtubule-dependent transport (e.g. Rab6 effector Rabkinesin-6, Echard et al. 1998) and vesicle docking and membrane fusion (see below). After inactivation by specific GAPs, Rab proteins can be extracted from the membrane by GDI and recycled back to the cytosol, or, in the case of delivery of a vesicle from one compartment to another, to the donor compartment (Pfeffer & Aivazian 2004).

One of the most extensively studied Rab GTPase, Rab5, is required for the homotypic early endosome fusion (Stenmark et al. 1994) via activation of several different effectors, including Rabaptin-5/Rabex complex (Horiuchi et al. 1997) and PI3K (Li et al. 1995, Christoforidis et al. 1999b). The Rab5 effector EEA1 is recruited to endosomal membranes through interactions with PI3P. EEA1 binds both to PI3P and Rab5 via its FYVE domain (Lawe et al. 2000) and mediates the docking and fusion of early endosomes through interactions with two distinct target-membrane associated SNAREs, Syntaxin 6 and Syntaxin 13 (Christoforidis et al. 1999a, Simonsen et al. 1999, McBride et al. 1999). In addition to early endosome fusion, Rab5 is involved in the heterotypic CCV-early endosome fusion (Zerial & McBride 2001). Furthermore, Rab5 mediates the PI3K-dependent motility of early endosomes along microtubules (Nielsen et al. 1999).

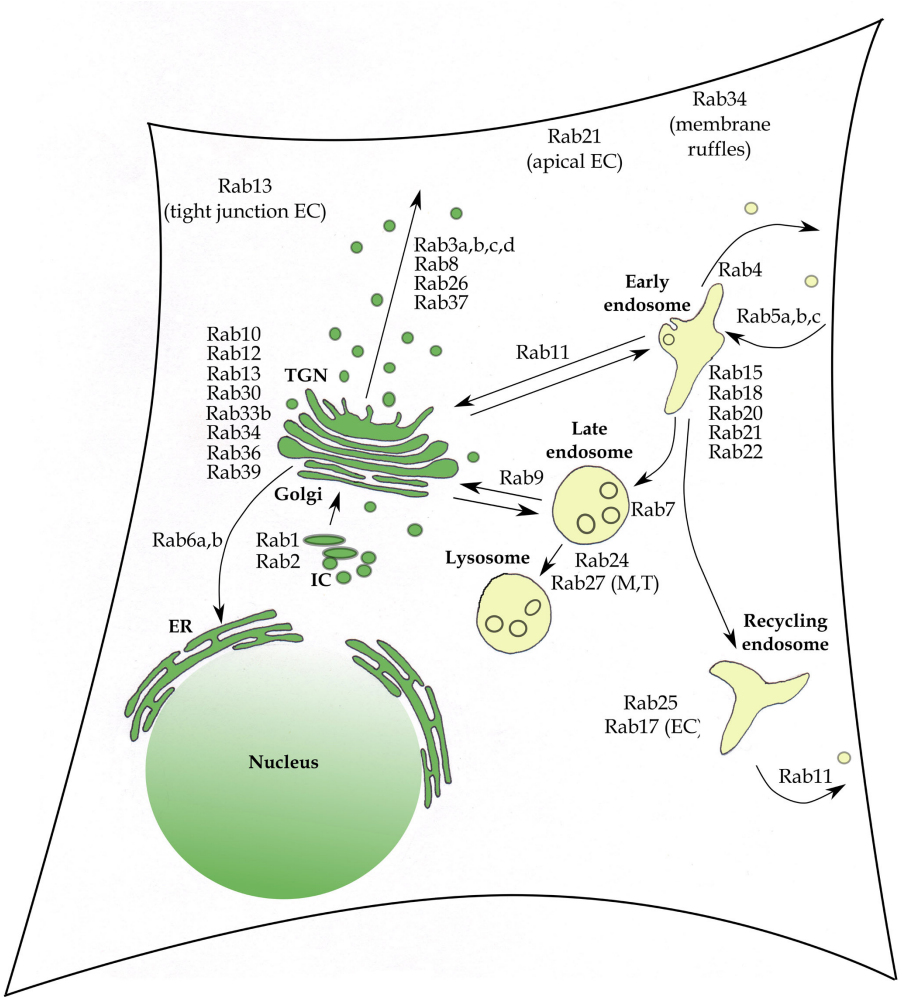

FIGURE 6 Intracellular localization of selected Rab GTPases. Some Rab GTPases are cell- or tissue-specific, or the localization may be cell type-dependent. EC, epithelial cells; IC, ER—Golgi intermediate compartment; M, melanosomes; T, T-cell granules. Modified from Stenmark & Olkkonen (2001) and Zerial & McBride (2001).

In vesicle tethering, Rab proteins and their effectors can interact with SNARE proteins (Cai et al. 2007). In humans, 36 members of SNAREs form a family of membrane-associated proteins that are differentially distributed among intracellular membranes. SNAREs regulate fusion reactions and vesicle trafficking to ensure proper targeting and delivery of specific membrane proteins and soluble cargo (reviewed in Jahn & Scheller 2006). Complementary SNAREs at both vesicle (v-SNAREs) and target membranes (t-SNAREs) provide a mechanism for fusion by forming a tightly packed tetrameric bundle of coiled α-helices (three derived from the t-SNARE and the fourth from the cognate v-SNARE, Sutton et al. 1998) that draws the membrane surfaces together (Jahn & Scheller 2006). Most SNAREs are anchored to the cytoplasmic side of the membrane via a single C-terminal transmembrane anchor that is connected to the SNARE motif by a short linker. Many SNAREs have also independently folded domains in N-terminus that vary between the subgroups of SNAREs. In membrane fusion, the energy released during the SNARE complex formation is

enough to overcome the repulsive forces that keep membranes apart. After the fusion, SNARE complex is disassembled by the N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs), and SNAREs can be recycled for further rounds of transport (reviewed in Hong 2005, Jahn & Scheller 2006, Malsam et al. 2008).

Recycling from early endosomes is mediated by different Rab GTPases. Rab4 has been shown to localize at early endosomes and to regulate fast recycling pathways from early/sorting endosomes to the plasma membrane (Daro et al. 1996), whereas Rab11 mediates slower recycling pathways through the recycling endosomes (Ullrich et al. 1996). However, the functions of Rab5, Rab4 and Rab11 are likely to overlap, thereby forming different Rab domains on the same endosome. Sonnichsen and colleagues (2000) suggested that cargo enters the cell via Rab5-containing structures, but it is then rapidly sorted into Rab4-containing domains within the same organelle. Rabaptin-5, which has been shown to interact with both Rab5 and Rab4 (Vitale et al. 1998), could link the different domains both structurally and functionally (Zerial & McBride 2001). From the Rab5/Rab4-positive structure, the cargo could then enter perinuclear membranes dominated by Rab4 and Rab11 domains (Sonnichsen et al. 2000). However, only Rab11 has been shown to fuse with the plasma membrane during exocytosis, suggesting that Rab4 and Rab11 segregate from each other and thereby generate different compartments (Ward et al. 2005).

The later steps in endocytosis are mediated mainly by Rab7 and Rab9. Rab7 is involved in transport of cargo from early to late endosomes (Feng et al. 1995) as well as in late endosome/lysosome fusion (Bucci et al. 2000). Furthermore, Rab7 is recruited to the phagosomal membrane and promotes the phagosome/late endosome fusion via interaction with Rab7 effector, Rab-interacting lysosomal protein (RILP) (Harrison et al. 2003). RILP is also needed for the microtubule-dependent transport from late endosomes to lysosomes (Cantalupo et al. 2001, Jordens et al. 2001). In addition to Rab7, also Rab9 is located at the late endosomes, where it functions, through interactions with the effector protein p40 (Diaz et al. 1997), in cargo transport from late endosomes to the *trans*-Golgi network (Lombardi et al. 1993, Riederer et al. 1994). Analogously to Rab5, Rab4 and Rab11 in early and recycling endosomal compartments, also Rab7 and Rab9 have been shown to define distinct membrane domains in late endosomes (Barbero et al. 2002).

Also many other Rab GTPases have been associated with early endocytic pathways, although their precise roles are not well understood. Besides localizing at the Golgi complex (Wang & Hong 2002), Rab34 has been shown to be involved in the formation of macropinosomes by closing membrane ruffles through reorganization of the actin cytoskeleton (Sun et al. 2003). Also, Rab15 (Zuk & Elferink 1999), Rab 17 (Zacchi et al. 1998), Rab22 (Kauppi et al. 2002) and Rab25 (Casanova et al. 1999) have been implicated in the regulation of endocytic pathways, mostly at the early/recycling endosome level.

2.3 Echovirus 1

Echoviruses (*e*nteric *c*ytopathogenic *h*uman *o*rphan viruses) belong to the family of *Picornaviridae* and to the genus *Enterovirus* in that group. At the moment, the *Picornaviridae* is divided into nine genera, the other genera being *Rhinovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus, Parechovirus*, *Erbovirus*, *Kobuvirus* and *Teschovirus* (Stanway et al. 2005). Each genus is further divided into species, e.g. the genus *Enterovirus* consists of five human virus species (human enteroviruses (HEV)A to D and poliovirus) and three nonhuman virus species (bovine enterovirus and porcine enteroviruses A and B). Echovirus 1 (EV1) is a member of the HEV-B group, which consists of 37 different virus serotypes. Altogether, the *Picornaviridae* comprises over 200 serotypes with different antigenic determinants (Stanway et al. 2005).

Among picornaviruses, enteroviruses cause the most severe human diseases. Poliomyelitis, caused by the three serotypes of poliovirus, is disappearing from the world due to efficient vaccination. Acute and chronic myocarditis and paralysis, as well as myocardial infarction are all severe diseases associated with enteroviruses. Enteroviruses cause also conjunctivitis and respiratory illnesses, as well as milder symptoms like rash, common cold and acute otitis. Furthermore, enterovirus infections have been associated with chronic illnesses, including type 1 diabetes (Andreoletti et al. 1997, Clements et al. 1995, Roivainen 2006). The enterovirus under study, EV1, can cause meningitis, encephalitis and mild respiratory and enteric infections in humans (Grist et al. 1978).

2.3.1 General properties of EV1

2.3.1.1 Virion structure

The EV1 capsid, similar to other picornavirus capsids, has an icosahedral symmetry (approximately 30 nm in diameter) and it consists of single copies of four viral proteins, VP1-VP4, arranged in 60 repeating protomeric units (Filman et al. 1998). Five protomers form a pentamer, and twelve pentamers create the viral capsid (Figure 7). VP1, VP2 and VP3 form the outer side of the capsid, whereas VP4 is located at the inner surface of the capsid, being closely associated with the RNA core. VP1-3 share a common core structural motif, an eight-stranded β-barrel, connected by loops. VP1 proteins form the five-fold axis of the virion, whereas the three-fold axis is connected with VP2 and VP3 (Hogle et al. 1985). A deep cleft, called a canyon, surrounds each five-fold axis and operates as the receptor binding site (Rossmann et al. 2002).

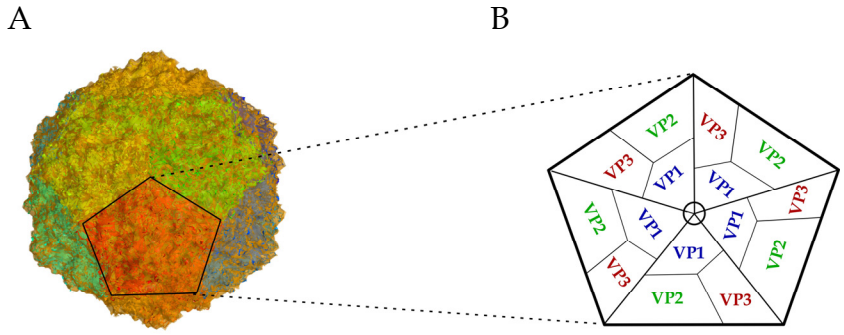

FIGURE 7 EV1 structure. (A) Twelve pentamers form the icosahedral capsid of EV1. Different pentamers are illustrated with different colors. The figure was generated using Jmol version 11.6.4, and the atomic coordinates were published by Filman et al. (1998). The coordinates were obtained from the Protein Data Bank, Brookhaven National Laboratory. (B) Blow-up picture of one pentamer shows the locations of different capsid proteins. VP1-VP3 form the outer surface of the capsid whereas VP4 is hidden in the inner surface (not shown). One canyon is indicated with a ring. Modified from Smyth & Martin (2002).

