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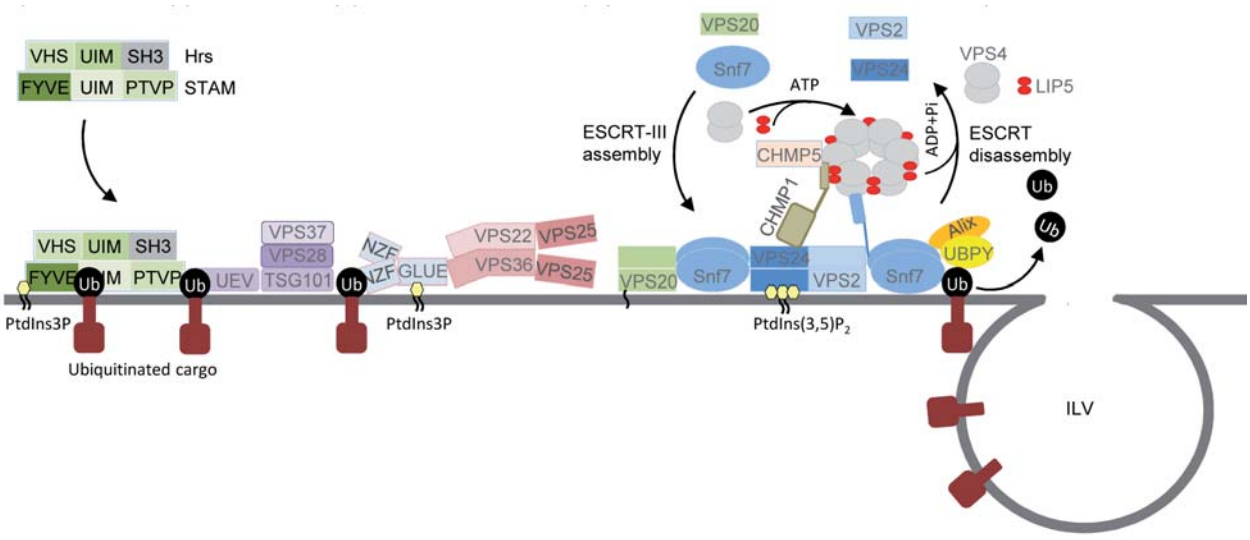
227

Mikko Karjalainen

Echovirus 1 infectious entry via novel multivesicular bodies



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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2011

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ABSTRACT

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Echovirus 1 is a small non-enveloped virus that belongs to the Picornaviridae family of RNA viruses. It is a human pathogen and is able to cause a variety of diseases, such as meningoencephalitis, carditis and rashes, as well as mild respiratory and enteric diseases. Here, the early internalization and post-entry trafficking of EV1 were studied in detail. The majority of the early uptake was shown to be dependent on factors regulating macropinocytosis, including p21-activated kinase (Pak)1, Rac1, class I phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC) & actin. Instead, caveolin-1, clathrin & flotillin mediated routes, as well as the pathway used by glycosylphosphatidylinositol anchored proteins had a negligible role in EV1 internalization. The EV1 receptor α2β1 integrin, clustered by antibodies or by EV1 binding, internalized into tubulovesicular structures & this step seemed to require a functional ubiquitination machinery. Endosomes matured further into larger multivesicular bodies within 15 min to 2 h & this process was dependent on an endosomal sorting complex required for transport (ESCRT). EV1/α2β1 integrin positive endosomes were shown to be devoid of markers of late endosomes and lysosomes, such as Rab7, CD63, LAMP-1, CI-MPR, Dil-LDL and LBPA and did not markedly acidify. Furthermore, they did not accumulate activated epidermal growth factor receptors (EGFR) that are known to be internalized into multivesicular bodies. Over-expression of dominant negative VPS4 and studies with pharmacological agents that inhibit macropinocytosis (EIPA) and ubiquitination (PYR-41) showed that the structural maturation of endosomes and the continued activity of ubiquitinating enzymes were important for successful infection. It was also shown that clustered α2β1 integrin is associated with an active form of calpain-1. The clustered and internalized integrin does not recycle back to the plasma membrane but instead it undergoes enhanced down-regulation that is independent of the proteosome and lysosomes, but is promoted by activated neutral calpains.

Keywords: Echovirus 1, endocytosis, ESCRT, multivesicular body, integrin.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

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, Pak-1-regulated entry participates in α2β1 integrin-dependent sorting to caveosomes. *Molecular Biology of the Cell*, 19:2857-2869.

II Karjalainen M., Rintanen N., Lehkonen M., Kallio K., Mäki A., Hellström K., Siljamäki V., Upla P. & Marjomäki V. 2011. Echovirus 1 infection depends on biogenesis of novel multivesicular bodies. (in press)

III Rintanen N., Karjalainen M., Paavolainen L., Mäki A., Nissinen L., Cheng H.R., Upla P. & Marjomäki V. 2011. Calpains promote α2β1 integrin down-regulation after integrin clustering triggered internalization. (submitted manuscript)

* equal contribution

RESPONSIBILITIES OF MIKKO KARJALAINEN IN THE ARTICLES OF THIS THESIS

Article I: I conducted the experiments together with Elina Kakkonen. I was responsible for the studies on α2β1 internalization in experiments concerning caveolin, SV40 and dextran. I was also responsible for the EV1 infection assays in experiments with macropinocytosis inhibitors and dynamin together with Heli Paloranta. I conducted the siRNA assays and studies with mutant constructs. I was responsible for the confocal microscopy and data analysis together with Elina Kakkonen. I prepared the figures for publication and participated in the writing of the article together with Varpu Marjomäki and Elina Kakkonen.

Article II: I was responsible for planning the experiments together with Varpu Marjomäki. I performed the immunofluorescence studies, live-microscopy and EV1 infection assays with ESCRTs, siRNAs and mutant constructs. I also did the EM imaging and subsequent quantification of the data. I did the experiments with EGF together with Anita Mäki and Moona Lehkonen. I constructed the figures and I wrote the article together with Varpu Marjomäki.

Article III: I conducted the confocal imaging of samples in degradation assays based on fluorescence together with Nina Rintanen. I designed the data analysis protocol and analyzed the data in fluorescence-based degradation and recycling assays. I participated in writing and finalizing the article together with Nina Rintanen and Varpu Marjomäki.

ABBREVIATIONS

A549	human lung carcinoma cell line
AP	adaptor protein
ATP	adenosine triphosphate
BARS	brefeldin A-ADPribosylated substrate
Cav1	caveolin
CCP	clathrin coated pit
CCV	clathrin coated vesicle
CI-MPR	cation-independent mannose-6-phosphate receptor
CLIC	clathrin independent carrier
CME	clathrin-mediated endocytosis
CPE	carboxypeptidase E
CR3	C3bi receptor
CtBP-1	carboxy-terminal-binding protein-1
CTxB	cholera toxin B
DN	dominant negative
DUB	deubiquitinating enzyme
ECM	extracellular matrix
EE	early endosome
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EM	electron microscopy
EPS	EGFR-pathway substrate
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
EV1	echovirus1
FAK	focal adhesion kinase
FC	focal contact
FcR	Fc-receptor
FGFR	fibroblast growth factor
GEEC	GPI-anchored protein enriched early endosomal compartment
GFP	green fluorescent protein
GHR	growth hormone receptor
GPI	glycophosphatidylinositol
GTPase	guanoside triphosphatase
IgG	immunoglobulin G
IL2	interleukin 2
ILV	intraluminal vesicle
LBPA	lysobisphosphatidic acid
LE	late endosome
ME	maturing endosome
MVB	multivesicular body

NEM	n-ethylmaleimide
Pak1	p21-activated kinase 1
PDGF	platelet derived growth factor
PI3K	phosphatidyl inositol 3-kinase
PIP2	phosphatidylinositol (4,5)-bisphosphate
PKC	protein kinase C
RTK	receptor tyrosine kinase
SAOS	human osteosarcoma cell line
siRNA	short interfering RNA
SV40	simian virus 40
t-BOC	fluorogenic calpain substrate: 7-amino-4-chloromethylcoumarin, *t*-BOC-l-leucyl-l-methionine amide
TGN	*trans*-Golgi network
UIM	ubiquitin interacting motif
VEGF	vascular endothelial growth factor

1 INTRODUCTION

The evolution of organisms and viruses are closely linked. There is still a debate on which came first: are viruses just pieces of genome that accidentally escaped from a cell or did cells actually evolve from some primitive viruses? The fact is that all cellular life can be infected by viruses. Viruses are obligatory parasites that need the cellular machinery of the host organism in order to replicate. The variety of viruses is enormous and their size can vary from less than 30 nm up to 750 nm and their shape can be anything from an icosahedron to the complex structures of bacteriophages. Regardless of their nature, all viruses need to cross a critical barrier, the plasma membrane, in order to enter cells. To overcome this barrier, viruses have learned to utilize naturally occurring mechanism present in animal cells. Endocytosis is a carefully regulated means for cells to take up liquids, nutrients and other solutes. By taking advantage of this system, a virus gains access to the cell. For scientists, studying viral mechanisms of infection not only provides a lot of valuable information about the virus, but also helps us understand the biology of the host cell.

In this thesis, the early infection pathway of a small human pathogen, echovirus 1 was studied in detail. When measured in size, echovirus 1 is among the smallest of viruses with a diameter of 30 nm. But size is not everything in the world of viruses and cells & echovirus 1 can effectively take over a cell thousands of times larger than itself. A part of this process was unveiled in this thesis and the internalization pathway of echovirus 1 was shown to have some unique characteristics. Also, this study provided new information on integrin internalization and downregulation

2 REVIEW OF THE LITERATURE

Viruses are sometimes called "the ultimate cell biologists" as they are able to manipulate cells and take control of the cellular machinery. For this reason they are invaluable tools for studying cells. In this thesis studies on echovirus 1 (EV1) revealed new aspects about the role of integrins, endosomes and protein down-regulation in endocytosis. In the following chapters I will introduce the players of this thesis α2β1 integrin and EV1 & summarize the latest knowledge of the best characterized endocytic pathways in mammalian cells.

2.1 Echovirus 1

2.1.1 General properties of echovirus 1

EV1 is a human pathogen belonging to the genus Enterovirus of the Picornaviridae family. It causes a variety of diseases, including meningitis, encephalitis, carditis, rashes as well as mild respiratory and enteric diseases (Grist et al. 1978). It is a non-enveloped virus a with single-stranded (+)RNA genome of 7 – 8 kb, packed in a small protein capsid (30 nm in diameter). The structure of the protein shell is similar to other picornaviruses and consists of 60 heteromeric units, each composed of four capsid proteins, VP1-VP4 whicht arrange into 12 pentamers, which in turn are organized along an icosahedral symmetry (Filman et al. 1998). VP1-VP3 build the outer side of the capsid, VP4 in turn is hidden on the inner capsid surface and is in close association with the viral RNA (Hogle et al. 1985). VP1 molecules form pentamers around icosahedral 5-fold axes, whereas VP2 and VP3 form heterohexamers around icosahedral 3-fold axes (Hogle et al. 1985) (Fig. 1). Like most picornaviruses EV1 has a canyon along the 5-fold axes that acts as the receptor binding site (Rossmann et al. 2002). All five binding sites around an EV1 5-fold axis can be occupied by receptor molecules (Xing et al. 2004). The infection cycle of EV1 probably follows generally similar stages as do those of other picornaviruses: first, the virus binds to cell surface receptors, inducing signaling and the

subsequent internalization of the virus and the receptor & later the release of the genome into the cytoplasm. How the EV1 genome is released is currently not known but in the cytoplasm the viral RNA is translated to produce a virus-coded RNA synthesizing machinery, initiating replication and finally leading to the release of new virions by cell lysis (Fig. 2).

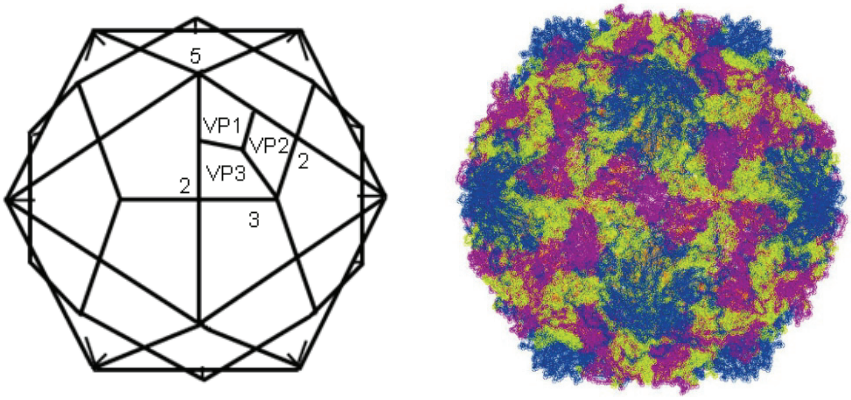

FIGURE 1 Echovirus 1 particle structure. Cartoon representation of the EV1 icosahedron showing the locations of 2-, 3- and 5-fold axes of symmetry and VP1–3 (left). Molecular structure of EV1 showing different amino acid chains. The structure was created using Jmol version 12.0.41 and the atomic coordinates were downloaded from Protein Data Bank, Brookhaven National Laboratory (right).

2.1.2 EV1-receptor interaction

2.1.2.1 Integrins

Integrins are a superfamily of cell adhesion receptors that mediate interactions with the extracellular matrix (ECM) and cell-surface ligands & also with some soluble ligands (Hynes 2002). The ECM substrates include laminin, collagen, vitronectin and fibronectin (Humphries et al. 2006). Integrins are also receptors for many viruses, including adenovirus, EV1, hantavirus & foot and mouth disease viruses (Stewart et al. 2007). Integrins form transmembrane αβ heterodimers (Fig. 3) & currently 18 α and eight β subunits are known and 24 different heterodimers have been found in humans (Hynes 2002). By interacting with the ECM, integrins attach cells to their environment or to each other. They control many cellular processes, such as migration and proliferation that are important during development and in the progression of diseases such as cancer (Schwartz 2001, Hynes 2002). Besides their role in cell adhesion, by sensing their environment through binding (or unbinding) to the matrix, integrins can activate many intracellular signaling pathways through their connections with the cytoskeleton. They act as bi-directional transducers that can mediate signals from the outside to the inside of cells or vice versa (Schwartz 2001). These signal transduction pathways are complex and there are many similarities to those triggered by growth factor receptors. Indeed, integrin

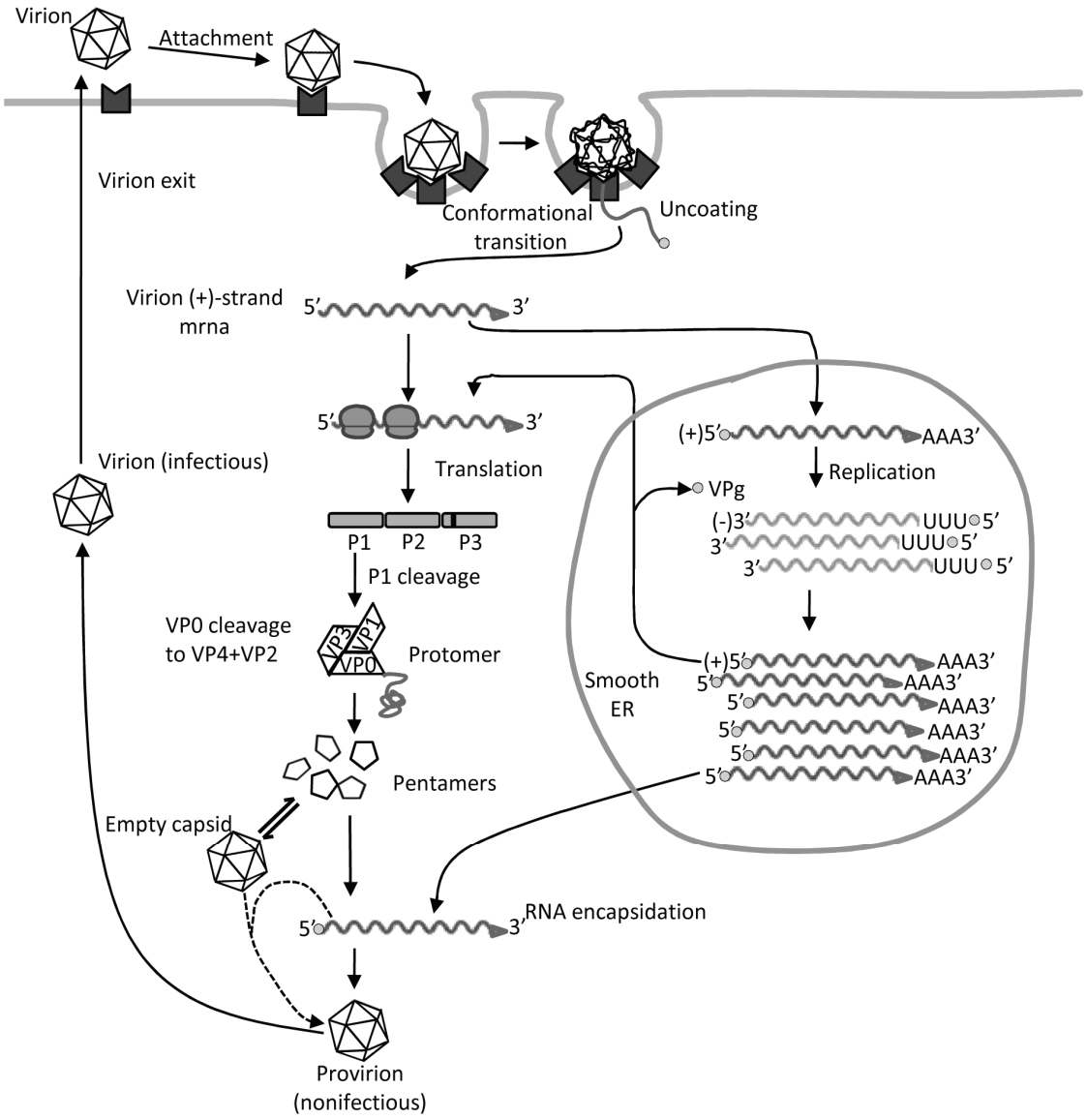

FIGURE 2 The infectious cycle of picornaviruses. Infection is initiated when the virus bindsto the receptor. Conformational changes in the virus result in the release of the viral RNA into the cytoplasm where it is replicated. These progeny RNAs are then translated into a long polyprotein that is processed by viral proteases. New viruses are assembled in stages, including a protomer, a pentamer, an empty capsid, a provirion & finally the fully infectious virus. Redrawn from Hogle (2002).

and growth factor receptor activation are closely coupled as integrins can influence the way growth factor receptors such as epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) respond to their ligands (Walker et al. 2005). Integrins recruit also other signaling molecules such as focal adhesion kinase (FAK) to initiate "outside-in" signaling and modulate cellular responses to extracellular events (Caswell et al. 2009). The intracellular signaling pathways triggered by the binding of extracellular ligands to integrins control two important functions: organization of the

cytoskeleton and regulation of cellular properties such as survival, differentiation and growth (Cabodi et al. 2010).
When integrin function is controlled from within the cell, it is called "inside-out' signaling" (Hynes 2002). The conformational state of the integrins, controlled by proteins associated with the β-integrin cytoplasmic tail, affects how integrins bind their ligands (FIG. 3). Talin and kindlin have an important role in switching integrins from a low-affinity conformation to a high-affinity active conformation. This process is important for platelet activation and for the coordination of adhesion during the migration of various cell types (Wegener et al. 2007, Harburger et al. 2009).

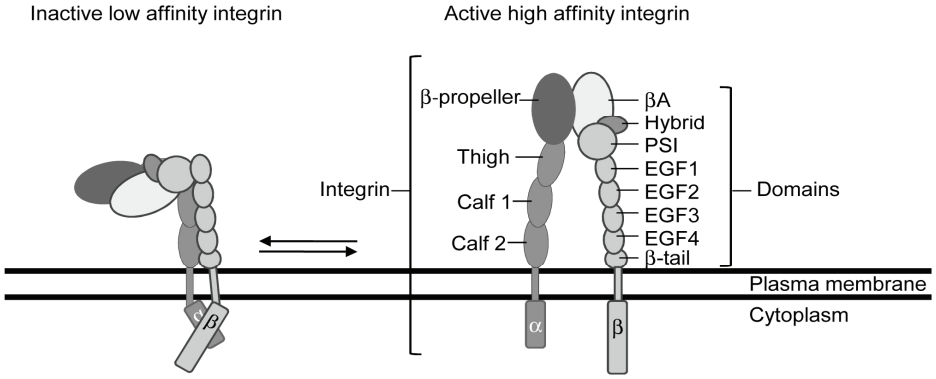

FIGURE 3 Integrin architecture and schematic representation of integrin activation. Upon activation, the cytoplasmic tails of the α and β -subunits separate, allowing the assembly of multiprotein signalling complexes at the β-cytoplasmic tail. EGF, epidermal growth factor; PSI, plexin–semaphorin–integrin domain. Modified from Moser et al. (2009), Smith et al. (2010).

2.1.2.2 α2β1 integrin as an EV1 receptor

The primary cell surface receptor for EV1 cell entry is α2β1 integrin, more specifically, an inserted domain in the α2 subunit (α2I) (Bergelson et al. 1992, Bergelson et al. 1993). α2β1 integrin is expressed in a variety of cell types, including platelets, epithelial cells and mesenchymal cells, such as fibroblast and osteoblasts. The main natural ligands of this integrin are collagen and laminin that also bind to the α2I-domain (Elices et al. 1989). However, there are differences between collagen and EV1 binding to integrin: the two ligands bind to slightly different sequences in the α2I-domain (King et al. 1995) & unlike collagen, EV1 does not need divalent cations for attachment (Bergelson et al. 1993). Structural studies have revealed that collagen and EV1 cannot bind simultaneously to the α2I domain due to a partial overlap in binding spaces (Xing et al. 2004), but the binding affinity of EV1 is ten times higher compared to collagen, helping it to compete for free α2β1 integrin (Xing et al. 2004). In addition, because all five binding sites in the 5-fold axis of the viral capsid can be occupied, EV1 avidity increases markedly and results in very tight binding.

Unlike many other picornaviruses whose interactions with their receptors destabilize the virion structure and leads to RNA release (Rossmann et al. 2002),

the binding of EV1 to α2β1 does not induce uncoating (Marjomäki et al. 2002). On the contrary, it is believed that the viral capsid is stabilized upon receptor binding (Xing et al. 2004), possibly preventing untimely genome release.

2.1.2.3 Internalization of EV1

In order to infect cells, viruses need to get access to the cytoplasm of the host cell and take use of the cellular machinery to make up new copies of the virus. One means to cross the plasma membrane is a process called endocytosis (discussed in more detail below). Multiple attachments of EV1 to receptors causes the clustering of α2β1 integrins, which in turn leads to signaling, followed by the rapid internalization of the virus together with the receptor (Upla et al. 2004). EV1 capsid proteins are seen in the perinuclear vesicular structures together with α2β1 integrin within 15 min to 2 h post infection (p.i.). The initial internalization is dependent on protein kinase C (PKC)α activation (Upla et al. 2004), however the exact means of the uptake has remained obscure and there are some contradictory results (Marjomäki et al. 2002, Pietiäinen et al. 2004, Upla et al. 2004). EV1 is internalized into endosomes that accumulate caveolin-1 (Cav1), a marker molecule for the slow endocytic pathway & some of the virus can be found in caveolae (Marjomäki et al. 2002, Upla et al. 2004. However, the primary entry pathway seems to be independent of Cav1 (Pietiäinen et al. 2004). This is supported by the fact that EV1 uptake is fast and independent of actin, unlike the caveolar uptake of simian virus 40 (SV40) (Pelkmans et al. 2001, Pietiäinen et al. 2004) and the majority of EV1 do not colocalize with caveolin on the plasma membrane (Pietiäinen et al. 2004).