2.3.1.2 Genome organization and replication

The genome of picornaviruses is composed of a 7 – 8 kb, single-stranded (+)RNA that serves as a template for both viral protein translation and RNA replication. A single open reading frame encodes a large polyprotein that is processed by post-translational modifications and viral-encoded proteases into three precursor proteins (P1-P3), which are further cleaved into the capsid proteins and nonstructural proteins, as well as to the enzymes necessary for replication and translation (Figure 8). Picornavirus RNA genome acts directly as a viral mRNA. In contrast to cellular mRNAs, the 5'end of the viral genome lacks a 7-methyl guanosine cap, and instead, the translation is directed by an internal ribosome entry site (IRES) (Jang et al. 1988). The 5'end also contains a small, covalently linked protein involved in the initiation of viral replication, called VPg (Lee et al. 1977). Picornavirus replication takes place in membrane-associated replication complexes in the cell cytoplasm (Salonen et al. 2005). The genomic (+)RNA is copied into complementary negative-strand (-)RNA by 3D RNA polymerase, after which the (-)RNAs can serve as templates for the production of (+)RNA genomes. The (+)RNAs can then be packaged into new virions or serve as templates for the synthesis of viral proteins. A large number of RNA sequences and cellular proteins have been implicated in picornavirus replication (for a review, see Bedard & Semler 2004). After the spontaneous assembly of the capsid proteins and (+)RNA genome packaging into the capsids, progeny virus are released by host cell lysis.

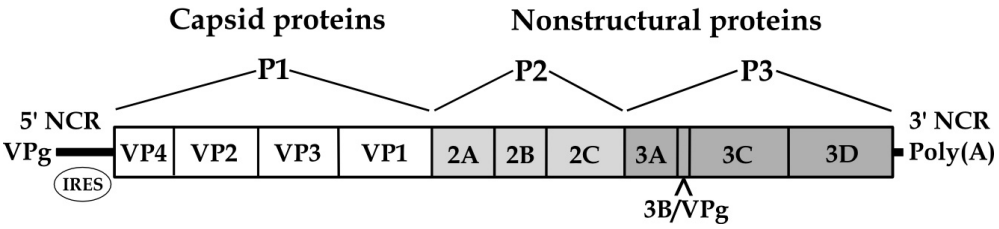

FIGURE 8 Genomic structure of EV1. Three precursor proteins (P1-P3) are cleaved into structural capsid proteins (VP1-VP4) and non-structural proteins (2A-3D). The long 5′ noncoding region (5′ NCR) contains both the protein needed for viral RNA synthesis (VPg) and the region necessary for translation initiation (IRES). The short 3′ NCR and the poly(A) tract are thought to be involved in RNA replication and translation. Modified from Bedard & Semler (2004).

2.3.2 EV1—receptor interactions

2.3.2.1 Integrins

The interaction of picornaviruses with their cell membrane receptor(s) may trigger endocytosis or lead to conformational changes that induce the viral entry and RNA release (Rossmann et al. 2002). The picornavirus receptors are all type I transmembrane glycoproteins with two to five extracellular immunoglobulin domains. Integrins form a group of picornavirus receptors, employed e.g. by human parechovirus 1 (αVβ1, Pulli et al. 1997; αVβ3, Joki-Korpela 2001), coxsackievirus A9 (αVβ3, Roivainen et al. 1994; αVβ6, Williams et al. 2004) and foot-and-mouth disease virus (αVβ3, Berinstein et al. 1995; α5β1 Jackson et al. 2000a; αVβ6, Jackson et al. 2000b; αVβ1, Jackson et al. 2002).

Integrins (Figure 9) are a family of transmembrane, noncovalently bound heterodimeric glycoproteins that consist of α and β subunits. There are 18 α and 8 β subunits, which form 24 different integrin dimers. Integrins contribute to numerous cellular processes, including cell-cell and cell-extracellular matrix (ECM) interactions, and signal transduction pathways that impact cell morphology, migration, proliferation and apoptosis (Ruoslahti 1991, Hynes 1992, Montgomery et al. 1994). In addition to pathogens, integrins can bind to numerous physiological ligands, including extracellular matrix proteins (e.g. collagens, vitronectin, laminins, tenascin, talin and fibronectins) and intercellular adhesion proteins ICAMs (Ruoslahti 1991, Hynes 1992). Many ligands contain an arginine-glycine-aspartatic acid tripeptide that is specifically recognized by certain integrins (Ruoslahti & Pierschbacher 1987). Integrin activation can be mediated by interactions of integrin cytoplasmic tails with intracellular proteins, leading to conformational changes in the ligand binding site and to increased ligand-binding affinity (inside-out signaling). ECM-integrin binding induces then integrin association with the actin cytoskeleton, initiating signal transduction cascades inside the cell (outside-in signaling) (Dedhar & Hannigan 1996).

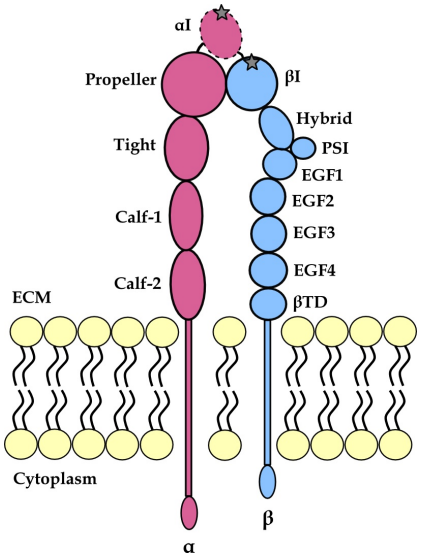

FIGURE 9 A schematic representation of integrin structure. Integrins are heterodimers that consist of α and β subunits ending in a pair of single-pass transmembrane helices and short cytoplasmic tails. Nine α subunits contain an additional I domain that has a central role in ligand binding. In integrins lacking an αI domain, the propeller and βI domain are the major ligand binding sites. Both αI and βI domains bind divalent cations through MIDAS (grey stars). Different domains of the subunits are illustrated. Modified from Avraamides et al. (2008).

2.3.2.2 Interaction of EV1 with α2β1 integrin

EV1 uses α2β1 integrin for efficient cell entry (Bergelson et al. 1992). This integrin is expressed in several cell types, including epithelial cells, endothelial cells, fibroblasts, platelets and chondrocytes (Zutter & Santoro 1990), and it is known to regulate the mitogen-activated protein kinase (MAPK) signaling pathways (Heino 2000). The specific binding site for both the natural ligand of α2β1 integrin, collagen, and for EV1 is mapped within the inserted, independently folding I domain (Figure 9) at the N-terminus of the α subdomain (residue 140-349; the residues 199-201, 212-216 and 289 are essential for EV1 binding) (Bergelson et al. 1994b, Tuckwell et al. 1995, King et al. 1997, Dickeson et al. 1999). The α2I domain binds mainly within the outer canyon wall of the EV1 capsid, in sequences that are distinct from the metal-ion dependent adhesion site (MIDAS) required for collagen binding. Thus, EV1 binding is not dependent on divalent cations (Bergelson et al. 1993, King et al. 1997). Cryo-electron microscopy studies have also revealed that simultaneous binding of EV1 and collagen to the α2I domain is impossible due to their partially overlapping binding spaces, thereby forcing the ligands to compete for binding (Xing et al. 2004). The binding affinity of EV1 has been shown to be ten times higher compared to collagen. Furthermore, the binding is considerably tight, since all five binding sites on the viral pentamer are able to bind five different α2I domains simultaneously, leading to multiple receptor binding (Xing et al. 2004).

Multivalent EV1 attachment to α2β1 integrin initiates receptor clustering, which leads to rapid internalization of the receptors and receptor-bound virus

(Xing et al. 2004). In contrast to several picornaviruses whose interactions with their receptors destabilize the virus and initiate the RNA release (Rossmann et al. 2002), the interaction of EV1 with α2β1 integrin does not induce viral uncoating. Instead, the interaction is believed to stabilize the virus (Xing et al. 2004). This further ensures that, before genome release, the virus is endocytosed to the appropriate location close to the replication site, which is reached after penetration through the endosome membrane.

2.4 Baculoviruses

Baculoviruses are a major group of arthropod viruses that replicate mainly in insects. They are well known for their potential as agents of biological control in regulating the size of insect populations, and they are also widely used as expression vectors in biotechnology. Baculoviruses comprise a large and diverse group of viruses, of which members have been isolated from over 600 host species mainly from the insect orders *Lepidoptera*, *Diptera* and *Hymenoptera*. The family *Baculoviridae* consists of two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). While GVs contain only one nucleocapsid per envelope, the NPVs can contain either a single nucleocapsid (single-nucleopolyhedroviruses; SNPVs) or multiple nucleocapsids (multiple nucleopolyhedroviruses; MNPVs) per virion (Blissard & Rohrmann 1990). Both NPVs and GVs can produce two different viral phenotypes: occlusion derived virus (ODV), which is responsible for transmitting infections between insect hosts, and budded virus (BV), which spreads the infection from cell to cell within an infected insect (Summers & Volkman 1976). These phenotypes have identical DNA but different protein composition (Figure 10), and they are produced at different times and different locations during infection (Blissard and Rohrmann 1990, Blissard 1996). Although baculoviruses do not replicate in vertebrates, they are still capable of transducing various mammalian cells, thereby being promising candidates for gene delivery applications (Hofmann et al. 1995, Boyce & Bucher 1996, Shoji et al. 1997, Ho et al. 2004).

2.4.1 Virion structure

Baculoviruses are double-stranded DNA viruses, the viral genome ranging in size from 80 to 200 kb. The genome is packaged into 30–300 nm long, rod-shaped nucleocapsids that assemble in the nucleus. Among the numerous baculoviruses, *Autographa californica* multiple NPV (*Ac*MNPV) is the most extensively studied. *Ac*MNPV (approximately 60 nm in diameter and 266 nm in length, Transfiguracion et al. 2007) has a circular double-stranded DNA genome of 134 kb, with 154 open reading frames (Ayres et al. 1994). In both baculovirus phenotypes (ODV and BV), the genome is surrounded by a small, basic DNA-associated protein (p6.9) involved in the condensation of the viral genome inside the nucleocapsids. Both forms also have the major capsid protein, vp39,

and other capsid proteins, p80 and p24, as well as the protein that encloses the capsid structure (orf1629) (Blissard 1996). However, the lipid and protein components of their envelopes differ (Blissard 1996). In BVs, the major envelope protein is the viral glycoprotein gp64 (Braunagel & Summers 1994), which is required for virus binding to the cell surface (Hefferon et al. 1999) and for cell-to-cell transmission of infection (Monsma et al. 1996). The protein is also responsible for the low-pH-mediated envelope fusion with endosomes (Blissard & Wenz 1992) and virion budding from the plasma membrane (Oomens & Blissard 1999). In baculoviruses lacking the gp64 protein, the pH-dependent membrane fusion during viral entry is triggered by the F protein (IJkel et al. 2000, Pearson et al. 2000).

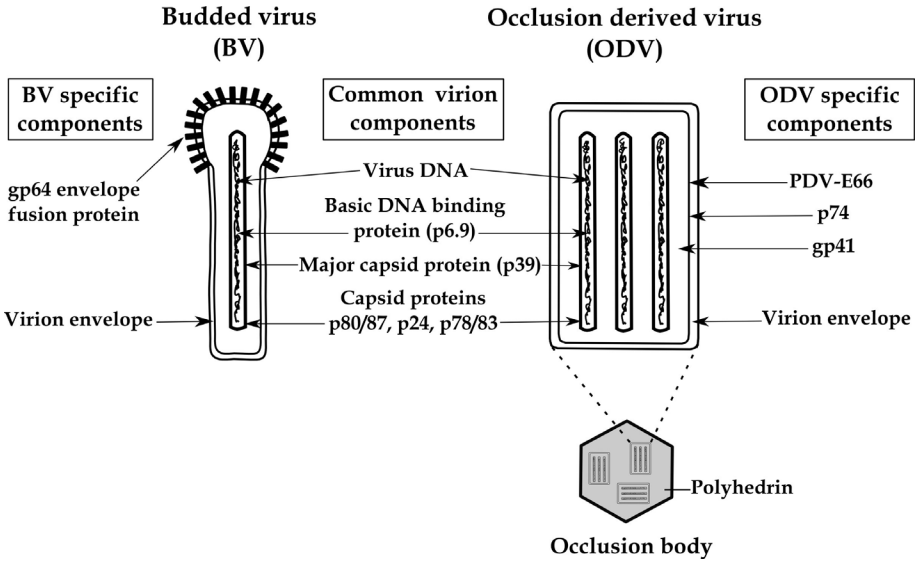

FIGURE 10 Baculovirus structural components. The two baculovirus phenotypes (BV and ODV) are illustrated with shared and phenotype-specific components. ODV represents the multicapsid NPV group. Modified from Blissard (1996).