2.2 Overview of the endocytic pathways in mammalian cells

Eating and drinking, or ingestion, are essential for every living being in order to stay alive. This feature is not only relevant at the level of the whole organism, say an elephant, but every single cell of that elephant needs to "eat and drink" too At the cellular level this process of ingesting compounds is called endocytosis. For a cell, endocytosis is more than just taking up nutrients: it is a means of regulating the lipid and protein composition of the plasma membrane, which in turn determines how the cell interacts with its environment. It is also becoming clear that endocytosis has an important role in organizing cell signaling both spatially and temporally (Scita et al. 2010). As our knowledge of endocytosis grows, it is becoming evident that there are several different endocytotic pathways that are differentially regulated. Most of the greatest advancements in the field have become from studying viruses which, in the course of evolution, have learned to take advantage of endocytic processes in order to invade cells. (Fig. 4).

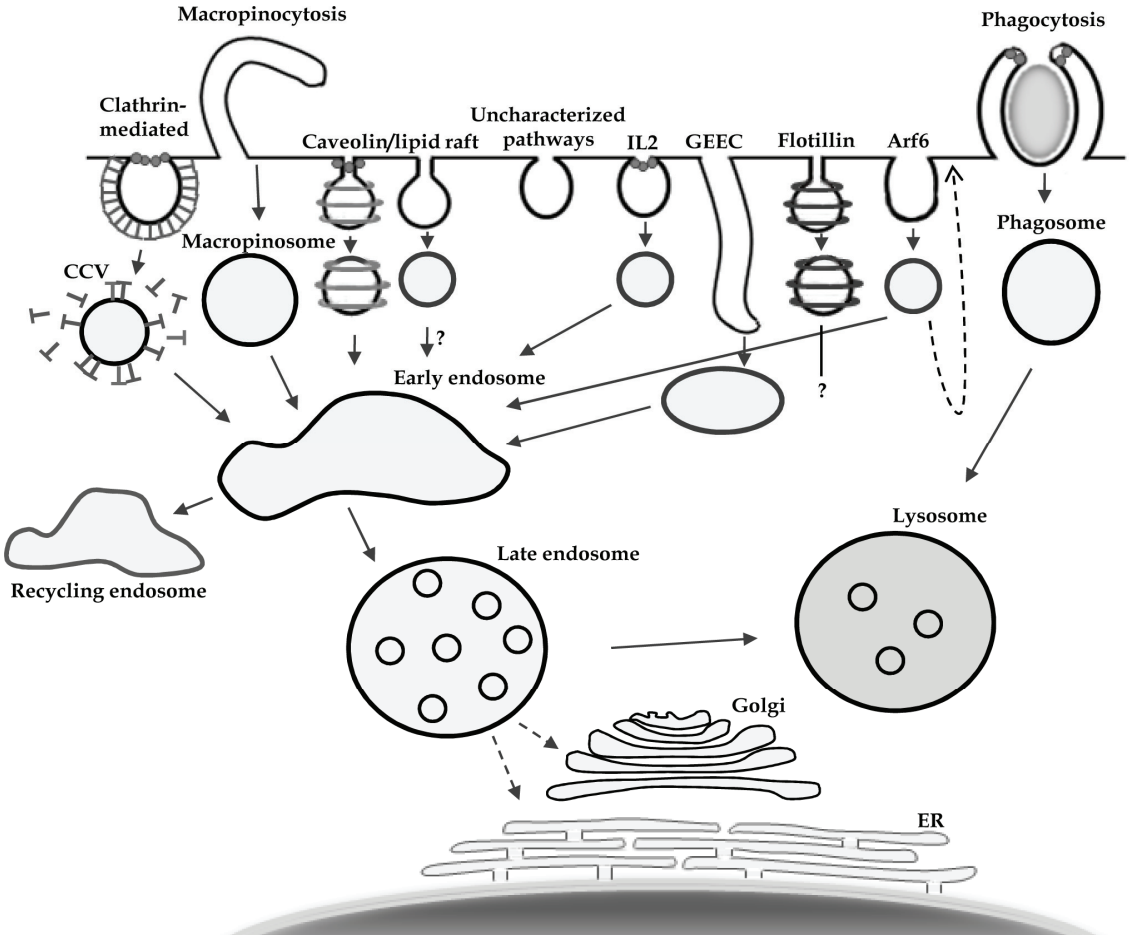

FIGURE 4 Endocytic pathways and intracellular trafficking of endocytosed material in mammalian cells. CCV, clathrin coated vesicle; ER, endoplasmic reticulum; IL2, interleukin-2; GEEC, GPI-anchored protein enriched early endosomal compartment. Modified from Marsh et al. (2006), Mayor et al. (2007), Doherty et al. (2009), Mercer et al. (2010).

2.2.1 Clathrin-mediated endocytosis

The clathrin-mediated endocytosis (CME) pathway is considered the major pathway for ligand internalization in mammalian cells. It is responsible for the uptake of nutrients, growth factors, receptors, antigens and pathogens. The key element of the CME pathway is a soluble multimeric clathrin complex that consists of three heavy chains and three light chains and takes the shape of a triskelion. The clathrin triskelia are recruited from the cytoplasm to the plasma membrane and assemble into a polygonal lattice to form clathrin coated pits (CCP) at the sites of membrane budding. The CCPs then pinch off from the membrane to the cytoplasm through the action of the GTPase dynamin, forming a clathrin-coated vesicle (CCV) (Kirchhausen 2000, Pucadyil et al. 2008). Shortly after internalization CCVs ares uncoated and fuse with the early endosome. From there the endocytosed cargo is either sorted to late endosomes

and subsequently lysosomes for degradation, or alternatively recycled back to the plasma membrane. Along the degradation pathway, the endosomal pH decreases from the mildly acidic conditions of early endosomes (pH 6.5-6.0), to more acidic late endosomes (pH 6.0-5.0) and lysomes (pH 5.0-4.5). The regulation of luminal acidity is achieved by the regulation of V-ATPase activity and specific targeting and trafficking of V-ATPase as well as other proteins involved in adjusting intraendosomal pH, such as channels and exchangers (for example Na^+/H^+ exchanger), to organelles for review, see (Marshansky et al. 2008).

Based on theoretical calculations, it is unlikely that clathrin polymerization alone is sufficient to create membrane curvature (Nossal 2001). Indeed, there are several accessory and adaptor proteins coordinating CCV formation. The adaptors link the membrane proteins with the clathrin lattice that forms the outer layer of the coat. Together with accessory and regulatory molecules, cargo proteins, adaptors and clathrin co-assemble & the growing coated pit invaginates. It has been proposed that there are different subtypes of CCVs in the cell. These are separated by distinct adaptor and accessory proteins that control the internalization of distinct cargoes (Benmerah et al. 2007). Different specialist proteins are probably required to assemble each pit subtype. The best characterized adaptors are a family of closely related proteins called the adaptor proteins (APs) comprising AP1, AP2, AP3 and AP4, of which the AP2 adaptor is associated with receptor-mediated endocytosis (Collins et al. 2002, Owen et al. 2004). AP2 interacts with the plasma membrane through binding sites for the common plasma membrane lipid phosphatidylinositol (4,5)-bisphosphate (PIP2). Internalized receptors contain short sequence motifs in their cytoplasmic domains that are recognized by APs, like the YTRF motif of the transferrin receptor (Collawn et al. 1990).

There are several known accessory proteins that assist membrane deformation: N-BAR and BAR domain-containing proteins, such as SNX9 (Lundmark et al. 2003) and amphiphysin (David et al. 1996) aid in generating and stabilizing membrane curvature. They also bind both clathrin and AP2 & recruit dynamin to the neck of the budding vesicle (Lundmark et al. 2004, Yoshida et al. 2004). Another accessory protein, the EGFR-pathway substrate (EPS)15, localized predominantly to the growing edges of the coated pit, function as a scaffolding protein and clusters AP2 appendages through its long and flexible C-terminal tail (Schmid et al. 2006). Among the plethora of other accessory proteins are epsin1, AP180, CALM, NECAP, intersectin & stonin, as well as several kinases that may play important roles during the endocytic process (Zhang et al. 1998, Morgan et al. 1999, Legendre-Guillemin et al. 2004, Schmid et al. 2006). After the dynamin driven membrane scission, the clathrin is quickly released by the ATPase Hsp70 and the coat component auxilin (Lemmon 2001, Newmyer et al. 2001). The APs and accessory proteins are probably uncoated separately, possibly with the help of synaptojanin which weakens the attachment of coat components with the vesicle membrane

through its lipid phosphatase activity (Cremona et al. 1999, Verstreken et al. 2003).

2.2.2 Caveolae / raft-mediated endocytosis

Caveolae/raft-dependent endocytosis refers to dynamin-dependent raft pathways, mediated by caveolae or morphologically equivalent non-caveolin vesicular intermediates. These pathways are important for the internalization of albumin, sphingolipids, GPI-anchored proteins, the autocrine motility factor (AMF), endothelin, growth hormone and interleukin 2 (IL2) receptors. They also mediate the entry of some viruses (e.g. simian virus 40, SV40), bacteria and bacterial, sphingolipid binding toxins, such as cholera and shiga toxins (Minshall et al. 2000, Nichols et al. 2001, Duncan et al. 2002, Johannes et al. 2002, Pelkmans et al. 2002, Conner et al. 2003, Cheng et al. 2006). Further, it has been suggested that the clustering and activation of β1-integrins can trigger caveolar endocytosis with the subsequent removal of β1-integrins from the plasma membrane (Sharma et al. 2005).

Caveolae are morphologically identifiable lipid domains, seen as flask- or cup-like invaginations of the plasma membrane (Razani et al. 2002, Richter et al. 2008, Schlormann et al. 2010). Caveolae are enriched in sphingolipids, cholesterol, caveolin (Cav) proteins and the recently found cavins (Rothberg et al. 1992, Ortegren et al. 2004, Hill et al. 2008). There are three mammalian caveolin proteins: Cav1 and Cav2 are found in abundane in almost all cells except muscle cells, Cav3 is muscle specific and not found in other cell types. Only neurons and leukocytes seem to lack caveolae. Caveolins and cavins are essential for caveolae formation and cells devoid of caveolin are incapable of forming structures morphologically determined as caveolae (Razani et al. 2002, Hill et al. 2008). Cholesterol dependent lipid rafts are also shown to be needed for caveolae formation and it is possible that by binding to cholesterol (Murata et al. 1995) caveolin oligomerization induces the coalescence of lipid rafts into these membrane invaginations (Razani et al. 2002, Sharma et al. 2004), forming a subtype of lipid rafts characterized by proteinaceous Cav1/cavin coat.

Our knowledge on how caveolae form has substantially increased in recent years. The caveolar coat is composed of two major layers of protein: The inner layer in non-muscle cells is decorated with Cav1 and -2. Cav1 forms a hairpin like structure, embedded in the membrane, both its N and C termini are exposed to the cytoplasm (Dupree et al. 1993, Monier et al. 1995). Cavins in turn make up an outer peripheral layer facing the cytosol probably stabilizing the caveolin scaffold and thereby regulating membrane curvature and/or budding of caveolae (Hill et al. 2008, Liu et al. 2008a, Hansen et al. 2009). In addition, high-resolution EM analysis of caveolar ultrastructure has revealed thet precence of a ringlike density around the caveolar neck (Richter et al. 2008).