2.4.2 Baculovirus life cycle

The baculovirus infection cycle consists of primary and secondary infections (Figure 11). In the primary infection, ODVs enter the insect's midgut epithelial cells by fusion with the plasma membrane microvilli, followed by nucleocapsid transport to the nucleus for viral DNA uncoating, gene expression and replication. Some progeny nucleocapsids are transported to the plasma membrane, where they bud through from the basolateral side and acquire the specific envelope of BVs that further spread the infection within the host's cells (reviewed in Blissard & Rohrmann 1990 and Blissard 1996). In this secondary infection, BVs enter the cells via adsorptive endocytosis (Volkman & Goldsmith 1985, Wang et al. 1997), after which the nucleocapsids are released from the endosome, triggered by acidification of the endosome and subsequent fusion with the virion envelope. Nucleocapsids are then transported to the nucleus for viral gene expression, DNA replication and assembly (Blissard 1996). Some nucleocapsids are enveloped in the nucleus and become occluded within

polyhedral-shaped occlusion bodies, composed of the occlusion body protein, polyhedrin (Rohrmann 1986). Occlusion bodies are released to the environment when the virus lyses the cell and destroys the host. The primary infection starts again when an insect larva ingests the viruses, and the nucleocapsids of ODVs are liberated due to solubilization of the polyhedrin matrix by the alkaline pH of the insect midgut (Blissard 1996).

The infectious cycle of baculovirus can be divided into three phases. During the early phase (0-6 h post infection, p.i.), genes involved in the manipulation of the host cell (e.g. cytoskeletal rearrangements, Lanier & Volkman 1998) and in the replication of the virus are expressed. In the late phase (6-24 h p.i.), shutdown of the host cell protein synthesis, viral DNA replication, late gene expression and BV production occurs. During the very late phase (>20 h p.i.), virions become occluded by the polyhedrin protein and the occlusion bodies are released by cell lysis (Blissard 1996, Okano et al. 2006).

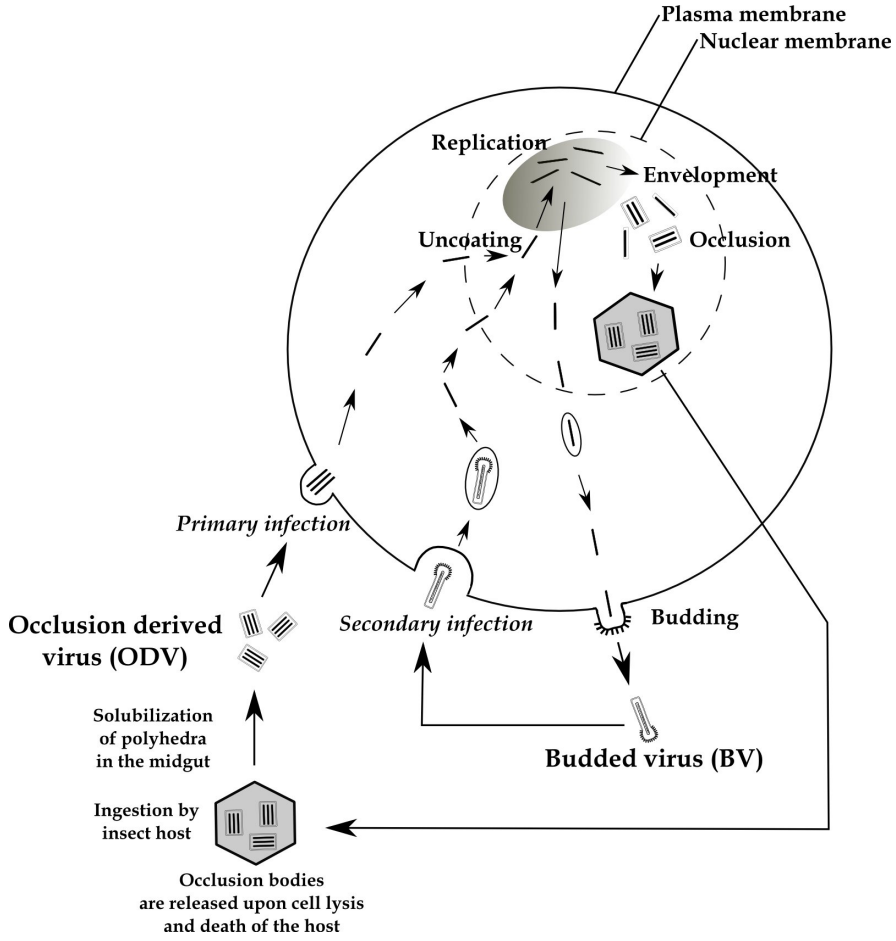

FIGURE 11 Typical life cycle of a baculovirus in an infected insect cell. Primary infection starts when the insect ingests occluded virions (ODV) which become solubilized in the midgut. Released virions fuse with the cell and the nucleocapsids are transported to the nucleus for viral uncoating, gene expression and replication. Budded viruses (BV) promote secondary infection of adjacent cells. Some progeny nucleocapsids become enveloped within the nucleus, followed by occlusion within polyhedrons. Occlusion bodies are released into the environment upon cell lysis and insect death. Modified from Blissard & Rohrmann (1990).

3 AIM OF THE STUDY

Viruses have evolved to use a variety of endocytic mechanisms for efficient cell entry. In this thesis, we have carefully followed two different endocytic pathways, utilized by echovirus 1 and baculovirus. To characterize these pathways in detail, the aims of this research were:

1. To define the entry route of echovirus 1 and the regulators of its early entry.

2. To study the cholesterol dependency of echovirus 1 internalization and infection.

3. To study the functional entry route of baculovirus (*Ac*MNPV) into human cells.

4 SUMMARY OF MATERIALS AND METHODS

The materials and methods used in this thesis are summarized in the table below. Detailed descriptions are found in the publications indicated by Roman numerals.

TABLE 3

Material/Method	Publication
Virus production and purification	I, II, III, IV
EV1 infection and α2β1 integrin clustering	I, II, III
Baculovirus transduction	IV
Transfection	I, II, IV
Small interfering RNA (siRNA) experiments	I, II, IV
Drug treatments	I, III, IV
Immunofluorescence labeling	I, II, III, IV
Internalization assay	I, II, III, IV
Electron microscopy	I, II, III, IV
Confocal microscopy	I, II, III, IV
Dextran uptake assays	I, II, IV
Transferrin uptake assays	I, IV
Microinjection	II
Live-cell imaging	II, IV
Macropinocytosis assay	II
HRP uptake assay	IV
Flow cytometry	IV
SDS-PAGE and immunoblotting	I, IV
Analyses of EV1 uncoating	III
BioImageXD data analysis	I, II, III, IV

5 REVIEW OF THE RESULTS

5.1 Regulation of EV1 entry

5.1.1 Early entry of EV1

Studies on EV1 entry were performed in SAOS-α2β1 cells (human osteosarcoma cells transfected with α2 integrin cDNA) using confocal and electron microscopy and biochemical approaches. In order to evaluate the involvement of the plasma membrane caveolae in EV1 entry, studies on dynamin and caveolin were performed. SAOS-α2β1 cells transfected with a dominant-negative dynamin-2 had no effect either on EV1 infection (I, Fig. 3A) or on the internalization of clustered α2β1 integrin (the receptor of EV1; I, Fig. 3C). α2β1 integrin, clustered by primary and secondary antibodies, was also used to study the colocalization of the integrin with caveolin-1. During the early steps of internalization (5-15 min) the colocalization was scarce, but after that it increased gradually (I, Fig. 1A and Suppl. Fig. 1A). The colocalization experiment was also performed in HeLa MZ cells (human cervical adenocarcinoma cells that express endogenous α2β1 integrin), and similar results were obtained (I, Fig. 1B). Furthermore, colocalization with EV1 and caveolin-GFP in SAOS-α2β1 cells resulted in the same observation (I, Fig. 1B).

To further study the involvement of caveolin in EV1 entry, a dominant-negative mutant of caveolin-3 (KSY) was tested. Cav^{KSY} is known to inhibit the entry of SV40 (Roy et al. 1999), a well-known virus that utilizes the caveolar pathway (Pelkmans et al. 2001). In the Cav^{KSY} transfected SAOS-α2β1 cells, EV1 infection was totally inhibited (I, Fig. 3D). However, differential labeling of surface-bound versus intracellular EV1 revealed that the virus was not halted on the plasma membrane, but it was internalized in a similar manner as in wild-type caveolin-3 transfected cells (I, Fig. 3E). Also, α2β1 integrin showed normal internalization after transfections with another caveolin-3 mutant, DGV (I, Fig. 3F), that acts as a dominant-negative inhibitor of endogenous caveolin and inhibits the entry of SV40 (Roy et al. 1999). These results thus showed that even

if caveolae were not involved in the first events of entry, caveolae seem to be involved later in the pathway.

To further study the involvement of the caveolar pathway in the later steps of EV1 and α2β1 integrin entry, studies on SV40 and caveosomes were performed. Caveosomes are caveolin-1 positive, multivesicular and pH neutral organelles, where SV40 is known to accumulate before the virus enters the ER and the nucleus (Pelkmans et al. 2001). Previous studies have suggested that also EV1 and α2β1 integrin enter caveosomes during the first 2 h of infection (Pietiäinen et al. 2004). SV40-Alexa Fluor 568 and clustered α2β1 integrin were allowed to internalize into the cells simultaneously, the further route of SV40 from caveosomes to endoplasmic reticulum being inhibited by nocodazole treatment. Since SV40 is internalized slowly to the caveosomes, we started the internalization 90 min before the internalization of α2β1 integrin. The colocalization was rather scarce after 5 min of internalization, but it increased significantly over time (I, Fig. 1C). Similar results were obtained for the colocalization between α2β1 integrin and caveolin-1 in the same experiment (I, Fig. 1C). Labeling with caveolin-1 showed that the structures positive for α2β1 integrin and SV40 were also positive for caveolin-1, verifying that these structures were caveosomes (I, Fig. 1C). Altogether, these results are in line with previous live-cell imaging studies that suggested that EV1 is not mainly internalized via caveolae but enters caveosomes during the viral infection (Pietiäinen et al. 2004).

5.1.2 EV1 is internalized along with fluid-phase markers into tubulovesicular structures that mature into multivesicular bodies

Next, the involvement of fluid-phase entry pathways in EV1 internalization was studied. Nearly all structures positive for α2β1 integrin became positive with horseradish peroxidase (HRP) during the co-internalization assay (I, Fig. 2A). Similarly, 10-kDa dextran showed colocalization with α2β1-clustered, caveolin-1 positive vesicles (I, Figs. 2B and 2C). Without integrin clustering, only a small amount of dextran colocalized with caveolin-1 positive (I, Fig. 2C) or SV40-positive (I, Fig. 2D) vesicles, indicating that dextran is targeted to the default lysosomal pathway without α2β1 integrin clustering. The structures formed without integrin clustering showed a significant loss of fluorescence after the treatment with the acid-sensitive dextran-FITC (I, Fig. 2D), suggesting that these structures were acidic in nature. In contrast, structures formed after integrin clustering showed no sensitivity to dextran-FITC and therefore were not acidified (I, Fig. 2D). This observation was also verified by live imaging with internalized integrin and labeled LysoTracker, and no colocalization was detected during the internalization period of 3 h (I, Suppl. Fig. 3B).

As EV1 and clustered α2β1 integrin entered a fluid-phase pathway that was independent of clathrin and caveolin, other possible pathways involved in their entry were tested. First, the involvement of the GEEC pathway used by GPI-anchored proteins (Sabharanjak et al. 2002) was tested. Colocalization of green fluorescent protein tagged GPI-APs with clustered α2β1 integrin stayed

low during the whole internalization period (0-2h) (I, Fig. 4A). In contrast, without integrin clustering the colocalization was rather high on the plasma membrane, suggesting that the clustering initiates redistribution of the integrin from GPI-AP positive areas. It has previously been shown that α2β1 integrin locates at the plasma membrane raft areas positive for the GPI-AP binding bacterial toxin aerolysin (Upla et al. 2004). A similar colocalization experiment with cholera toxin B subunit (CTxB) and clustered α2β1 integrin resulted only in minor colocalization, showing a small peak after 30 min (I, Fig. 4A). A portion of CTxB is known to pass through cavesomes during transport to the Golgi complex (Nichols 2002). Involvement of the raft-derived pathway followed by flotillin (Glebov et al. 2006) was also tested. However, no colocalization was perceived (I, Suppl. Fig. 2B).

The presence of classical endosomal markers during EV1 entry was also tested. Neither EEA1, CD63, cation-independent mannose-6-phosphate receptor (CI-MPR) (I, Suppl. Fig. 3A) nor LAMP-1 (Rintanen et al., unpublished data) showed colocalization with EV1 or α2β1 integrin. Instead, after 5-15 min of clustering and/or EV1 infection, EV1 and α2β1 integrin entered smooth-surfaced tubules that lacked the typical clathrin cage coating and were larger in size than caveolae (I, Fig. 4B). These structures were first tubulovesicular in nature, but they gradually matured into multivesicular structures. After two hours, most of the structures were multivesicular in nature (I, Fig. 4B). To conclude, these results indicate that EV1 is endocytosed with fluid-phase markers into tubulovesicular structures that mature into large multivesicular structures (α2-MVBs). Furthermore, these structures are notably different from the multivesicular endosomes belonging to the classical endocytic pathway, namely 1) α2-MVBs do not colocalize with CD63, CI-MPR or LAMP-1, 2) α2-MVBs are not acidic in nature, and 3) they colocalize with caveolin-1.

5.1.3 Macropinocytic regulators in EV1 entry

Due to the colocalization of HRP and dextran with EV1 during the cell entry, markers and regulators of macropinocytic entry were studied. Macropinocytosis is associated with different regulators, including PI3K, PLC, PKC and amiloride-sensitive Na^+/H^+ exchanger. Previously we have shown that PKCα activation is needed for rapid internalization of EV1 and α2β1 integrin (Upla et al. 2004). To test the involvement of PI3K, PLC and amiloride-sensitive Na^+/H^+ exchanger in EV1 entry, different inhibitors were used. All the inhibitors (LY294002 for PI3K; U73122 for phosphatidylinositol-specific PLC (PI-PLC, Amyere et al. 2000); EIPA for amiloride-sensitive Na^+/H^+ exchanger, West et al. 1989) blocked EV1 infection when added 30 min before or directly after EV1 binding (I, Fig. 5A). At later time points, EIPA proved to be a more efficient inhibitor, whereas U73122 inhibited the infection only during the early EV1 entry: some EV1 infected cells were detected when U73122 was added 1 h p.i., whereas EIPA completely blocked the infection when added after 2 h p.i. (I, Fig. 5A). Monitored by confocal microscopy, EV1 and α2β1 integrin internalization were arrested at the plasma membrane in U73122 treated cells (I,

Figs. 5C and 5D), whereas the LY294002 treatment allowed the virus to enter deeper into the cytoplasm (I, Fig. 5C). These results are in line with dextran-uptake assays performed with these inhibitors (I, Suppl. Fig. 4B). Both confocal images and electron microscopy studies with ruthenium red (a dye specific for acid mucopolysaccharides present in the cell coat) showed that EIPA did not block the internalization, since 95% of the structures were intracellular and not connected to the plasma membrane, but further transport from the peripheral cytoplasm was arrested (I, Fig. 5D and Suppl. Fig 4D). Studies with electron microscopy also revealed that EIPA prevented maturation of tubulovesicular structures into multivesicular bodies, most of the α2β1 integrin positive structures being tubulovesicular at 2 h after EIPA treatment (I, Fig. 6A). Also caveolin-1 entry into these structures was blocked by both EIPA and U73122 (I, Figs. 6B and 6C).

To further test the putative involvement of macropinocytosis in EV1 entry, the roles of macropinocytic regulators, Pak1 and Rho GTPases, were studied. Dominant-negative (DN) mutant of Pak1 (AID) inhibited EV1 infection totally, whereas highly kinase active mutant (T423) showed values similar with control transfections (wild-type, WT; mock-plasmid, N1-YFP) (I, Fig. 7B). In confocal images, total inhibition of EV1 infection was seen by AID mutant at both 2 h and 6 h p.i. (I, Fig. 7C). WT, AID and T423 constructs were also tested for their influences on dextran and transferrin uptake, dextran uptake being inhibited only by AID (I, Fig. 7A). Activation of Pak1 early in EV1 infection (5-30 min) was observed with antibodies against activated phospho-Pak1 (I, Fig. 8D).

Since the Rho GTPases Rac1 and Cdc42 are regulated upstream of Pak1, they were also tested for their involvement in EV1 infection. siRNAs against Rac1, Cdc42 and RhoA were tested in EV1 infection, but only the siRNA against Rac1 inhibited infection despite of efficient knockdown achieved with the siRNAs (I, Fig. 8A). Similar results were obtained from experiments carried out with transfected DN or WT constructs, which showed that only DN Rac1 had an inhibitory effect on EV1 infection (I, Fig. 8B). Both the DN and WT constructs of RhoA showed lowered levels of EV1 infection, but there were no difference in the levels of EV1 infection between the RhoA constructs. These results suggest that Rac1 is involved in EV1 entry and may be the upstream regulator of Pak1. Because the actin cytoskeleton is regulated by Rho GTPases and Pak-kinases, the role of actin dynamics in EV1 infection was studied using actin depolymerizing (cytochalasin D) and stabilizing (jasplakinolide) drugs. Both drugs inhibited EV1 infection (I, Suppl. Figs. 5B and 5C), cytochalasin D more efficiently after longer preincubation times. Furthermore, in confocal images actin stress fibres were seen to depolymerize shortly after EV1 internalization (I, Fig. 8C), suggesting that actin dynamics is needed for EV1 entry and infection. Taken together, the involvement of PI3K, PLC, amiloride-sensitive Na^+/H^+ exchanger, Pak1, Rac1 and the actin cytoskeleton in EV1 entry strongly suggests that EV1 uses macropinocytosis for efficient cell entry.

5.2 CtBP1/BARS in endocytosis

5.2.1 Role of CtBP1/BARS in macropinocytosis

Previously, CtBP1/BARS has been suggested to be involved in dynamin-independent, fluid-phase endocytosis. Bonazzi and colleagues (2005) showed that dextran internalization was inhibited in different cell lines treated with siRNAs against CtBP1/BARS, as well as in cells overexpressing a dominant-negative mutant of CtBP1/BARS. Here, to determine whether CtBP1/BARS has a role in macropinocytosis, human A431 epidermoid carcinoma cells were used. First, EGF-stimulated macropinocytosis was tested for dextran uptake, and it was shown to efficiently enhance dextran internalization (II, Figs. 1A and 1B). EGF-stimulated macropinocytosis was shown to be dependent on CtBP1/BARS, since siRNAs against CtBP1/BARS reduced dextran internalization significantly (II, Figs. 1C and 1D). Similarly, dextran internalization was inhibited by microinjection of two recombinant deletion mutants of CtBP1/BARS (SBD-GST and NBD-GST) (II, Figs. 1E and 1F), as well as by microinjection of blocking anti-CtBP1/BARS antibody (II, Figs. 1G and 1H). Interestingly, dextran internalization was recovered following CtBP1/BARS-GST microinjection into the CtBP1/BARS-depleted cells (II, Figs. 1C and 1D), indicating that EGF-stimulated macropinocytosis was restored due to CtBP1/BARS addition. Inhibition or depletion of CtBP1/BARS showed no effects on actin dynamics (II, Suppl. Fig. 1D), nor did enhanced levels of cellular CtBP1/BARS alter EGF-stimulated macropinocytosis. Altogether, these results provide evidence for CtBP1/BARS involvement in macropinocytosis.

To investigate the location of CtBP1/BARS during macropinocytosis, live-cell imaging was used. Due to EGF stimulation, a fluorescent-tagged construct of CtBP1/BARS was detected to move onto the membrane ruffles and the cup-shaped structures following the ruffling (II, Suppl. Movies 1 and 2). The macropinosomes formed moved rapidly into the cell, CtBP1/BARS having detached from the cup before its internalization (II, Fig. 2C). Similar results were obtained from confocal images that showed strong CtBP1/BARS recruitment to the F-actin rich membrane ruffles upon EGF stimulation (II, Fig. 2B). Furthermore, immuno-EM experiments showed CtBP1/BARS enrichment in ruffling areas in EGF-stimulated cells (II, Fig. 2D).

In order to clarify the exact mechanism of CtBP1/BARS in macropinocytosis, FITC-conjugated dextran was applied. These experiments were performed without a pre-fixing wash, so that fully sealed macropinosomes could be distinguished from macropinocytic cups still connected to the plasma membrane. The fixed cells were then incubated either in PBS (pH neutral) or in acidic medium (pH 5.0), since FITC-dextran is quenched below pH 5.5. Compared to control cells that were microinjected with GST after EGF stimulation, the cells microinjected with a dominant-negative mutant of CtBP1/BARS (NBD-GST) showed fluorescence bleaching from dextran-positive, macropinosome-like structures, indicating that these

structures were still connected with the extracellular space (II, Figs. 3A and 3C). This result was confirmed with another experiment using TRITC-(tetramethylrhodamine isothiocyanate) and biotin-conjugated dextran, which was stained with streptavidin-633 to reveal the macropinosomes connected to the extracellular space (II, Fig. 3D and 3E). In addition, live-cell imaging with TRITC-dextran treated cells resulted in similar findings (II, Suppl. Movies 3 and 4, and Fig. 3F), confirming the role of CtBP1/BARS in macropinosome closure.

CtBP1/BARS phosphorylation by Pak1 affects the cellular localization and corepressor activity of the protein (Barnes et al. 2003). Here, the role of this phosphorylation was found to be essential for the macropinocytic cup fission. EGF-stimulated macropinocytosis was inhibited both by transfecting cells with a dominant-negative mutant of Pak1 (AID) and with siRNAs against CtBP1/BARS (II, Figs. 5A and 5B). Pak1 phosphorylates CtBP1/BARS on serine 158 (Barnes et al. 2003), and two point-mutations on that serine were generated. The phospho-depleted mutant (S147A) strongly inhibited macropinocytosis, whereas the phospho-mimetic mutant (S147D) had no apparent effect (II, Figs. 6B and 6C). Upon EGF stimulation both mutants translocated to the plasma membrane ruffles (II, Fig. 6A), indicating that CtBP1/BARS phosphorylation by Pak1 is essential, particularly during macropinosome fission.

5.2.2 CtBP1/BARS in EV1 entry

Due to the involvement of several macropinocytic regulators in EV1 entry, the role of CtBP1/BARS was also studied. SAOS-α2β1 cells microinjected with deletion peptides of CtBP1/BARS (SBD-GST or NBD-GST, acting as dominant-negative mutants) showed a clear inhibition in EV1 entry (II, Fig. 4A). Confocal images showed EV1 accumulation close to the plasma membrane in SBD-GST-microinjected cells, whereas into control cells the virus internalized normally (II, Fig. 4C). The quantification of internalized versus total EV1 confirmed that in control cells microinjected with GST, the virus was internalized efficiently when compared to cells microinjected with the SBD-GST mutant (II, Fig. 4D). Similarly, EV1 infection was also inhibited by microinjection of anti-CtBP1/BARS antibody (data not shown), which further confirms the essential role of CtBP1/BARS in EV1 infection.

The EV1 receptor, α2β1 integrin, internalization was detected in more detail with electron microscopy experiments. In cells transfected with a mutant construct of CtBP1/BARS (NBD-YFP), the clustered integrin internalization was blocked at the plasma membrane or in small structures still connected to it (II, Figs. 4E and 4F). The plasma membrane connection of α2β1 integrin was confirmed by treatment with ruthenium red. These results are in line with the observations from the experiments with FITC- and TRITC-dextrans (II, Figs. 3A-F), indicating that α2β1 integrin internalization was blocked into these non-sealed macropinocytic cups. In control cells, the integrins were seen in fully sealed, ruthenium red-negative tubulovesicular structures (II, Figs. 4E and 4F). Due to the mutant construct of CtBP1/BARS, maturation of α2β1 integrin

positive structures into multivesicular bodies was significantly inhibited (II, Fig. 4F).

In control experiments performed with SV40 in CV-1 cells, the virus infection was not altered by microinjection of the CtBP1/BARS mutant (NBD-GST) (II, Fig. 4B). Similar results were obtained from transfection experiments with another dominant-negative construct of CtBP1/BARS (D355A) that allowed normal SV40 internalization (data not shown). These results thus suggest that the caveolar pathway is not regulated by CtBP1/BARS. In contrast, the D355A mutant of CtBP1/BARS in SAOS-α2β1 cells reduced the entry of EV1 and showed virus accumulation at the plasma membrane (data not shown). Rather, the pathway used by SV40 was slightly increased due to the CtBP1/BARS mutant (II, Fig. 4B). The role of CtBP1/BARS in clathrin-mediated endocytosis was excluded by experiments with the clathrin-mediated pathway utilizing coxsackievirus B3 (Chung et al. 2005), whose infectivity was not altered upon transfections with the CtBP1/BARS mutant (NBD-YFP) (data not shown). Altogether, these results show that CtBP1/BARS is an essential regulator of macropinocytic entry of EV1, and that the protein is required at the fission state of the entry process.