The function of caveolae is still somewhat obscure. They have been implicated in wide range of processes, including endocytosis, cholesterol and lipid metabolism and storage, mechanosensation and cellular signaling.

However, the physiological roles of caveolae seem to differ depending on the cell type and organ system examined (for reviews, see (van Deurs et al. 2003, Parton et al. 2007). Caveolae have been suggested to have an important role in cell signaling, because a variety cellular signaling molecules associate with them. These include receptor tyrosine kinases (RTKs) EGFR and platelet derived growth factor receptor (PDGFR), the non-receptor tyrosine kinases Src and FAK, receptor serine/threonine kinase TGFβ type I receptor, G-protein-coupled receptors, steroid hormone receptors and many more (Krajewska et al. 2004, Patel et al. 2008).

Caveolae-like invaginations can be found in cells that do not express caveolin. In lymphocytes, clustered GPI-anchored proteins accumulate in smooth invaginations morphologically equivalent to caveolae before endocytosis (Deckert et al. 1996). (Le et al. 2002) showed that inhibiting the budding of vesicles by expressing a dominant negative (DN) dynamin mutant, dynK44A in Abl-transformed NIH-3T3 cells that normally express little caveolin and few caveolae, results in smooth invaginations that are morphologically indistinguishable from caveolae induced in the same cells by the expression of Cav1. Treatment with methyl-b-cyclodextrin abolishes the dynK44A-induced smooth invaginations, indicating that they are derived from cholesterol-rich glycolipid raft domains. These caveolae like structures are very instable, short-lived and can only be visualized when budding is inhibited. It has been suggested that rather than being a determinant of caveolae invagination and internalization, Cav1 is actually a negative regulator that stabilizes caveolae at the plasma membrane, reducing the endocytic potential of caveolae/raft domains (Nabi et al. 2003). (Kirkham et al. 2005b) further elaborate the idea by suggesting that the underlying mechanisms for the lipid raft dependent, caveolar & caveolin-independent endocytic pathways are fundamentally similar. According to them the elementary pathway is lipid raft-dependent and is activated by cargo. Cav1 and dynamin just provide an additional level of regulation, most likely a negative one, as suggested also by Nabi & Le. This is supported by the fact that caveolae are normally stable structures (Thomsen et al. 2002, Tagawa et al. 2005) and because the expression of Cav1 reduce lipid raft-mediated endocytosis (Le et al. 2002, Kojic et al. 2007, Lajoie et al. 2007).

Flotillin proteins are structurally homologous to Cav1, which suggests that like caveolae, they play a role in the ordering of lipids. It has been proposed that flotillins are palmitoylated and form a similar hairpin structure at the membrane as Cav1 (Glebov et al. 2006). Although homologous to caveolin, flotillins are found oligomerized in distinct membrane domains (Frick et al. 2007). Flotillin-1 is marker for the dynamin-dependent but clathrin and Cav1-independent uptake of cell surface proteoglycans, which are then targeted to the specific late endosomes characterized by flotillin-1-positive compartments (Payne et al. 2007). It seems that flotillin-1 is responsible also for a portion of cholera toxin B (CTxB) uptake (Glebov et al. 2006) and is necessary for CD59 uptake (Frick et al. 2007).

2.2.3 Macropinocytosis

Macropinocytosis is a form of receptor-independent endocytosis and a major endocytic pathway in epithelial cells, fibroblasts, neutrophils & macrophages (Swanson et al. 1995, Nichols et al. 2001). According to the classical definition macropinocytosis is characterized by extensive plasma membrane ruffling and the subsequent formation of large vacuoles, commonly called as macropinosomes, which have a diameter of 0.5 – 10 μm and whose primary function is the non-selective internalization of fluids and membranes (Watts et al. 1992). While in some cells, such as macrophages and many tumor cells, macropinocytosis is a constitutive process, in other cell types it is initiated after external stimulation, such as by growth factors and phorbol esters (Swanson et al. 1995). In special cases, particles like bacteria, viruses, apoptotic bodies and necrotic cells can induce macropinocytosis and are internalized together with fluid into macropinosomes.

Macropinocytosis and the formation of a macropinosome are regulated by many factors and some of the molecules relevant for this thesis are discussed below. There are three types of plasma membrane protrusions involved in macropinocytosis: planar lamellipodia, circular ruffles and membrane blebs, the form of protrusion depending on the cell type and the nature of ligand. All of these are triggered by the small Rho GTPase Rac1 (Ridley et al. 1992, Mercer et al. 2008). Another important protein is the p21-activated kinase 1 (Pak1), a serine/threonine kinase, activated by Rac1 or Cdc42. Pak1 is a regulator of cytoskeletal dynamics and motility & is essential during all stages of macropinocytosis (Dharmawardhane et al. 2000, Liberali et al. 2008). Activated Pak1 relocates to the plasma membrane where it activates effectors needed for ruffling, blebbing and macropinosome formation (Galisteo et al. 1996, Dharmawardhane et al. 1999, Puto et al. 2003, Mercer et al. 2008). Pak1 also has a critical role in macropinosome closure through the activation of carboxy-terminal-binding protein-1/brefeldin A-ADP ribosylated substrate (CtBP-1/ BARS) (Liberali et al. 2008). PKC has shown to be crucial for macropinocytosis, although its exact function is yet to be resolved (Keller 1990). Macropinocytosis is very sensitive to amiloride inhibitors and their analogs, such as EIPA that block the function of Na^+/H^+ exchangers. This is at least partially explained by a recent finding that shows tht amiloride inhibitors prevent the induction of membrane ruffling by lowering submembranous pH and preventing Rac1 and Cdc42 signaling (Koivusalo et al. 2010). The submembraneous pH seems to be crucial for the activation of the GTPases that promote the actin remodeling important for macropinocytosis. Cholesterol is also important, possibly because it affects the localization Rac1 and other signaling molecules (Grimmer et al. 2002).

2.2.4 Clathrin, caveolin and dynamin independent pathways

In addition to the endocytosis events summarized above, there are many less well known internalization pathways. Cells depleted of both clathrin and

caveolin are still able to actively endocytose as mentioned in chapter 2.1.2. In addition to the clathrin and caveolin dependent pathways, there are dynamin independent but cholesterol dependent pathways, implying the existence of endocytic pathways that require specific lipid compositions. These pathways seem to be regulated by Arf6 or Cdc42. The major Cdc42 dependent pathway is known as CLIC/GEEC (clathrin independent carrier / GPI-anchored protein enriched early endosomal compartment) route. This dynamin-independent pathway seems to be the main non-clathrin, non-caveolar uptake mechanism for GPI-anchored proteins, CTxB, ricin and the Helicobacter pylori vacuolating toxin (Llorente et al. 1998, Fivaz et al. 2002, Sabharanjak et al. 2002, Gauthier et al. 2005). This Cdc42-regulated endocytosis has long and relatively wide surface invaginations & is thus associated with the internalization of large volumes of fluid, contrasting to the small spherical carriers that are characteristic of the clathrin and caveolar pathways (Sabharanjak et al. 2002, Kirkham et al. 2005a). The intracellular destination for material endocytosed through the CLIC/GEEC pathway differs between cell types, possible targets include lysosomal and pericentriolar recycling compartments (Fivaz et al. 2002, Sabharanjak et al. 2002).

The Arf family GTPase Arf6 mediated endocytosis seems to be involved in the internalization of several proteins, including GPI-linked protein, β1 integrin, carboxypeptidase E (CPE), CD59 and major histocompatibility complex (MHC) class I (Brown et al. 2001, Arnaoutova et al. 2003, Naslavsky et al. 2004). Arf6 is also well-known regulator of a recycling pathway to the plasma membrane (Donaldson et al. 2009). Arf6-associated endocytosis is dynamin-independent and appears to be distinct from the CLIC/GEEC endocytic route although the latter can communicate with both transferrin-positive compartments and the Arf6-dependent recycling pathway (Naslavsky et al. 2004).

2.2.5 Phagocytosis

Phagocytosis involves the engulfment and internalization of extracellular organisms such as bacteria & occurs mainly in specialized cells, such as macrophages, monocytes and neutrophils. It is also a form of eating, used by primitive single cell organisms like amoeba. Phagocytosis and macropinocytosis have some common regulatory components and factors and they also share other features, such as a large vacuole size, transient activation and dependence on actin (Swanson 2008). But despite these similarities, the underlying fundamental molecular mechanisms of phagocytosis and macropinocytosis are different. In phagocytosis a plasma membrane bound particle guides the actin-dependent formation of the phagosomes which is usually shaped by the cargo, forming a tight-fitting endocytic vacuole around the particle (Swanson 2008). In contrast, random ruffling seems to occur during macropinocytosis.

The best studied phagocytic process is Fc-receptor (FcR)-mediated phagocytosis, in which phagosomes form by a receptor-guided, zipper-like advance of the membrane and the cytoskeleton over particle surfaces. The

TABLE 1 Summary of endocytic pathways and their regulators. Modified from Mercer et al. (2010).

	Cellular factors				
Endocytic pathway	Coat proteins	Scission factors	Regulatory factors	Cytoskeleton	Trafficking
clathrin mediated	clathrin	dynamin-2	PI(3,4)P, PI(4,5)P2, cholesterol, cortactin, Arp2/3	actin, microtubules	Rab5, Rab7, Rab4, Rab11, Rab22
Cav1 mediated	Cav1, cavin	dynamin-2	tyrosine kinases, phosphatases, PKC, RhoA, cholesterol	actin, microtubules	Rab5
macropinocytosis	none	CtBP1 ?	tyrosine kinases, Pak1, PI3K, PKC, Ras, Rac1, Cdc42, Rab34, Na+/H+ exchange, cholesterol	actin, microtubules, myosins	Rab5, Rab7, Arf6
lipid raft	none	unknown	tyrosine kinases, Rho A, cholesterol	actin	unknown
GEEC pathway	unknown, GRAF1	unknown	Arf1, ARHGAP10, Cdc42, lipid rafts	actin	Rab5, PI3K
flotillin mediated	flotillin-1	unknown	Fyn kinase, lipid rafts	unknown	Rab5, PI3K
phagocytosis	none	dynamin-2	tyrosine kinases, PI(3)K, PKC, Ras, RhoA, RhoG, Rac1, Cdc42, Arf6, cholesterol	actin, microtubules, myosins	Rab5, Rab7

particles to be engulfed are opsonized with immunoglobulin G (IgG). The constant regions of antibodies interact with the FcRs, inducing the phagocyte to produce filopodial extensions around the particle in a Cdc42-dependent manner and subsequently the forming phagosome is internalized in Rac1-dependent manner (Massol et al. 1998, Chimini et al. 2000). Actin polymerization is essential in phagocytic membrane remodeling where Rac1 and Cdc42 recruit N-WASP and the actin nucleating and polymerizing Arp2/3 complex to the phagocytic membranes (May et al. 2000).

Phagocytosis of the complement component C3bi opsonized particles is morphologically distinct from FcR-mediated phagocytosis.The C3bi receptor (CR3) consists of the integrin chains αM and β2, which can bind and internalize C3bi-coated particles (Caron et al. 2000). The morphology of CR3-mediated phagosome formation varies depending on the cell type. In some cells, it resembles the zipper model (Dewitt et al. 2006) whereas in macrophages, the phagosome seems to sink into the cytoplasm, with actin organized as discrete patches in the phagocytic cup (Kaplan 1977, Allen et al. 1996). C3bi-opsonized particles have also been seen to be internalized in ruffles or loosely adherent phagocytic cups (Hall et al. 2006).