5.3 Cholesterol-dependent entry of EV1

5.3.1 Role of cholesterol in α2β1 integrin internalization

It has been shown previously that α2β1 integrin localizes at the plasma membrane in cholesterol-rich, detergent resistant domains (Marjomäki et al. 2002, Upla et al. 2004). To further investigate the role of cholesterol and lipid rafts in EV1 and α2β1 integrin internalization, the cholesterol-aggregating drugs filipin and nystatin were applied. Confocal images showed strong inhibition in internalization of clustered integrin by both drugs (III, Fig. 1A). Quantification of the plasma membrane associated versus intracellular integrin showed a strong α2β1 integrin accumulation at the cell surface (III, Fig. 1B). The accumulation was in a similar range as in the control cells clustered on ice (III, Figs. 1A and 1B) indicating that α2β1 integrin internalization is critically dependent on the plasma membrane cholesterol.

To study the existence of cholesterol and lipid rafts also in the multivesicular structures formed following α2β1 integrin clustering and internalization (α2-MVBs), cold Triton X-100 treatment (a treatment widely used in studying the existence of lipid rafts; Brown & Rose 1992), was performed. To this purpose, clustering with antibodies was carried out both to α2β1 integrin, and as a control, to αV integrin. αV integrin internalization has been shown to occur via clathrin-mediated endocytosis (Upla et al. 2004). Integrin internalizations were allowed to proceed for 2 h before the detergent treatment. According to our electron microscopy measurements, at that time point ~70% of the α2 structures are already multivesicular in nature (I, Fig. 4B).

Quantification of the segmented structures with the segmentation tool of the BioImageXD software showed that the Triton X-100 treatment did not have any effect on the amount of the α2-positive structures formed, whereas the amount of the αV integrin structures was reduced (III, Figs. 2A and 2B). The quantifications also showed that due to the Triton X-100 treatment, the intensity of the αV-positive structures was greatly reduced (III, Fig. 2C). The mean size of the structures formed was also affected; the α2-positive structures were slightly smaller, whereas the αV-positive structures were significantly larger after the Triton X-100 treatment (III, Fig. 2D).

Filipin treatment inhibited α2β1 integrin internalization, whereas αV integrin entry was not affected (III, Fig. 2A). The influence of filipin was further monitored by quantifications of confocal data that showed reduction in the size of α2-positive structures, as well as a slight increase in the amount of the vesicles (III, Figs. 2B and 2D). In contrast, αV-positive structures were only slightly affected by filipin (III, Figs. 2B and 2D). Altogether, these results indicate that in addition to the plasma membrane, cholesterol and possibly lipid rafts are also present in α2-MVBs.

In order to study the effects of filipin and nystatin in more detail, electron microscopy experiments were performed. When compared to control cells, filipin and nystatin greatly reduced the amount of multivesicular bodies formed during the early (10-30 min) internalization period of α2β1 integrin and EV1 (III, Fig. 4C). The drug treatments (drugs added at different time points p.i.) also resulted in a high number of single, gold-labeled α2 and EV1 particles outside the endosomes, suggesting that a part of the already formed α2-positive endosomes had broken down due to the filipin and nystatin treatments (III, Fig. 4C). To further verify whether the drugs had broken down some of the structures, internalization experiments with the fluid-phase marker FluoSpheres co-internalized with clustered α2β1 integrin were performed. Confocal images showed strong colocalization of FluoSpheres with internalized clustered integrin after 2 h (III, Fig. 4D). However, after filipin treatment the intensity level of the endosomes was reduced by 28% on average (III, Fig. 4D), indicating that FluoSpheres had partially leaked out of the cholesterol-aggregated structures, possibly because of vesicle fragmentation.

5.3.2 Cholesterol in EV1 entry and infection

The formation of plasma membrane ruffles and macropinosomes is dependent on intact cholesterol (Grimmer et al. 2002). Due to the macropinocytic entry of EV1 and the lipid raft involvement in α2β1 integrin internalization, cholesterol-dependency of EV1 entry and infection was studied. Preincubation with filipin and nystatin totally blocked EV1 infection (III, Fig. 3A), which was further judged by immunofluorescence labeling of EV1 (III, Fig. 3B). Similarly, these drugs totally inhibited the infection when added at 5 min p.i. (III, Fig. 4A). Analysis of the samples by confocal microscopy showed that the virus accumulated on the plasma membrane when the drugs were added 30 min before infection or 5 min p.i. (III, Figs. 3B and 4A). Interestingly, filipin and

nystatin still inhibited the infection when added 1 to 3 h p.i. (III, Fig. 4B). Drug additions later than 3 h p.i. led only to a partial inhibition of infection, suggesting that the drugs did not directly inhibit replication of the virus. Previously it has been shown that EV1 replication starts around 3 h p.i. (Upla et al. 2008). These results thus suggest that an intact cholesterol environment is needed for efficient EV1 internalization and infection, and that the cholesterol dependency continues until the virus genome is released from the α2-MVBs for replication.

To investigate the involvement of cholesterol-aggregating drugs on EV1 uncoating, experiments with light-sensitive, neutral-red (NR) labeled virus were performed. This method is based on the neutral red dye which is light-sensitive and, upon exposure to light, causes cross-linking of the labeled genome with the surrounding capsid, thus inhibiting the uncoating of the virus (Brandenburg et al 2007). If the uncoating has not occurred before the light reaction, there will be no newly synthesized virus in the assay. On the other hand, the uncoated virus will proceed with infection despite the later light reaction. Here, the control cells treated with neutral-red labeled EV1 followed by a strong light treatment 5-15 min p.i. showed no infection after 7 h, indicating that in those cells no virus was uncoated (III, Fig. 5A). After 30 min p.i. some of the cells were infected, suggesting that uncoating has started. Later, 1 to 4 h p.i., the infection level of the control cells (no light treatment) was reached (III, Fig. 5A). Filipin was administrated to the cells 15 min p.i., thus before the viral uncoating but after the time point when the majority of the α2/EV1-structures were already intracellular (data not shown), and ~25% of the structures were multivesicular in nature (I, Fig. 4B). When evaluating the cells in which the light exposure had been performed after filipin addition, no infected cells were found during 30 min to 4 h p.i. (III, Fig. 5B), suggesting that EV1 was unable to uncoat due to the filipin treatment. Similar results were obtained from sucrose gradient sedimentation experiments applied with [35S]methionine-labeled EV1. In the gradient, 160S particles (EV1 that still contains RNA) sediment in the bottom part of the gradient, whereas the release of the RNA genome leads to the formation 80S particles (light, empty capsids) that sediment in the upper part of the gradient (Marjomäki et al. 2002). These results showed that filipin treatment resulted in a clear inhibition of viral uncoating (III, Fig. 5E), since in filipin treated cells the sedimentation of RNA-lacking 80S particles was decreased by four-fold when compared to control, untreated cells (III, Fig. 5C). Nystatin had also an inhibitory effect on viral uncoating, although the effect was not as efficient as with filipin (III, Fig. 5D). This is in line with previous results, which showed that nystatin blocked the EV1 infection in a slightly weaker manner than filipin (III, Figs. 4A and 4B). To conclude, these results show evidence that intact cholesterol domains in α2-MVBs are essential for EV1 uncoating.

5.4 Regulation of early baculovirus uptake

5.4.1 Baculovirus is not internalized through clathrin-dependent and macropinocytosis pathways

Baculovirus entry was defined in highly permissive human cell lines (HepG2 and 293) using confocal microscopy, electron microscopy and flow cytometry experiments. The uptake of baculovirus in insect cells was shown to occur via adsorptive endocytosis (Volkman & Goldsmith 1985). In mammalian cells, clathrin-mediated pathway has been suggested to be involved in baculovirus uptake (Long et al. 2006). Also our earlier results showed that baculovirus accumulated at a slow pace in EEA1-positive compartments, suggesting that pathway(s) such as the clathrin-mediated pathway leading to early endosomes could be involved. Our present studies showed, however, that colocalization between clathrin and baculovirus was not detected during early entry (5-15 min) of the virus (IV, Figs. 1A-C), and no visible clathrin coat was detected on the baculovirus-containing vesicles (IV, Figs. 2C and 2D). Similarly, transferrin did not colocalize with baculovirus (IV, Suppl. Fig. 1A). However, the dynamin inhibitor dynasore (Macia et al. 2006) blocked baculovirus entry by 67% (IV, Fig. 1D), suggesting that dynamin was needed in baculovirus entry.

The involvement of macropinocytosis was studied using the inhibitor of amiloride-sensitive Na^+/H^+ exchanger (EIPA), which had no effect on baculovirus gene-expression (IV, Fig. 3C). Other regulators associated with macropinocytosis, e.g. Rab34 and Pak1 (IV, Fig. 3D), Rho GTPase Rac1 (IV, Suppl. Figs. 3A and 3B) and CtBP1/BARS (data not shown) did not have any effect on baculovirus entry either, since the virus was internalized efficiently in every case. Interestingly, as monitored by confocal microscopy, the virus caused ruffle formation on the cell surface (IV, Fig. 2A), which was further confirmed by quantification (IV, Fig. 2B). In addition, the fluid-phase marker HRP was detected in baculovirus-filled endosomes (IV, Fig. 2D), the uptake of HRP, however, not being induced by virus transduction (IV, Fig. 2E). Therefore, these results suggest that macropinocytosis is not involved in baculovirus entry, but the virus still induces membrane ruffling and is internalized together with fluid-phase markers.

Since fluid-phase pathways are often derived from the plasma membrane raft areas, the raft disturbing drug filipin was used. Differential labeling of the intracellular and surface-bound baculovirus in filipin treated cells showed that the drug inhibited baculovirus entry efficiently (IV, Fig. 3A), suggesting that baculovirus uptake originated from cholesterol-sensitive and possibly lipid raft-enriched plasma membrane areas. Other raft-derived pathways were also studied in baculovirus transduction, but neither GEEC nor flotillin pathway markers showed colocalization with baculovirus during early transduction (IV, Fig. 3B).

5.4.2 Phagocytosis-like uptake of baculovirus

While searching for regulators of baculovirus entry, we found two GTPases involved in baculovirus internalization, namely Arf6 and RhoA. Arf6 dominant-negative (DN) as well as constitutively active (CA) mutants inhibited baculovirus internalization, in contrast to the wild-type (WT) construct that allowed efficient internalization (IV, Fig. 4A). Quantification of the internalized versus plasma membrane associated virus revealed that in cells expressing the DN Arf6, baculovirus internalization was reduced when compared to wild-type transfected cells (IV, Fig. 4A). Also the nuclear localization of baculovirus was reduced (IV, Fig 4C). In addition, baculovirus-mediated luciferase expression was inhibited both in CA and DN Arf6-transfected cells (IV, Fig. 4B). Involvement of RhoA in baculovirus entry was detected in an internalization assay demonstrating that CA RhoA mutant inhibited baculovirus entry efficiently (IV, Fig. 5B). Also siRNA experiments verified the involvement of RhoA in the virus entry (IV, Fig. 5C). Of note, the RhoA-dependent IL-2 pathway was not found to be involved in baculovirus entry (IV, Suppl. Fig. 3C).

Since Arf6 and RhoA have both been associated with clathrin-independent and phagocytic-like entry (Caron & Hall 1998, Niedergang et al. 2003) the role of phagocytosis in baculovirus uptake was further elucidated. For this purpose, *E.coli* bioparticles were used as markers for phagocytosis. Interestingly, baculovirus and *E.coli* colocalized extensively during the early entry (5-10 min) when they were administrated simultaneously (IV, Fig. 6A and 6B). The cellular uptake of *E.coli* increased significantly in the presence of baculovirus (IV, Fig. 6C), indicating that baculovirus is able to induce *E.coli* entry into non-phagocytic human cells. Also, when baculovirus was allowed to internalize for 15 min and then *E.coli* for the next 60 min, the cells did not internalize the bacteria efficiently (IV, Fig. 6C). This suggests that simultaneous uptake is needed for baculovirus-triggered *E.coli* entry. Furthermore, dynasore and filipin reduced the baculovirus/*E.coli* entry efficiently, suggesting that also *E.coli* entered the cells from cholesterol-enriched plasma membrane areas in a dynamin-dependent manner (IV, Fig. 6D). To conclude, involvement of actin (Salminen et al. 2005), dynamin, RhoA and Arf6, as well as membrane ruffling induced by baculovirus, and baculovirus induced uptake of *E.coli* altogether suggest, that baculovirus entry occurs via a mechanism that is reminiscent of phagocytosis.

6 DISCUSSION

Possibly all the endocytic pathways of mammalian cells are utilized by different pathogens. This remarkable feature of viruses, bacteria and toxins has inspired researchers over decades to define the pathways used, and particularly, to find the various regulators employed. However, these attempts have not been made easy, due to the variety of endocytic pathways and the high number of different regulators involved. For example, cellular lipids are a large group of regulators whose importance in pathogen entry has gained an increasing amount of attention, and many of the endocytic pathways are indeed associated with plasma membrane areas enriched in cholesterol and lipid rafts. In the present thesis, we have contributed to this task to elucidate viral entry by studying the pathways exploited by a human pathogen, echovirus 1, and an insect pathogen and promising gene therapy vector, *Ac*MNPV-baculovirus.

6.1 EV1 entry

6.1.1 Early entry of EV1

Although the entry pathways utilized by members of the large *Picornaviridae* family are still quite poorly characterized, the clathrin-mediated pathway is probably used by most of the viruses. For example human rhinovirus 14 (DeTulleo & Kirchhausen 1998), coxsackievirus B3 (Chung et al. 2005) and human parechovirus 1 (Joki-Korpela et al. 2001) have been reported to use clathrin coated vesicles in efficient cell entry. Despite these observations, no connection between EV1 and clathrin-mediated endocytosis has been detected (Marjomäki et al. 2002, Pietiäinen et al. 2004, Upla et al. 2004). Also in the present study, monitored by electron microscopy, no visible clathrin coat was observed (I). Another route that is also involved in viral entry, the caveolar pathway, was also excluded since no colocalization between caveolin-1 and clustered α2β1 integrin was detected at the plasma membrane or during the first 5 min of internalization (I). However, the colocalization of clustered α2β1

integrin and caveolin-1 increased gradually, and finally the integrin was shown to partially colocalize with SV40 in multivesicular structures (I). The colocalization between α2β1 integrin and caveolin-1 was shown to increase over time (I). These results suggest that α2β1-containing vesicles fuse with caveolae/caveosomes, thereby forming structures called α2-MVBs. Crosstalk between different endocytic pathways is common (e.g. clathrin coated vesicles and caveolae can fuse with early endosomes) and recently, also clathrin- and caveolin-independent carriers have been suggested to fuse with early and recycling endosomes (Kirkham et al. 2005, Mayor & Pagano, 2007). Although caveolin-1 on the plasma membrane is not required for EV1 internalization, it may have a role in the EV1 entry process into α2-MVBs. The affinity of caveolins for cholesterol may be crucial for the virus infection, which was shown to be dependent on intact cholesterol domains in α2-MVBs (III). In addition, the cav^{DGV} mutant, which has been shown to cause abnormal lipid accumulations and imbalance in intracellular cholesterol (Pol et al. 2001), inhibited EV1 entry totally, although the virus internalization was not affected (I). Thus, the caveolin and/or cholesterol domains seem to be important at the later stages in EV1 infection.

Several clathrin- and caveolin-independent pathways originate from plasma membrane areas enriched in lipid rafts. These pathways can be dynamin-dependent (e.g. the IL-2 pathway, Lamaze et al. 2001) or dynamin–independent (e.g. the GEEC pathway; Sabharanjak et al. 2002, the CTxB pathway; Massol et al. 2004, and the flotillin pathway; Glebov et al. 2006). EV1 and α2β1 integrin entry was shown to be dynamin-independent (I), although dynamin's role in EV1 entry may be cell type-dependent. Dynamin mutant (K44A) was shown to inhibit EV1 infection in CV-1 cells (Pietiäinen et al. 2004), in contrast to SAOS α2β1-cells (I). α2β1 integrin is known to be located at the plasma membrane in raft-enriched regions (Pietiäinen et al. 2004). From those areas α2β1 integrin and EV1 were shown to internalize into tubulovesicular structures that gradually matured into multivesicular structures. Compared to the multivesicular structures formed in the classical endocytic pathway (Gruenberg 2001), α2-MVBs were pH neutral and they did not colocalize either with the late endosomal markers CD63 and CI-MPR (I) or with the lysosomal marker LAMP-1. Also raft-derived GPI-APs can be found in tubulovesicular endosomes (Sabharanjak et al. 2002), but no colocalization between α2β1 integrin and GPI-AP, CTxB or flotillin was detected during the internalization process (I). Only CTxB showed a small colocalization peak after 30 min internalization, probably due to its passage through caveosomes on its way to the Golgi complex (Nichols 2002). Since unclustered α2β1 integrin showed a rather high colocalization with GPI-AP at the plasma membrane, the integrin had to be translocated away from the GPI-AP-enriched areas upon clustering. Interestingly, a number of enteroviruses bind to raft-derived GPI-anchored proteins during cell entry (Bergelson et al. 1994a, Bergelson et al. 1995, Karnauchow et al. 1996), emphasizing the role of plasma membrane lipid rafts in (picorna)viral internalization.

6.1.2 Macropinocytosis in EV1 entry

Generally, macropinocytosis is considered as a non-specific internalization pathway that is not dependent on ligand binding to a specific receptor. Macropinosome formation occurs after cell stimulation, followed by rapid lamellipodia formation at the sites of membrane ruffling. Due to the strict dependency of the actin cytoskeleton, inhibitors of macropinocytosis often include drugs that disturb the actin dynamics. Here, EV1 entry was shown to be inhibited by cytochalasin D and jasplakinolide, and when triggered by virus entry, actin stress fibers depolymerized rapidly (I). However, the specificity of actin inhibiting drugs is not limited to macropinocytosis, since functional actin cytoskeleton is needed for the majority of endocytic pathways currently known (Kirkham & Parton 2005).

The induction of macropinocytosis in cell entry is not especially common among viruses, although the ability of macropinosomes to intersect with other endocytic vesicles and to become acidified (Racoosin & Swanson 1992) makes them conceivable targets for a variety of viruses. Adenoviruses (Ad2 and Ad5) have been shown to trigger macropinocytosis coincident with their primary, clathrin-mediated entry pathway (Meier et al. 2002, Meier & Greber 2004). However, in those cases macropinocytosis is not used for efficient uptake, but for viral escape from the endosomes (Meier et al. 2002). In the present study, EV1 internalization was shown to be dependent on Rac1, Pak1 and CtBP1/BARS (I, II). The Rho GTPase Rac1 is also indicated in phagocytosis (Caron & Hall 1998), whereas Pak1 and CtBP1/BARS have not been found to be involved in other endocytic pathways. Furthermore, inhibition of PI-PLC and PI3K, as well as treatment with EIPA prevented EV1 infection (I), demonstrating the important role of macropinocytic regulators in EV1 infection. Also, the fluid-phase markers HRP and dextran were sorted into late caveosomes together with EV1 (I). Since in the absence of α2β1 integrin clustering dextran was sorted to another, default pathway, the integrin clustering most likely triggers the α2-dependent pathway and targets dextran into α2-MVBs.

It is of interest to note that a small virus such as EV1 (~30 nm in diameter) would use macropinocytosis, which engulfs rather large (0.2–5 μm) macropinosomes (Swanson 2008). Larger viruses, such as adenoviruses (~90 nm) and vaccinia virus (~70-100 nm) (Mercer & Helenius 2008) may use macropinocytosis due to the capability of macropinocytosis to internalize particles that are too large for other viral endocytic mechanisms. Interestingly, in a recent study, adenovirus 3 was shown to utilize a similar, macropinocytosis-like pathway as EV1, but also another, still not elucidated clathrin- and dynamin-dependent pathway (Amstutz et al. 2008). Ad3 was endocytosed with its receptor CD46 and αV integrin coreceptors into macropinocytic vesicles in an actin, Rac1, Pak1 and CtBP1/BARS-dependent manner. Dextran uptake was also triggered by Ad3, but in this case the dextran pathway followed the normal route to lysosomes (Amstutz et al. 2008).

Similarities in the EV1 and Ad3 entry pathways indicate that macropinocytic uptake might be a common viral pathway for efficient cell entry.

To conclude, in this study we have shown that EV1 and its receptor α2β1 integrin exploit macropinocytosis in efficient cell entry. The uptake occurs with fluid-phase markers into multivesicular endosomes that further fuse with caveolae or caveosomal structures, thereby forming late caveosomes. The entry pathway is summarized in Figure 12.

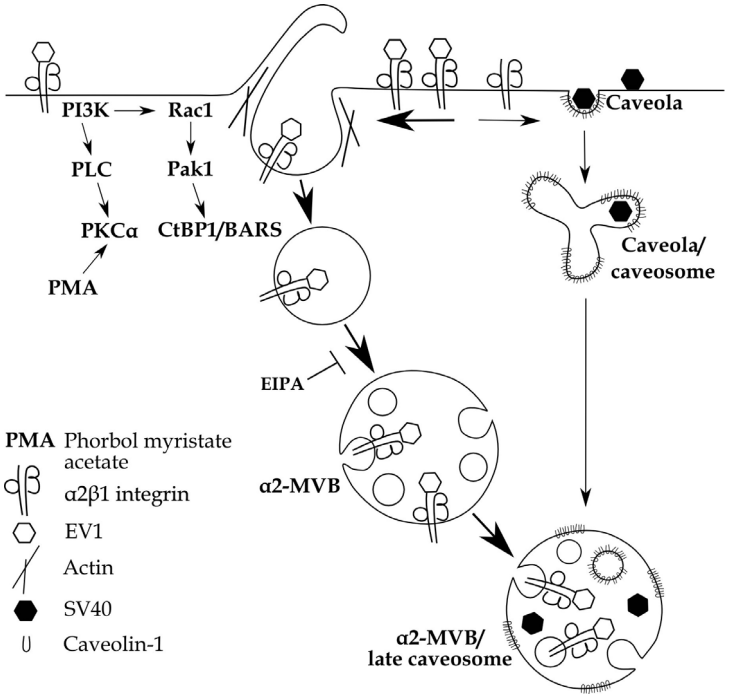

FIGURE 12 The proposed model for entry of EV1 and clustered α2β1 integrin into cells. Internalized tubulovesicular structures mature into multivesicular bodies, α2-MVBs. α2-MVBs are able to fuse with internalized caveolae or caveosomal structures containing caveolin-1 and SV40. The resulting fused structures may be considered as late caveosomes.

6.2 CtBP1/BARS in endocytosis

6.2.1 Role in macropinocytosis

For a long time now, the large GTPase dynamin has dominated the studies concerning fission of cellular membranes. Dynamin is shown to be involved in numerous fission and trafficking events, e.g. in the caveolae-mediated endocytosis, in the clathrin-mediated pathway, in some clathrin- and caveolin-independent endocytic pathways (Hinshaw 2000, Sever et al. 2000b, Conner & Schmid 2003) as well as in the formation of secretory vesicles from the TGN (Cao et al. 2000, Kessels et al. 2006). However, due to the existence of several endocytic pathways that are independent of dynamin (Sabharanjak et al. 2002,

Massol et al. 2004, Glebov et al. 2006), other proteins operating in membrane fissions have also been investigated. In the present study, mutant constructs of CtBP1/BARS showed a clear inhibitory effect in dynamin-independent, EGF-stimulated macropinocytosis. Upon CtBP1/BARS inhibition, dextran internalization was decreased markedly, both in siRNA-experiments and when using dominant-negative mutants of CtBP1/BARS (II). CtBP1/BARS is a member of a dual-function protein family involved both in gene transcription (Chinnadurai 2002) and in membrane fission (e.g. in formation of post-Golgi transport carriers, Bonazzi et al. 2005). Here, the inhibitory effects of the protein did not result from other functions than inhibiting endocytosis, since the dominant-negative forms of CtBP1/BARS did not disturb the trafficking out of the Golgi complex, and their effects were too fast to be mediated by transcriptional activities. These results confirm the previous suggestion, which indicated that CtBP1/BARS might be involved in a clathrin- caveolin- and dynamin-independent endocytic pathway (Bonazzi et al. 2005).

In normal (untreated) cells the location of CtBP1/BARS was both nuclear and cytoplasmic. Upon activation of macropinocytosis by EGF, the protein moved to the forming plasma membrane ruffles and the cup-shaped structures developed from ruffles, and colocalized there with actin (II). Interestingly, CtBP1/BARS is phosphorylated by p21-activated kinase Pak1, which stimulates the actin polymerization via the LIMK1-cofilin pathway (Edwards et al. 1999), and thus possibly contributes to the formation of membrane ruffles. Pak1 has also the ability to mediate the subcellular localization of CtBP1/BARS and also to prevent the nuclear corepressor activity of the protein (Barnes et al. 2003). Since Pak1 activation by EGF (Singh et al. 2005) or by EV1 (I) leads to rapid nuclear localization of Pak1, it is possible that activated Pak1 moves to the nucleus in order to inactivate there the corepressor activity of CtBP1/BARS, which then is 'free' to transfer to the plasma membrane ruffles. Membrane ruffling was also detected by electron microscopy demonstrating a strong enrichment of CtBP1/BARS in ruffling areas (II). Furthermore, Pak1-mediated phosphorylation of CtBP1/BARS was shown to be necessary for EGF-stimulated macropinocytosis (II), confirming the essential role of Pak1 in macropinocytosis.