Once a particle is internalized, the resulting intracellular vacuoles, or phagosomes, begin to mature in a process greatly resembling the progression of the classical endocytic pathway. Through a series of fission and fusion events that modify the composition of their limiting membrane and of their contents, accompanied by the acidification of the structure, the phagosomes fuse with an acidic lysosomal structure (Desjardins et al. 1994, Beron et al. 1995, Tjelle et al. 2000, Swanson 2008). The most common endocytic pathways and their regulators are summarized in Table 1.

2.3 Intracellular trafficking of endocytosed material

The trafficking and fate of the endocytosed material after internalization was only briefly discussed above. As an essential part of my studies, intracellular trafficking of plasma membrane receptors deserves its own chapter. Here, the emphasis will be on molecules that have an important role in this thesis, integrins and EGFR. One of the best known and most studied ligands internalizing through clathrin-coated pits is EGFR. Deciphering EGFR endocytosis has been invaluable for the modeling of the morphology, kinetics and mechanisms of endocytic pathways in general – in fact the EGFR endocytosis model has become an archetype for the endocytosis of other RTKs. The post-endocytic compartments, often called “classical endosomes”, found after CME have reached a paradigm status extending to other endocytic pathways.

2.3.1 Endosomal network

The above-mentioned classical endosomes, the main organelles of the endocytic pathway, can be subclassified into early endosomes (EEs), maturing endosomes (MEs), late endosomes (LEs), recycling endosomes (REs) & lysosomes (Mercer et al. 2010). The MEs and LEs have a multivesicular appearance and are termed multivesicular bodies (MVBs). Internalized cargo reaches the EEs, located in cell periphery, in less than two minutes, the perinuclear MEs/LEs in 10-12 minutes and the lysosomes in 30-60 minutes (Mukherjee et al. 2004, Lakadamyali et al. 2006, Mercer et al. 2010). The different classes of endosomes are heterogeneous in composition & are distinguished by their morphology, level of acidification and distinct, more or less specific marker molecules, such as different types of Rabs and phosphatidyl-inositides (Fig. 5).

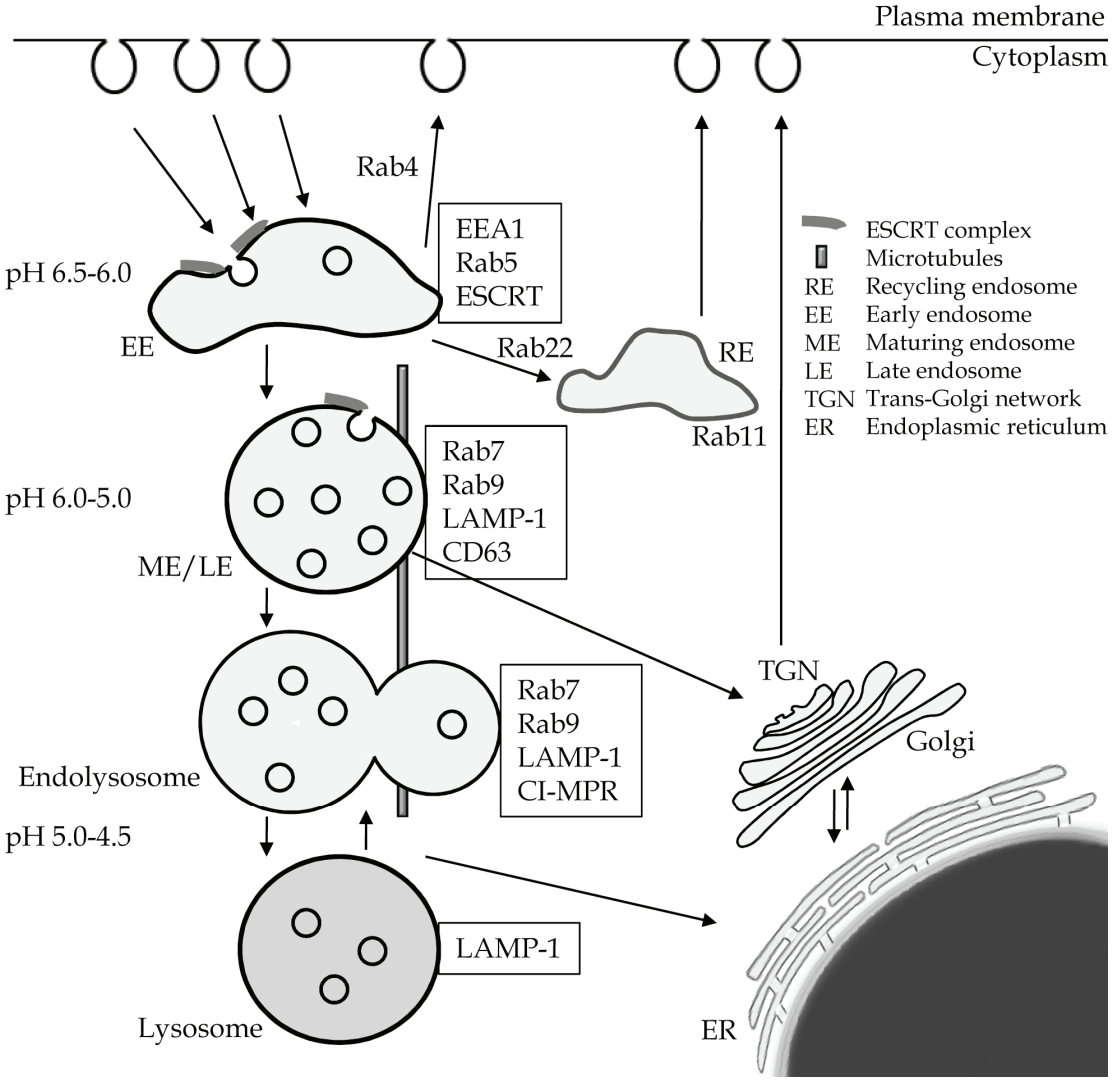

FIGURE 5 Main organelles of the endocytic pathway. The endocytic compartments can be distinguished by their morphology, specific marker molecules and level of acidification. ME and LE are usually referred to as multivesicular bodies due to their appearance. Modified from Mercer et al. (2010).

EEs are complex organelles characterized by several tubular and vacuolar domains, which are needed for Rab mediated molecular sorting and trafficking (Marsh et al. 1986, Zerial et al. 2001, van Meel et al. 2008). From EEs the cargo is selectively transported to the plasma membrane (Rab4), LEs (Rab7), REs (Rab22), or the *trans*-Golgi network (TGN) (Rab9) (Zerial et al. 2001, Bonifacino et al. 2006). Transport to perinuclear MEs and LEs is microtubule-mediated, dynein-dependent movement. MEs are intermediate organelles that contain both early and late endosomal markers, including Rab5 and Rab7 (Rink et al. 2005, Vonderheit et al. 2005). With further acidification they gradually mature into LEs, which then can fuse with each other and with lysosomes, eventually forming actively degradating organelles called endolysosomes (Nishi et al. 2002, Luzio et al. 2009). The end station of transport and degradation resides in a dense core lysosome, a home for lysosomal enzymes and membrane proteins awaiting fusion with incoming LEs (Luzio et al. 2007). A large portion of internalized membrane components is usually recycled back to the plasma membrane or integrated into a structural element of an endosome. However, transmembrane proteins destined for degradation are tagged with monoubiquitins and sequestered to intraluminal vesicles (ILVs) by the endosomal sorting complex required for transport (ESCRT) -machinery, as discussed later. The continuos formation of ILVs by inward vesiculation in EEs and LEs gradually forms MVBs that eventually fuse with lysosomes (Luzio et al. 2009)

2.3.2 Epidermal growth factor receptor endocytosis and degradation pathway

Even without activation, EGFR is constitutively internalized at a rate comparable to basal membrane recycling (Wiley et al. 1991, Sorkin et al. 2008). Inactive receptors are usually recycled back to the plasma membrane (Wiley 2003, Madshus et al. 2009). There are some differences in the half-lives of inactivated EGFRs depending on the cell type and the expression level of the receptor ($t_{1/2}$ 6-24 h) (Beguinot et al. 1984, Stoscheck et al. 1984, Sorkin et al. 2008). However, binding of EGF to EGFR results in the accelerated uptake of the receptor (Wiley et al. 1991). Most of the current experimental evidence supports internalization through clathrin coated pits but under special circumstances (high receptor and/or ligand concentration) secondary internalization pathways may be activated (Sigismund et al. 2005, Sigismund et al. 2008, Sorkin et al. 2008).

EGFR can sit in or outside of lipid rafts. Ligand binding induces dimerization and activation of the receptor, initiating signaling that eventually leads to internalization by endocytosis. Activated EGFR phosphorylates clathrin and causes the redistribution of clathrin at the cell periphery and clathrin dependent internalization of EGFR (Lamaze et al. 1995, Mineo et al. 1999). EGFR internalization is at least partially dependent on the clathrin-interacting adaptor, epsin & its partner proteins EPS15 and EPS15-related protein EPS15R (Chen et al. 1998, Confalonieri et al. 2000, Huang et al. 2004). Curiously, this dependency is evident also in the clathrin-independent uptake of EGFR,

possibly through the action of ubiquitin (Sigismund et al. 2005). A crucial mediator of EGFR internalization is the RING-finger E3 ubiquitin ligase Cbl, that ubiquitinates the receptor at multiple sites (Haglund et al. 2003). It is still under debate to what extent internalization is dependent on ubiquitination and phosphorylation (for review, see (Madshus et al. 2009). After internalization EGF-receptor complexes can be rapidly recycled from EEs or sorted into MVBs and LEs. Ubiquitin has an essential role in MVB sorting: ubiquitinated EGFR is recognized by the ubiquitin binding domains of ESCRT-0 and EPS15b, other ESCRT proteins then incorporate the receptor into internal vesicles of MVB (Roxrud et al. 2008, Raiborg et al. 2009). Sequestering EGFR to internal vesicles of MVBs effectively prevents further signaling and EGFR is eventually degraded when MVBs fuse with lysosomes.

The purpose of receptor endocytosis is not only to silence signaling; endosomes are also important sites for receptor-initiated signal transduction. There is evidence that signaling from endosomes is qualitatively distinct from plasma membrane derived signaling (Vieira et al. 1996, Wu et al. 2001, Lampugnani et al. 2006).

The factors that regulate the initial internalization of EGFR are still under extensive investigation. Most of the current data suggest that ligand-induced receptor dimerization, with the subsequent autophosphorylation of tyrosine residues in the EGFR tail, is required for EGFR endocytosis (Huang et al. 2005, Sorkin et al. 2008), although some studies state that dimerization alone is sufficient for internalization (Wang et al. 2005). The role of EGFR ubiquitination in endocytosis is still somewhat obscure. Based on findings with EGFR mutants that are ubiquitinated inefficiently, it seems that EGFR ubiquitination is not necessary for its internalization (Huang et al. 2007). However, endocytosis of an EGFR mutant with impaired kinase activity was rescued when two main ubiquitination sites were reconstituted (Huang et al. 2007). This in turn indicates that ubiquitination enhances EGFR internalization, at least under conditions of impaired EGFR kinase activity.

2.3.3 Ubiquitin as a sorting signal

Ubiquitin is a small 8.5 kDa protein expressed ubiquitously in eukaryotic cells and serves as an important signal for protein degradation. Other ubiquitin connected processes, like DNA repair and ribosomal protein synthesis will not be discussed here.

There are two parallel ubiquitination based sorting systems in a cell: Tagging proteins with chains of ubiquitin (polyubiquitination) targets proteins to proteosomal degradation (Pickart 2000). Single moieties of ubiquitin (monoubiquitination), in turn, direct protein to lysosomal degradation via the MVB pathway (Katzmann et al. 2002). There is also growing evidence that ubiquitin may have a role in selective autophagy, targeting protein aggregates, mitochondria, peroxisomes and invading bacteria to lysosomes (Komatsu & Ichimura 2010). In any case, ubiquitination involves three steps, resulting in a covalent linkage of a C-terminal glycine of ubiquitin to lysine residues in the

target protein. First, ubiquitin is activated by an E1 ubiquitin activating enzyme, next ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme & finally an E3 ubiquitin ligase forms an isopeptide bond between the target protein and ubiquitin (Glickman et al. 2002). Polyubiquitination can be achieved by ubiquitinating any of the seven lysines found in ubiquitin itself (Peng et al. 2003). Ubiquitination is a hierarchical cascade in similar to many kinase pathways – one E1 can bind dozens of E2s, which can bind hundreds of E3 ligases, thus amplifying the response several fold (Glickman et al. 2002). Ubiquitin itself does not particape in the degradation process, instead it merely tags proteins for degradation and is recycled through the function of deubiquitinating enzymes (DUBs) (Nijman et al. 2005). Ubiquitin is recognized by various proteins involved in degradation. In proteasome 26S there are specific ubiquitin receptors that bind proteins destined for proteosomal degradation (Hartmann-Petersen et al. 2004). As the main function of the proteasome is to degrade unnecessary or damaged intracellular proteins, this text will focus on pathway leading to lysosomal degradation of plasma membrane proteins. This is mediated by the ESCRT machinery that recognizes and captures ubiquitinated cargo.

2.3.4 Endosomal sorting complex required for transport (ESCRT)

The endosomal sorting complex required for transport machinery is conserved from Archaea to complex multicellular organisms and is responsible for a variety of cellular events related to membrane deformations: it catalyzes the scission of membrane necks (Wollert et al. 2009) in the biogenesis of MVBs (Piper et al. 2007), cytokinesis (Carlton et al. 2007, Morita et al. 2007, Samson et al. 2008) and the budding of enveloped viruses such as HIV-1 (Morita et al. 2004, Fujii et al. 2007). ESCRT-mediated scission is topologically the opposite to that mediated by the dynamin family of membrane-scission proteins. Cleavage by ESCRT occurs from the surface of the membrane that is contiguous with the inside of the membrane neck, whereas dynamin cleaves membrane necks by constricting them from the outside. Here I will focus on the function of ESCRTs in the formation of multivesicular bodies.

The ESCRT machinery consists of the four protein complexes ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III & several accessory complexes, the most important one being Vps4-Vta1 (Saksena et al. 2007, Hurley 2008, Raiborg et al. 2009) (Fig. 6, Table 2). ESCRT-0, ESCRT-I, ESCRT-II and Vps4-Vta1 are soluble complexes that cyle between cytosolic and membrane-bound states. ESCRT-III proteins, on the other hand, are soluble monomers that assemble on membranes into tightly bound filaments (Ghazi-Tabatabai et al. 2008), tubes (Hanson et al. 2008, Lata et al. 2008) and spirals (Hanson et al. 2008) that cannot detach from membranes without the ATP-dependent action of Vps4-Vta1. The roles of ESCRTs in MVB biogenesis can be separated into three tasks: recognition of ubiquitinated cargoes and prevention of their recycling or retrograde trafficking; deformation of the endosomal membrane and sorting of the cargo into endosomal invaginations; and finally, generation of intralumenal vesicles

by pinching off the endosomal invaginations containing the cargo (Raiborg et al. 2009). The cargo varies from misfolded proteins at the plasma membrane to activated growth factor, hormone and cytokine receptors (Marchese et al. 2001, Rocca et al. 2001, Shenoy et al. 2001).

The ESCRT-based sorting of ubiquitinated cargo can be summarized as follows: ESCRT-0 is most likely responsible for the initial capture of the cargo through its multiple ubiquitin interacting motifs (UIMs). The ESCRT-0 protein Hrs then bind to endosomal membrane lipid phosphatidylinositol 3-phosphate (PIP3) through its FYVE domain and concentrates cargo at the endosomal membrane; ubiquitinated cargo is then delivered to ESCRT-I and ESCRT-II, both of which have only one ubiquitin-binding domain (Raiborg et al. 2009). The final step of the sorting process is the deubiquitination of the cargo by DUBs and their packaging into intraluminal vesicles that are formed by the action of ESCRT-III. Subsequent to membrane scission, Vps4 hydrolyzes ATP to drive the recycling of ESCRT-III subunits back into their soluble monomeric form, enabling additional rounds of budding (Raiborg et al. 2009, Wollert et al. 2009). The resulting endosomal structure, characterized by internal vesicles, is termed the multivesicular body, which is discussed next.

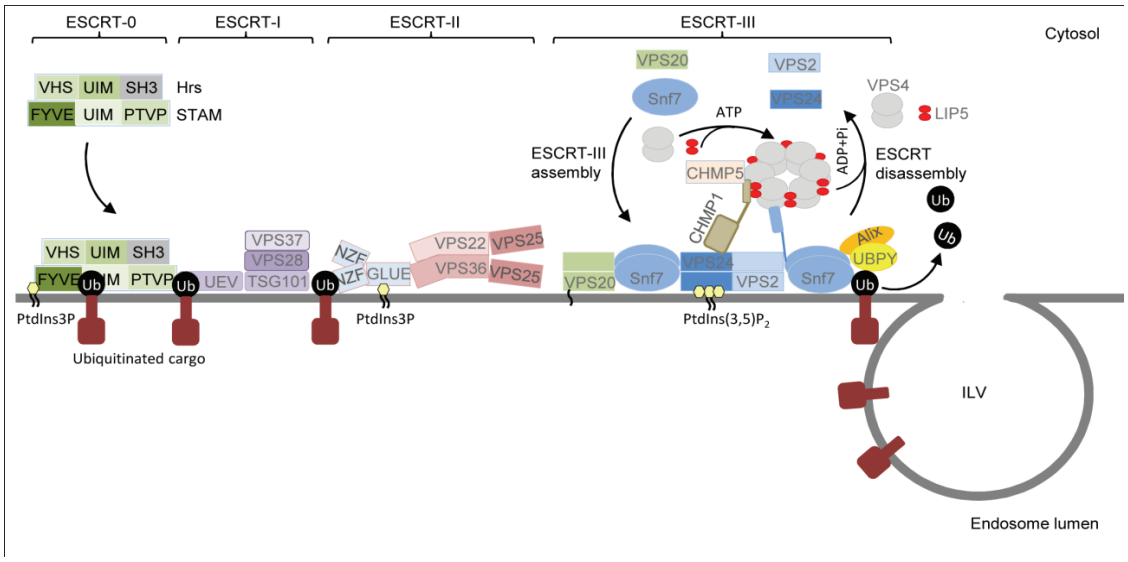

FIGURE 6 ESCRT machinery. Each ESCRT complex is composed of several interacting proteins. ESCRT-0 recruits ubiquitinated cargo to the endosomal limiting membrane. Cargo is delivered to ESCRT-III and subsequently deubiquitinated and sequestered to a budding intraluminal vesicle. ATP, adenosine triphosphate; ESCRT, endosomal sorting complex required for transport; ILV, intraluminal vesicle; PtdInsP, phosphatidyl inositol phosphate; Ub, ubiquitin; UIM, ubiquitin interacting motif; FYVE, 'Fab1, YOTB, Vac1, EEA1'; Hse, has symptoms of class E mutants; SH3, Src-homology 3; NZF, Npl4 zinc finger; PTVP, proline threonine valine proline; Snf, sucrose non-fermenting; STAM, signal transducing adaptor molecule; UEV, Ub E2 variant; VHS, 'Vps27, Hrs, STAM'; Vps, vacuolar protein sorting; CHMP, charged multivesicular body proteins. Modified from Hurley et al. (2006), Nickerson et al. (2007), Williams et al. (2007).

TABLE 2 Class E vacuolar protein sorting (Vps) and other selected proteins required for multivesicular body cargo sorting. Modified from Nickerson et al. (2007) and Hurley (2008).

Complex	Yeast	Mammal (synonym)	Binds
ESCRT-0			
	Vps27	Hrs	Ub, PI(3)P, Vps23
	Hse1	STAM1, 2	Ub, Rsp5
ESCRT-I			
	Vps23	TSG101	Ub, Vps27
	Vps28	VPS28	Vps20, Vps36
	Vps37	VPS37A, B, C, D	–
	Mvb12	–	–
ESCRT-II			
	Vps36	EAP45 (VPS36)	Ub, PI(3)P, Vps28
	Vps22	EAP30 (VPS22)	–
	Vps25	EAP20 (VPS25)	Vps20
ESCRT-III			
	Snf7 (Vps32)	CHMP4A, B, C (SNF7)	Bro1, Vps4, PI(3)P
	Vps20	CHMP6 (VPS20)	Vps28, Vps25, Vps4
	Vps2	CHMP2A, B (VPS2)	–
	Vps24	CHMP3 (VPS24)	Did2, PI(3,5)P_2
Other			
	Bro1 (Vps31)	Alix	Doa4, Snf7
	Rsp5	Nedd4	Hse1
	Doa4	AMSH, UBPY	Ub, Bro1
	Did2	CHMP1A, B	Vps4, Vps24, Vta1
	Vps60	CHMP5	Vta1
	Vta1	LIP5	Vps4, Did2, Vps60
	Vps4	VPS4A, B (SKD1)	Did2, Snf7, Vps20, Vta1

2.3.5 Multivesicular bodies

A distinct organelle that consists of limiting membrane enclosing internal vesicles of 40-90 nm was found by early electron microscopists already in the 1950s (Palade 1955, Sotelo et al. 1959, Katzmann et al. 2002). Nowadays these structures are called multivesicular bodies, or shortly MVBs, representing endocytic intermediates that are formed from early endosomes and contain molecules that have been internalized by endocytosis (Gruenberg et al. 2004).

MVBs also have a linkage to the *trans*-Golgi network wherefrom they receive biosynthetic cargo such as precursors of lysosomal enzymes (Klionsky et al. 1989, Raiborg et al. 2002). In most cell types, early endosomes mature into or fuse with multivesicular late endosomes. During maturation, portions of the limiting membrane of the endosome invaginate and then bud into the lumen of the endosome, forming ILVs. The MVBs eventually fuse with the lysosomes and the ILVs and their contents are degraded (Luzio et al. 2007, Luzio et al. 2009). As mentioned above, ESCRTs are involved both in the sorting of ubiquitinated cargo into ILVs and in the morphogenesis of the ILVs themselves.

Why is it then necessary to sort transmembrane proteins into topologically distinct limiting and intraluminal membranes? There are actually at least three important factors as summarized by (Staub et al. 2006): if the transmembrane proteins were sorted merely to the limiting membrane, only the small, extensively glycosylatedand thus protease-resistant luminal region of proteins would be exposed to lysosomal degradation. Cargo sorting to intraluminal membranes will make proteins more susceptible to proteases. Another point is that in some cells ILVs serve as storage sites for the transmembrane proteins. This specialized vesicle, the exosome, is able to fuse with the plasma membrane and its ILVs can then be released from the cell by a regulated exocytosis. Finally, in theory, receptor signaling is still possible from the limiting membrane of MVBs, but incorporating the receptor to ILVs sequesters it from the cytoplasm, leading to silencing of the signaling. In summary, sorting into MVBs directs both the delivery of transmembrane proteins to lysosomes and the extracellular space & determines the signaling potential of endocytosed receptors.