Although the exact function of dynamin is still a matter of debate (Sever et al. 2000b), it is well agreed that dynamin function (conformational changes driven by GTP-hydrolysis) is needed for the 'pinching off' of the invaginating vesicles from the plasma membrane in dynamin-dependent pathways. Here, the function of CtBP1/BARS was reasoned to be either in the formation or the fission of forming macropinosomes, since no effects on actin ruffling were detected upon inhibition of CtBP1/BARS. Upon CtBP1/BARS inhibition, macropinocytic cup formation was detected but their development into complete macropinosomes was blocked. Studies with FITC- and TRITC-dextran showed that the macropinocytic cups formed were still connected to the extracellular space (II), suggesting that CtBP1/BARS is essential for the fission step to continue properly. Similar observations were obtained in studies with

EV1 and α2β1 integrin, wherein the integrin was shown to accumulate close to the plasma membrane in structures that were still connected to the plasma membrane (II).

The possible functional analogy of CtBP1/BARS to dynamin is of great interest. Although the molecular mechanisms underlying CtBP1/BARS-mediated membrane fission are not clear, it is possible that Pak1 phosphorylation of CtBP1/BARS favors the protein's monomeric conformation, which has actually indicated to be the case when acyl-CoA binds to CtBP1/BARS in COPI-coated vesicle fission (Nardini et al. 2003, Yang et al. 2005). The monomers might then induce fission e.g. by binding cofactors that are responsible for the final fission (Yang et al. 2005). To conclude, in the present study CtBP1/BARS was shown to be an essential player in EGF-stimulated macropinocytosis. Since the protein's function was traced at the macropinosome fission, it thus broadens our knowledge of proteins acting at the fission steps of the plasma membrane derived vesicles.

6.2.2 CtBP1/BARS in EV1 entry

Regulation of EV1 entry by components associated with macropinocytosis (I) prompted the question whether CtBP1/BARS could also be involved in EV1 entry. Indeed, inhibition of CtBP1/BARS by inhibitory constructs, antibodies and inhibitory peptides resulted in a clear block of EV1 infection (II), confirming the status of macropinocytosis in EV1 entry. Involvement of CtBP1/BARS in the caveolar pathway was excluded by studies with SV40, where no inhibitory effect was detected in cells microinjected with CtBP1/BARS mutant. Interestingly, SV40 infection was slightly increased upon inhibition of CtBP1/BARS (II). This may be caused by an upregulation of the caveolar pathway as a consequence to the block of macropinocytosis. In CtBP1/BARS-knockout mice, dynamin has been shown to possess the ability to compensate for the loss of CtBP1/BARS by introducing a dynamin-dependent internalization mechanism (Bonazzi et al. 2005). A similar compensatory effect has been detected also in HeLa cells, wherein the loss of clathrin-mediated endocytosis was rapidly compensated by implementation of a clathrin- and dynamin-independent endocytic pathway (Damke et al. 1995).

By FITC- and TRITC-dextran assays, CtBP1/BARS was observed to act at the fission step of macropinosome closure. The unsuccessful macropinosome formation due to CtBP1/BARS inhibition was also observed with EV1 entry, where electron microscopy images showed an accumulation of α2β1 integrin close to the plasma membrane (II). Revealed by ruthenium red staining, the α2β1 integrin-positive tubular structures were still connected to the plasma membrane. Furthermore, the maturation of the tubulovesicular structures into multivesicular bodies was reduced remarkably due to the inhibition of CtBP1/BARS (II). The differences in size of the macropinosome formed by α2β1 integrin-induced or EGF-induced macropinocytosis may be cell type -dependent, or may be due to the different stimuli used to trigger macropinocytosis. In general, macropinosomes are highly heterogenous in size

and it is possible that there is no regulation regarding the size of the enclosed macropinosomes (Swanson & Watts 1995, Jones 2007).

Finally, upon microinjection with CtBP1/BARS mutant, confocal microscope images showed EV1 accumulation at the plasma membrane, which was further affirmed by quantification (II). Altogether, these results obtained with EV1 and α2β1 integrin internalization further confirm the role of CtBP1/BARS in the fission stage of macropinocytosis and in EV1 infection.

6.3 Cholesterol in EV1 entry

6.3.1 Role of cholesterol in α2β1 integrin entry

During the past decade, growing interest has emerged towards distinct domains within the cellular lipid bilayers, in which specific lipids are concentrated (Simons & Ikonen 1997, Brown & London 2000, Ikonen 2001, Helms & Zurzolo 2004). Cholesterol is one of the key components of these dynamic, sphingolipid-enriched plasma membrane microdomains, lipid rafts. Besides being involved e.g. in signal transduction (Simons & Toomre 2000) and modulation of kinase activity (Young et al. 2003, del Pozo et al. 2004), lipid rafts have also been found to offer an entry site for numerous pathogens (reviewed in Manes et al. 2003 and Lafont & van der Goot 2005). The high concentration of signaling molecules in lipid rafts makes them suitable targets for pathogens to hijack the host cell.

The set of proteins associated with plasma membrane rafts includes α2β1 integrin, which has been shown to reside in GPI-AP-positive domains at the plasma membrane (Upla et al. 2004, Pietiäinen et al. 2004). In the present study, α2β1 integrin internalization was inhibited by the cholesterol-aggregating drugs filipin and nystatin. Both drugs caused an integrin accumulation close to the plasma membrane, and the accumulation was in a similar range as in control cells subjected to α2β1 integrin clustering on ice (III). This indicates a role for cholesterol and possibly lipid rafts in α2β1 integrin entry. Previously, it has been shown that the ability of integrins to form clusters can be influenced by modulating the plasma membrane cholesterol level (Green et al. 1999, Gopalakrishna et al. 2000). However, due to the lack of palmitoyl modifications typical of many raft-associated proteins (Melkonian et al. 1999, Resh 1999), it is unclear whether integrins associate with lipid rafts directly. Indirect association has been reported e.g. for αVβ3 integrin, which localizes to lipid rafts in a signaling complex with trimeric G proteins and integrin-associated proteins (Green et al. 1999).

Lipid rafts have been generally defined as detergent-insoluble glycolipid domains, due to their resistance to solubilization by nonionic detergents such as Triton X-100 at low temperature (Brown & Rose 1992). Here, the multivesicular structures formed following α2β1 integrin clustering and internalization (α2-MVBs) remained intact despite the detergent treatment. Neither the amount nor

the intensity of α2-MVBs was affected, indicating that no structures were solubilized, which further suggests that lipid rafts may exist in α2-MVBs. The presence of lipid rafts in many intracellular organelles has already been suggested, including recycling endosomes (Gagescu et al. 2000), lysosomes (Simons & Gruenberg 2000), and exosomes (Wubbolts et al. 2003). In late endosomes, both the limiting and intraluminal membranes have been shown to be enriched in raft-like domains, with different compositions and properties (Sobo et al. 2007a). Whether lipid rafts are also present in both the limiting and the intraluminal membranes of α2-MVBs, requires further investigation.

Here, the control structures that were marked by the αV integrin subunit were significantly affected by the Triton X-100 treatment. In addition, the cholesterol-aggregating drug filipin clearly contributed to the amount and volume of α2-positive structures, whereas αV-positive structures survived nearly intact (III). Recently, the clathrin-mediated pathway used by αV integrin (Upla et al. 2004) was proposed to be an internalization route for some raft-derived proteins (Abrami et al. 2003, Hansen et al. 2005, Deinhardt et al. 2006), and endosomes in the clathrin pathway are also enriched in cholesterol and other raft lipids (Gagescu et al. 2000, Sobo et al. 2007a). Thus, in the present study, the different influences of the detergent treatment and cholesterol-aggregating drugs on α2- and αV-positive structures may be due to different amounts of lipid rafts in the structures, or perhaps different susceptibility of the integrins to the detergent and the drug. Nevertheless, these results suggest that cholesterol and possibly lipid rafts are present in α2-MVBs.

6.3.2 Role of cholesterol and lipid rafts in EV1 entry

The importance of cholesterol and/or lipid rafts has been related to several different aspects of viral entry. For example, lipid rafts may serve as a platform to concentrate virus receptors, to transport the virus to the appropriate cell compartment, or to affect conformational changes in the virus envelope thus enabling specific interactions during the fusion process (Chazal & Gerlier 2003, Manes et al. 2003). Cholesterol depletion from the plasma membrane has been shown to inhibit the entry of numerous viruses, both enveloped (e.g Ebola virus; Yonezawa et al. 2005, dengue virus; Lee et al. 2008, West Nile virus; Medigeshi et al. 2008) and nonenveloped (e.g. echovirus 11, Stuart et al. 2002). Here, preincubations with filipin and nystatin resulted in total inhibition of EV1 internalization and infection, and confocal images showed virus accumulation at the plasma membrane. This is in line with the known ability of cholesterol-sequestering drugs to inhibit e.g. macropinocytosis (Grimmer et al. 2002, Imelli et al. 2004), the endocytosis of raft-derived GPI-linked proteins (Deckert et al. 1996), and the SV40 entry through lipid rafts (Anderson et al. 1996).

Interestingly, the EV1 infection was inhibited also when the drugs were added two hours p.i. (III), after the time point at which the multivesicular structures enriched in EV1 are already formed (~15 min) (Pietiäinen et al. 2004). When monitoring the effects of nystatin and filipin by electron microscopy, it was observed that the amount of multivesicular structures was greatly reduced,

and the drugs were apparently able to break down some of the already formed α2-positive endosomes (III). Also, the co-internalization assay with the fluid-phase marker FluoSpheres and clustered integrin suggested that the reduction of the fluorescence intensity level of the endosomes after filipin treatment might be due to FluoSpheres leaking out from the damaged vesicles (III). These results imply that the integrity of these structures enriched in cholesterol and possibly lipid rafts is essential for the progression of EV1 infection. Recently, we have shown that EV1 release from the multivesicular structures and the viral replication in the cytoplasm occurs around 2.5-3 h p.i. (Upla et al. 2008). Here, the cholesterol-aggregating drugs were not able to arrest the viral infection if they were added at 3 h (or later) p.i., thus indicating that the drugs could only inhibit EV1 infection if the viral RNA was not yet released for replication. Indeed, studies on EV1 uncoating revealed that nystatin and especially filipin inhibited EV1 uncoating (III). Both in experiments performed with sucrose gradient sedimentation and neutral-red labeled virus (Brandenburg et al. 2007), EV1 uncoating was clearly inhibited compared to control cells, indicating a role for cholesterol and possibly lipid rafts in EV1 uncoating. Similar results have been observed with another, raft-associating picornavirus, echovirus 11, the uncoating of which was suggested to be blocked by a cholesterol-sequestering drug (Stuart et al. 2002). Previously we have shown that EV1 infection is dependent on cytosolic cysteine proteases, calpains (Upla et al. 2008). Although the exact role of calpains in EV1 infection is not clear, our previous study showed that calpains are present in α2-MVBs (Upla et al. 2008). Our recent unpublished results suggest that in addition to replication, calpains might be involved in EV1 uncoating as well.

In general, for enveloped viruses lipid rafts have been shown to play multiple roles during viral life cycle, e.g. in virion assembly and envelope-mediated viral budding (reviewed in Suomalainen 2002 and Chazal & Gerlier 2003). However, in the case of nonenveloped viruses, the requirement for lipid rafts at the later points of viral infection is unclear. Two nonenveloped viruses, rotavirus (Sapin et al. 2002) and bluetongue virus (Bhattacharya & Roy 2008), have been reported to depend on interactions between viral capsid proteins and lipid rafts during viral assembly, but mostly the requirement of lipid rafts in the case of nonenveloped virus concerns the early events at the plasma membrane. This study sheds more light on the role of normal cholesterol organization and a putative involvement of lipid rafts in nonenveloped virus infection. EV1 was shown to be dependent on cholesterol both at the plasma membrane and during the whole viral entry until the genome is released for replication. Furthermore, the EV1 uncoating was critically dependent on intact cholesterol domains. Dominant-negative caveolin-3 constructs have been shown to cause an imbalance in the cellular cholesterol distribution and to interfere with the cholesterol-regulatory role of caveolin (Roy et al. 1999, Pol et al. 2001). Here, the EV1 infection, but not the internalization, was shown to be inhibited by the dominant-negative constructs of caveolin-3 (I), further suggesting a possible role for cholesterol in EV1 infection.