Although MVB pathways are generally thought to finally lead to the degradation of receptors in lysosomes, not all MVBs fuse or exchange material with lysosomes: for example class II major histocompatibility complex (MHC) and tetraspanins accumulate in MVBs and form a specialized class II MHC compartment (MIIC) (van Niel et al. 2006). There are also further specialized, non-degradative MVBs, that are associated with exosomal pathways, such as the alpha granules of platelets and the Weibel-Palade bodies of endothelial cells packaged with von Willebrands Factor (Heijnen et al. 1999). Also the azurophilic granules of neutrophils have CD63 enriched ILVs (Cham et al. 1994, Cieutat et al. 1998). Melanosomes are yet another example of a non-degradative MVB (Theos et al. 2006).

2.3.6 Integrin trafficking

2.3.6.1 Function and regulation of integrin trafficking

The endocytic and exocytic cycle of integrins, or integrin trafficking, regulates important cellular processes, such as cell adhesion, migration and the maintenance of cell polarity, wherein the correct targeting of integrins and the dynamic remodelling of integrin-containing adhesion sites is crucial (Pellinen et al. 2006b, Caswell et al. 2009). Integrin trafficking has also a role in cell signaling:

Trafficking induces alterations in the signaling of integrins that in turn affect Rho GTPases (White et al. 2007). Integrins are also able to control the trafficking of receptor tyrosine kinases, such as vascular endothelial growth factor receptor 2 (VEGFR2) and EGFR (Caswell et al. 2008, Reynolds et al. 2009). In a simplistic model for integrin trafficking, adhesive contacts are disassembled and integrins are endocytosed from the retracting end of the cell, transported to and subsequently exocytosed at the leading edge of the cell where contacts are reassembled, providing fresh adhesion receptors and thus enabling cell movement. This model is supported by studies with a radioactively labeled fibronectin receptor α5β1 that was found to be recycled rather than degraded (Bretscher 1989, Bretscher 1992). Moreover, αVβ5 integrin has been found to colocalize with CCVs & dynamin localizes to focal contacts and is necessary for integrin disassembly (De Deyne et al. 1998, Ezratty et al. 2005), further supporting the notion that integrins are endocytosed and recycled.

Recent research has revealed more players in the integrin trafficking pathway and more a detailed model has begun to take shape. Based on current data (Pellinen et al. 2006b) proposed a model for integrin trafficking, where an integrin-ECM interaction leads to the clustering of integrins and the formation of focal contacts (FC), connecting integrins to the actin cytoskeleton through the FAK-Src complex and its substrates (Mitra et al. 2005). The integrin is then internalized, possibly in concert with FC disassembly. The connection with the actin cytoskeleton is lost and microtubules are targeted to internalization sites where they activate dynamin and therby endocytosis (Burridge 2005, Ezratty et al. 2005). The internalization of the prototype molecule used in their model, β1 integrin & the ECM proteins is regulated by PKCα through direct binding to the cytoplasmic tail of β1 integrin, (Ng et al. 1999), Rab5/Rab21, microtubules (Pellinen et al. 2006a) and Arf6. Internalized β1 integrin goes through EEs and then associates with Rab21-GTP and is recycled back to the plasma membrane through perinuclear recycling endosomes. This process is regulated by Rab11, Rab21 and Arf6 GTPase activities (Powelka et al. 2004, Roberts et al. 2004). Additionally, in response to growth-factor stimulation β3 integrins are diverted to a short-loop recycling pathway involving Rab4 and polycystin-1 (PKD1) (Woods et al. 2004). In fibroblasts, the exit of β1 integrins from recycling endosomes is also regulated by the PKCε mediated phosphorylation of vimentin (Ivaska et al. 2005). It has also been shown that the return of integrins to the plasma membrane is dependent on the activity of the PI-3-K-AKT-GSK3 pathway (Roberts et al. 2004).

Integrins can take either a clathrin dependent or independent internalization pathway. A given integrin heterodimer can follow more than one internalization route. For example α5β1 integrin can be internalized by both clathrin-dependent (Pellinen et al. 2008) and caveolar (Shi et al. 2008) endocytosis. On the other hand, αvβ3 integrin is recruited to caveolae and internalized in cholesterol and Cav1 -dependent manner (Galvez et al. 2004) and can also enter the cell in clathrin-coated structures (Nishimura et al. 2007). The trafficking of integrins is summarized in Fig. 7.

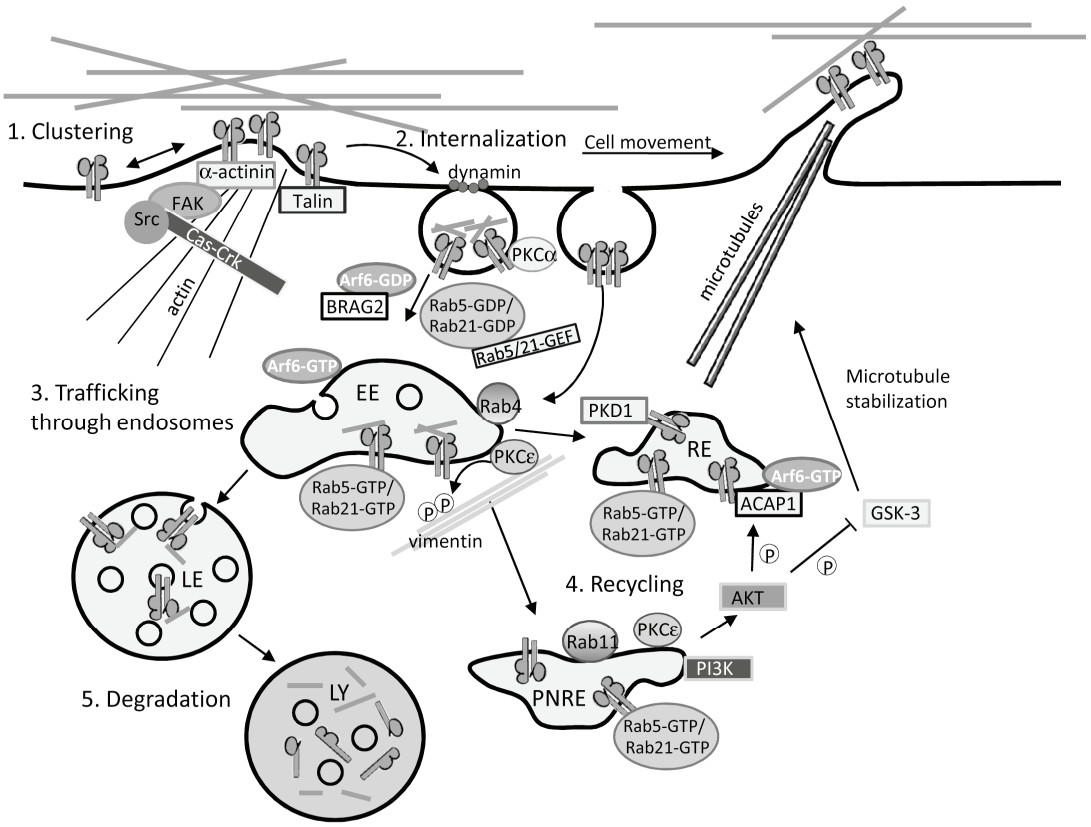

FIGURE 7 Integrin trafficking. The activation of integrins by extracellular ligands leads to the clustering (1.) and subsequent internalization of the integrin via dynamin dependent (i.e. caveolae or clathrin mediated endocytosis) or independent (raft-derived pathways, macropinocytosis) mechanism (2.). Internalized integrin trafficks through endosomes (3.) and is recycled back to the plasma membrane (4.) or degraded in lysosomes (5.). Modified from Pellinen & Ivaska (2006).

2.3.7 Integrin turnover and degradation

Protein turnover in general refers to the rate at which a given protein is degraded and resynthesized in cells. Proteins are continuously degraded and replaced even under in normal conditions and the term "half-life" is used to describe the time it takes for half of the amount of a specific protein in a cell to disappear after its synthesis. The half-lives of different proteins range from a few minutes or hours for some enzymes to the 223 days of histone H2B (Commerford et al. 1982) or the even longer half-life of collagen (Rucklidge et al. 1992, Thorpe et al.). The decay rate depends for example on the function of the protein, the phase of the cell cycle or the response to external stimuli.

Integrins are highly dynamic transmembrane proteins that are constantly endocytosed in migrating cells (Bretscher 1989, Bretscher 1992). As discussed above, the purpose of this internalization is to recycle rather than degrade integrins (Bretscher 1989, Bretscher 1992). Although most of the internalized integrin is recycled back to the plasma membrane, in some situations, however, it is beneficial for the cell to degrade rather than recycle the integrins. Normal turnover rates for integrins seem to vary according to the cell type and there are

studies showing half-lives from 8 h to 45 h in cultured cells (De Strooper et al. 1991, Dalton et al. 1992, Hotchin et al. 1992, Witkowski et al. 2000, Zargham et al. 2005). The impact of the cell type on turnover is clearly seen in studies with α6β4 integrin. In normal keratinocytes, the half-life of α6 is 42 h and of β4 45 h, whereas in carcinoma cells the biological half-lives of the α6 and β4 subunits are estimated to be 14 and 18 h, respectively (Witkowski et al. 2000).

Events such as loss of adhesion can lead to integrin downregulation by degradation. For example, β1 and β3 family integrins are lost from the cell surface in a few hours and degraded lysosomally after detachment in normal rat kidney (NRK) cells (Dalton et al. 1995). Similar results have been obtained with keratinocytes, also showing that unoccupied integrins are internalized and degraded in lysosomes (Hotchin et al. 1995). Recently, (Lobert et al. 2010) showed that α5β1 integrins can be degraded in an ESCRT and MVB dependent manner during the migration of fibroblasts. Ubiquitination of α5 targets it for degradation instead of recycling and this is essential for proper migration. Also external stimulation by angiopoietin may lead to the degradation of αVβ3 integrin in lysosomes in an ubiquitin-dependent manner (Thomas et al. 2010).

3 AIM OF THE STUDY

A detailed understanding of virus-host cell interactions is important in the battle against infectious viruses. Viruses are also tools for molecular medicine and potential devices for the delivery of combounds, for example drugs, into cells. In addition, viruses serve as important tools and model systems in the study of cell function. To date, the early infection pathway of EV1 has remained unresolved. The aims of this thesis were:

1. To characterize the regulators of the early internalization pathway of EV1 in more detail

2. To define the role and characteristics of the virus-induced MVBs in EV1 infection

3. To further study the EV1 induced pathway and to decipher the fate of theEV1 receptor α2β1 integrin after internalization.

4 SUMMARY OF MATERIALS AND METHODS

The materials and methods used in this thesis are summarized in the table below. Detailed descriptions of all other methods can be found in the publications indicated by Roman numerals. The ubiquitination studies are not included in the publications.

TABLE 3

Material / Method	
Virus production and purification	I
EV1 infection and α2β1 clustering	I, II, III
Transfections	I, II, III
Small interfering RNA experiments	I, II, III
Drug treatments	I, II, III
Immunofluorescence labeling	I, II, III
Internalization assay	I
Electron microscopy	I, II
Confocal microscopy	I, II, III
Dextran uptake assays	I
Transferrin uptake assays	I
Live cell imaging	II, III
SDS-PAGE and immunoblotting	I, II, III
Data analysis of light microscopy images	I, II, III
EGFR assays	II
Integrin recycling assays	III
Integrin degradation assays	III

4.1 Ubiquitination studies

4.1.1 Immunofluorescence and EM studies

α2β1 integrin was clustered as before (Upla et al. 2004). Briefly, antibody against α2 integrin was added to Dulbecco's modified eagle medium (DMEM) supplemented with 1 % fetal bovine serum (FBS), SAOS-α2β1 cells were incubated together with antibodies for 1 h on ice & washed to remove unbound antibodies. The cells were subsequently incubated with Alexa-conjugated goat anti-mouse IgG antibody on ice and washed. Clustering was induced by moving cells to 37°C. EV1 infection assays were performed as in (I). To test the effect of inhibition of ubiquitination to internalization of α2β1 integrin and EV1 infection, 50 μM PYR-41 (EMD Chemicals) was added for 15-30 min to SAOS-α2β1 cells before induction of clustering or infection. To detect the colocalization of α2β1 integrin and ubiquitin, cells were transfected with myc-ubiquitin and analyzed 24 h post-transfetion and integrin was clustered as above for different time periods. After fixation with 4 % paraformaldehyde (PFA), cells were permeabilized with 0.2 % Triton X-100 and labeled with rabbit anti-sera against myc (kindly provided by Dr. Harald Stenmark) and an Alexa-conjugated secondary antibody (Invitrogen). For cryo-EM, integrins were clustered as above; PYR-41 was added 30 min after induction of clustering. EM samples were prepared as in (II).