6.4 Regulation of baculovirus uptake

Baculoviruses are insect viruses that cannot replicate in mammalian cells. They are, however, capable of internalizing and effectively delivering genes into various mammalian cells (Hofmann et al. 1995, Boyce & Bucher 1996, Shoji et al. 1997, Ho et al. 2004), which has made them potential gene therapy vectors. To fulfil this purpose, the internalization mechanism(s) of baculoviruses has to be revealed. In insect cells, baculoviruses are thought to use adsorptive endocytosis for cell entry (Volkman & Goldsmith 1985), although the exact mechanism is still unclear. In mammalian cells, clathrin-mediated endocytosis has been suggested (Long et al. 2006). Previously, baculovirus was shown to accumulate into EEA1-positive endosomes at 30 min post transduction (Matilainen et al. 2005). However, in the present study, no association of prototype baculovirus (*Autographa californica* multiple nucleopolyhedrovirus, *Ac*MNPV) with the clathrin-mediated pathway was detected during the early internalization. The virus did not colocalize with markers of clathrin-mediated endocytosis, and no viruses could be detected in clathrin-coated vesicles (IV). Instead, baculovirus was observed to induce extensive membrane ruffling, and it was internalized into large vesicles containing fluid-phase markers. This, together with the observation that the clathrin-mediated pathway was not involved, suggested that some other endocytic pathway was utilized by the virus. Interestingly, uptake of the virus was shown to occur through cholesterol-enriched and possibly lipid raft-derived areas of the plasma membrane, since the cholesterol-aggregating drug filipin inhibited the virus entry efficiently (IV). Many clathrin-independent pathways, e.g. GEEC (Sabharanjak et al. 2002), IL-2 (Lamaze et al. 2001) and flotillin (Glebov et al. 2006) originate from the lipid raft-enriched areas of the plasma membrane. However, no colocalization between baculovirus and these pathways was detected (IV).

The rather large size of *Ac*MNPV (approx. 60 nm × 266 nm, Transfiguracion et al. 2007) may indeed exclude some endocytic pathways from the virus, although for example the clathrin-mediated pathway has shown remarkable flexibility by serving as an internalization route even for large bacteria (Veiga & Cossart 2005). Usually, to internalize particles large in size or to gain large amounts of extracellular fluids, cells have two intrinsically similar pathways, namely macropinocytosis and phagocytosis. They both are dependent on actin and a set of Rho GTPases for membrane ruffle formation (Swanson 2008). Here, baculovirus induced membrane ruffling was detected, but macropinocytic regulators, Rho GTPases Rac1 and Cdc42, as well as Rab34, Pak1 and CtBP1/BARS did not play any role in the virus entry (IV). Instead, Arf6 and RhoA were involved, indicating a possible role of a phagocytic-like mechanism in baculovirus entry. Arf6 is generally associated with clathrin-independent entry (Mayor & Pagano 2007). Previously, Arf6 has been shown to induce phagocytic uptake in macrophages by regulating the actin cytoskeleton

reorganization (Balana et al. 2005). Here, both DN and CA mutants of Arf6 inhibited baculovirus infection. The inhibitory effect of the CA mutant may have occurred through inactivation of RhoA, since previously, RhoA signaling was shown to be downregulated via activation of Arf6 (Boshans et al. 2000). Of note, whereas clathrin-mediated endocytosis is flexible enough to take up large bacteria, phagocytosis has been shown to be induced also by rather small particles, e.g. herpes simplex virus-1 (HSV-1) (Clement et al. 2006). Interestingly, the phagocytic uptake of HSV-1 into non-phagocytic human fibroblasts was regulated by RhoA but not Rac1 or Cdc42 (Clement et al. 2006).

In addition to RhoA and Arf6, the uptake of baculovirus seemed to be dependent on dynamin (IV). However, the role of dynamin in phagocytosis is rather controversial. Phagosome formation in macrophages has been reported to be dependent on dynamin (Gold et al. 1999), whereas Boleti and colleagues (1999) obtained opposite results. Furthermore, the internalization of *Chlamydia trachomatis* into epithelial cells was not affected by dynamin inhibition (Boleti et al. 1999). These differences may be due to the cell types used, or perhaps due to the presence of distinct mechanisms of phagocytosis (type I or type II), controlled by different Rho GTPases (Caron & Hall 1998). Interestingly, RhoA-regulated type II phagocytosis has been linked to complement-activated phagocytosis (Caron & Hall 1998), and activation of the complement system has also been detected after baculovirus administration (Hofmann & Strauss 1998).

To study phagocytosis, *E. coli* bioparticles have been widely used as phagocytic tracers. Here, baculovirus was shown to colocalize well with *E.coli* during the early entry. Since *E.coli* entry was extremely low in the absence of baculovirus, the virus obviously induced the uptake of the bacteria, which were not able to enter the non-phagocytic human cancer cells without baculovirus (IV). To conclude, our studies on baculovirus entry suggest that the virus induces ruffle formation on the plasma membrane and uses a phagocytic-like entry mechanism into non-phagocytic cells.

7 CONCLUSIONS

The main conclusions of this thesis are:

1. EV1 and α2β1 integrin internalization occurs together with fluid-phase markers into tubulovesicular structures that quickly mature into multivesicular bodies. The pathway is regulated by factors that have all been associated with macropinocytosis, namely PLC, PI3K, Rac1, Pak1 and CtBP1/BARS.

2. EV1 and α2β1 integrin entry is dependent on a normal organization on membrane cholesterol and possibly lipid rafts both at the plasma membrane and during the entry process. The viral uncoating and multivesicular structure formation are dependent on intact cholesterol domains in α2-MVBs.

3. *Ac*MNPV-baculovirus utilizes a clathrin-independent internalization mechanism that is regulated by Arf6 and RhoA. The virus induces membrane ruffling and is internalized together with fluid-phase markers. In addition, baculovirus triggers *E.coli* uptake into non-phagocytic human cells. The baculovirus entry is thus reminiscent of phagocytosis.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Echovirus 1:n ja bakuloviruksen soluun sisäänmenon reitit ja säätely

Solut ottavat sisäänsä materiaalia solun ulkopuolelta muodostamalla solukalvolle kuopan, joka lopulta kuroutuu rakkulana solun sisälle. Tällä tavalla solut saavat toimintansa kannalta elintärkeitä aineita, muun muassa ravintoaineita sekä immuunipuolustuksessa tarvittavia molekyylejä. Erilaisia kulkureittejä solun sisään tunnetaan useita, ja monille näistä reiteistä on myös löydetty molekyylejä, jotka toimivat reitin säätelytekijöinä. Useat virukset ja bakteerit hyödyntävät näitä samoja, solun normaaliin toimintaan kuuluvia reittejä soluun tunkeutumisessaan. Solukalvolta kuroutuvien vesikkelien sisällä virukset pääsevät syvemmälle solulimaan ja lopulta sellaiseen paikkaan solussa, jossa niiden genomin vapauttaminen, monistaminen ja uusien viruspartikkelien tuottaminen voi alkaa. Tässä väitöskirjassa tutkittiin kahden viruksen, echovirus 1:n ja bakuloviruksen mekanismeja tunkeutua solun sisään.

Echovirus 1 (EV1) on pieni, vaipaton RNA-virus, joka kuuluu laajaan pikornavirusten geeniperheeseen. Samaan geeniperheeseen kuuluvat mm. hyvin yleiset flunssan aiheuttajat, rinovirukset, sekä aikanaan laajaa tuhoa aiheuttanut poliovirus. Echovirukset voivat aiheuttaa ihmisillä aivokalvontulehdusta, sydänlihastulehdusta sekä hengitystieinfektioita. Väitöskirjan ensimmäisessä osatyössä tutkittiin EV1:n kulkureittiä solun sisään. Vaikka useat virukset pääsevät solun sisään joko klatriinivuoratuissa vesikkeleissä tai kaveoliinia sisältävissä vesikkeleissä, EV1 ei käyttänyt kumpaakaan näistä reiteistä. EV1:n huomattiin ensin kulkeutuvan putkimaisiin tai pyöreähköihin vesikkeleihin, jotka vähitellen kypsyivät suuremmiksi, sisävesikkeleitä sisältäviksi rakenteiksi. Näihin rakenteisiin päätyi myös kaveoliinireittiä pitkin simian virus 40 (SV40) ja kaveoliini-1, joten näiden kahden reitin todettiin yhdistyvän solun sisällä. EV1:n kulkureitillä toimivien säätelytekijöiden (PLC, PI3K, Rac1, Pak1) huomattiin olevan samoja, joiden on aikaisemmin havaittu toimivan makropinosytoosissa. Makropinosytoosi on solun tapa ottaa sisäänsä suuria (0,2-5 µm) partikkeleita, jotka makropinosomi-vesikkeleissä kulkeutuvat syvemmälle solulimaan. EV1:n havaittiin siis käyttävän makropinosytoosia soluun kulkeutumisessaan.

Väitöskirjan toisessa osatyössä tutkittiin CtBP1/BARS-proteiinin merkitystä makropinosytoosissa. Tämän proteiinin huomattiin olevan tärkeä säätelytekijä epidermaalisen kasvutekijän indusoimassa makropinosytoosissa, sillä makropinosomien kuroutuminen solun sisään oli täysin riippuvaista CtBP1/BARS:n toiminnasta: jos tämän proteiinin toiminta estettiin, makropinosomit alkoivat muodostua solukalvolla, mutta ne eivät päässeet kuroutumaan solukalvosta irti solun sisälle. Lisäksi CtBP1/BARS:n todettiin olevan tärkeä säätelijä EV1:n soluun sisäänmenossa. Tämä vahvisti osaltaan myös ensimmäisen osatyön tuloksen, jonka mukaan EV1 käyttää makropinosytoosia tehokkaassa soluun tunkeutumisessaan. CtBP1/BARS:n osallistuminen vesikkelien kuromiseen solun sisälle oli myös merkittävä havainto, sillä aikaisemmin

vain dynamiinin on havaittu toimivan vesikkelien sisäänkuroutumisessa solukalvolta.

EV1:n sekä sen reseptorin, α2β1-integriinin, soluun kulkeutumisen havaittiin myös olevan riippuvaista kolesterolista. Kolesteroli on solukalvojen olennainen rakenneosa, jota esiintyy solukalvoilla 50-90% (solutyypistä riippuen) koko solun sisältämästä kolesterolista. Solukalvolla kolesteroli, sfingolipidit ja glyserolipidit voivat muodostaa hyvin järjestäytyneitä, lateraalisia alueita, ns. lipidilauttoja, joita tarvitaan mm. proteiinien lajittelussa ja signaalikompleksien muodostamisessa. Useiden virusten on myös havaittu käyttävän lipidilauttoja hyväkseen esimerkiksi solukalvoon kiinnittymisessä ja soluun tunkeutumisessa. Väitöskirjan kolmannessa osatyössä havaittiin kolesterolin toimivan tärkeänä komponenttina α2β1-integriinin ja EV1:n soluun pääsyssä. Lisäksi niiden rakenteiden, joihin α2β1-integriini ja siihen sitoutunut EV1 solun sisällä kulkeutuivat, huomattiin sisältävän kolesterolia ja mahdollisesti lipidilauttoja. Näiden rakenteiden normaalin kolesteroliorganisaation tuhoaminen aiheutti EV1:n infektion estymisen. Myös EV1:n RNA-genomin vapautuminen endosomin sisällä oli riippuvaista ehjistä kolesterolidomeeneista.

Neljännessä osatyössä seurattiin hyönteisiä infektoivan bakuloviruksen soluun sisäänmenoa. Bakulovirukset ovat suuria DNA-viruksia, jotka pystyvät monistumaan vain niveljalkaisissa. Tästä huolimatta ne voivat päästä myös nisäkässolujen sisälle, vaikka näissä soluissa ne eivät voikaan tuottaa uusia viruspartikkeleita. Toisaalta, juuri tämän ominaisuuden takia bakulovirukset ovat potentiaalisia kuljettimia geeniterapiassa, jossa solujen perintöaineksen vaurioitunut tai puuttuva geeni pyritään korvaamaan. Jotta bakuloviruksia voitaisiin käyttää geeninsiirtovektoreina, niiden kulkureitti soluun on ensin tunnettava. Bakuloviruksen (*Autographa californica* multiple nucleopolyhedrovirus, *Ac*MNPV) todettiin indusoivan ihmisen maksasyöpäsolulinjassa solukalvojen poimuuntumista, mikä on ominaista sekä makropinosytoosille että fagosytoosille. Reittiä säätelevien molekyylien (Arf6 ja RhoA) huomattiin olevan samoja, jotka toimivat soluissa fagosytoosireitillä. Bakuloviruksen todettiin myös indusoivan fagosytoosia käyttävän *E.coli*n soluun sisäänmenoa, joten bakuloviruksen reitti solun sisälle muistutti huomattavasti fagosytoosia.

Jotta virusten aiheuttamia tauteja voitaisiin tehokkasti estää ja hoitaa, ensin on pyrittävä selvittämään virusten ja isäntäsolujen monimutkaisia vuorovaikutussuhteita. Geeniterapiassa puolestaan tarvitaan tietoa siitä, miten virusvektorit nisäkässoluihin päätyvät. Tutkimalla virusten soluun kulkeutumisen varhaisia vaiheita saadaan arvokasta tietoa paitsi itse virusten toiminnasta, myös virusten käyttämistä soluun sisäänmenon reiteistä ja niiden säätelytekijöistä.

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