4.1.2 Construction of the lysineless α2-tail mutant

The α2 integrin expression construct used for mutations is described in (Ivaska et al. 1999). Four lysines of the α2 cytoplasmic tail were replaced with arginines by site-directed mutagenesis. For this purpose the QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's protocol. The mutant oligos used were: sense CTTCAGAAGAAGATATGAAAGGATGACCAGAAATCCAGATGAGATTG, antisense
AATCTCATCTGGATTTCTGGTCATCCTTTCATATCTTCTTCTGAAG.
Successful mutagenesis was verified by sequencing.

4.1.3 Isolation of the integrin signaling complex with Dynabeads

Isolation of the integrin signaling complex was done using a modified protocol described previously (Humphries et al. 2009). EV1 or antibody was coupled to tosyl-activated paramagnetic Dynabeads M-450 (Invitrogen) according to the manufacturer's protocol. Coated beads were incubated with SAOS-2 cells, stably transfected with α2 integrin, in PBS containing 2 mM $MgCl_2$ for 5, 15 and 30 min at 37°C. DTBP crosslinker (Thermo Fisher Scientific) in PBS containing 2 mM $MgCl_2$ was added to the bead-bound cells to a final concentration of 2 mM

& samples were incubated for a further 5 min at 37°C. The crosslinker was quenched with 20 mM tris-HCl pH 7.5 before cell lysis in lysis buffer (100 mM N-octyl-β-D-glucopyranoside (Sigma), 150 mM NaCl, 25 mM tris-Cl pH 7.5, 2 mM $MgCl_2$ supplemented with 10 mM N-ethylmaleimide (NEM) to inhibit ubiquitin-conjugating enzymes & phosphatase and protease inhibitors (Roche PhosSTOP and Complete, mini, EDTA-free protease inhibitor cocktail tablets respectively)) for 30 min on ice with sonication (MSE Sonicator). After cell lysis, beads were washed with lysis buffer (without NEM and phosphatase and protease inhibitors) and PBS containing 2 mM $MgCl_2$ and 1 % Triton X-100. Proteins were eluted from the beads with SDS-sample buffer (Fermentas) containing 200 mM DTT and a magnet was used to separate the beads from the supernatant. Protein samples were separated by SDS-PAGE & analyzed by immunoblotting.

4.1.4 Immunoprecipitation and SDS-PAGE

To detect the ubiquitination status of proteins, cells were first washed twice in ice-cold phosphate-buffered saline and then scraped from 2 ml dishes into NEM lysis buffer (10 mM Na_2HPO_4, 1 mM EDTA, 100 mM NaCl, , 10 mM NEM, 100 mM octyl-β-D-glucopyranoside (Sigma-Aldrich)) supplemented with HALT phosphatase and protease inhibitor coctails (Thermo Scientific). Cells were solubilized on ice with occasional vortexing for 30 min. Lysates were cleared by centrifugation and incubated with rabbit antibodies against α2 integrin (ab1936, Millipore) integrin (Chemicon) and Protein A –sepharose 1 h at 4 °C to immuneprecipitate the α2.

The samples were separated in a 10% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Millipore). Ubiquitination was visualized by mouse or rabbit antisera against ubiquitin (Cell Signaling) followed by horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescent Super Signal substrate (Thermo Scientific).

5 REVIEW OF THE RESULTS

5.1 EV1 and α2β1 integrin enter cells by macropinocytosis

5.1.1 Characterization of the early uptake of EV1

Previous work had shown that EV1 positive endosomes accumulate Cav1 during the first 2 h after infection (Marjomäki et al. 2002, Upla et al. 2004), but EV1 does not colocalize with Cav1 at the plasma membrane, as was shown using live confocal microscopy (Pietiäinen et al. 2004). This indicates that the initial entry of EV1 is not caveolae-mediated. To study the early steps of internalization in more detail several approaches were taken: First, to verify that the entry of EV1 is truly independent of Cav1, a quantitative colocalization analysis was performed. In SAOS-α2β1 cells, colocalization of the EV1 receptor α2β1 integrin and endogenous Cav1 was initially low (< 10 %), increased over time up to 2 h (> 30 %), at which point the integrin was already in large cytoplasmic vesicles (I, Fig. 1A). The colocalization of EV1 and caveolin-GFP seemed to a follow a similar trend (I, Fig. 1B). The results were confirmed in another cell line, namely Hela MZ cells, which transiently expressed caveolin-GFP (I, 1B). It was also tested whether the DN mutants of Cav3, KSY and DGV, can prevent the internalization of EV1 and α2β1 integrin, but all of the observed cells showed internalized integrin and EV1 (I, Figs. 3E,F). These results thus confirm that EV1 entry is independent of caveolar uptake.

Further, to study whether EV1 internatization uses a clathrin-dependent pathway, we studied the effect of dominant negative dynamin-2 on EV1 infection. Cells were transfected with the mutant construct or alternatively a mock control plasmid for 48 h and infected with EV1 for 6 h. The effect of the dynamin mutant on α2β1 integrin clustering was also studied by clustering the integrin with antibodies for 2 h. The results showed that EV1 entry was independent of dynamin-2 in SAOS-α2β1 cells (I, Figs. 3A-C). When internalization was examined by EM, the samples with clustered integrin alone showed that 5–15 min after the clustering at 37°C, a large pool of α2β1 integrins

had entered the cell. Integrin α2β1 was often found in smooth-surfaced vesicles and tubules that were clearly larger than caveolae and lacked the typical clathrin cage coating (I, Fig. 4B). The mean size of these vesicles 5 min after internalization was 138 nm (SE 12 nm), whereas typical caveolae are 50-100 nm (Travis 1993). Integrin clustering was also able to retarget a 10 kDa dextran from the default lysosomal pathway to Cav1-positive endosomal structures (I, Figs. 2B-D). These observations thus indicated that a pathway other than the clathrin or caveolae one is involved in EV1 internalization.

5.1.2 EV1 entry is dependent on regulators of macropinocytosis

Earlier studies indicated that α2β1 integrin is located in raft-like membrane domains rather than in caveolae (Upla et al. 2004). Also the tubulo-vesicularity and rather large size of the internalized α2β1 integrin positive vesicles hinted toward a pathway originating from lipid rafts. Among these are macropinocytosis and pathways used by GPI-AP (the GEEC pathway) and CTxB. The latter two were ruled out by colocalization studies with clustered integrin (I, Fig. 5A). GPI-GFP and CTxB were internalized in the presence of clustered α2β1 integrin for 0 to 120 min. The colocalization of α2β1 with these markers was quantified from 30 cells in three separate experiments and the results showed negligible colocalization. Then the effect of different macropinocytosis inhibitors on infection was tested and the results showed that especially the amiloride analog EIPA, a Na^{2+}/H^{+} exchanger inhibitor that has been shown to prevent macropinocytosis & U73122 (an inhibitor of phospholipase C dependent processes, such as activation of Protein kinase C) were very potent inhibitors, blocking the infection completely when administered to cells from 30 min before to 1 h after infection. The phosphatidyl inositol 3-kinase (PI3K) inhibitor LY294002 was also an effective blocker of macropinocytosis, although it was not able to completely prevent infection (I, Fig. 5A). In EIPA and U73122 treated cells the integrin and EV1 seemed to remain at the plasma membrane or very close to it, indicating that the inhibition of infection was due to incomplete internalization (I, Figs. 5C, D). This was verified by EM studies: U73122 blocked internalization completely, while EIPA allowed internalization, but internalized structures remained close to the plasma membrane (I, Fig. 6A). In control cells internalized integrin was found in the perinuclear region 2 h after internalization (I, Fig. 6A). Ruthenium red staining in the EM verified that the tubulovesicular structures after EIPA treatment were truly intracellular and not connected to the plasma membrane (I, Fig. S4D).

Next, some known biological regulators of macropinocytosis were examined. Paks are a group of serine / threonine kinases known to associate with macropinocytosis (Dharmawardhane et al. 2000). Cells were transfected with dominant negative (Pak1 AID), highly kinase active (Pak1 T432E) or wild type (WT) Pak1 constructs, whose effect on infection was tested. Pak1 AID almost totally blocked the infection, whereas Pak1 T432E slightly increased

infection efficiency compared to WT or mock transfection (I, Figs. 7B, C). As a control also the effects of these different Pak1 constructs on transferrin and dextran uptake were analyzed. As expected, transferrin was internalized normally, whereas Pak1 AID blocked dextran entry efficiently, indicating a role for Paks in the uptake of large volumes (I, Fig. 7A). The role of Pak1 was further confirmed by following the activation of Pak1 during infection. The activation was seen as the emergence of phospho-Pak1. The fluorescent images showed that in SAOS-α2β1 cells, starved overnight, the level of phospho-Pak1 was low before infection. However, already 5 min after introducing of EV1 to cells, the levels were significantly higher & quantification revealed that phospho-Pak1 was even more active 15-30 min p.i. (I, Figs. 8D, S5A).

The involvement of the RhoGTPases Cdc42 and Rac1, upstream regulators of Pak1, in EV1 infection was also investigated. In addition, RhoA thich does not activate Pak1 was tested (Manser et al. 1994). Short interfering (si)RNA experiments showed significant inhibition of infection when Rac1 was knocked down (I, Fig. 8A). Similar results were obtained using dominant negative constructs of Cdc42 and Rac1, DN Rac1 expression causing significant inhibition of EV1 infection in SAOS-α2β1 cells (I, Fig. 8B). The results provide evidence that Pak1 regulates EV1 entry and infection and is itself activated very early. Furthermore, the results suggest that the Rho GTPase Rac1 may be the upstream regulator of Pak1 in this process.

5.2 Clustering of α2β1 integrin by EV1 induces formation of a novel neutral multivesicular body, α2-MVB

5.2.1 EV1 enters non-acidic endosomes negative for markers of classical endosomes

The plasma membrane proteins that are internalized through canonical pathways are usually targeted to the aforementioned classical endosomes. Those include early endosomes (EE), late endosomes (LE) and lysosomes. The pH of endosomes decreases along the pathway from the mildly acidic conditions of EEs (pH 6.5-6.0), to the more acidic LEs (pH 6.0-5.0) and lysosomes (pH 5.0-4.5). The pH of α2 positive structures was measured by first binding primary antibody against α2 & then treating cells with two different secondary antibodies, the first one conjugated with the acid sensitive dye FITC & the second with the acid insensitive Alexa 555. The binding of the secondary antibody initiates the clustering and internalization of α2β1 integrin and this was followed live with confocal microscopy. The ratio of the intensities of the two dyes was compared to a standard curve acquired using buffers with known pH in the presence of ionophore nigericin. The results revealed that during the 6 h imaging period, α2 positive structures did not markedly acidify (II, Figs. 2A, 2B; III, Fig. 4A). The functionality of the protocol was confirmed by measuring

the pH of endosomal structures labelled with the Vesicular stomatitis virus (VSV) G protein fused to the plasma membrane of Baby hamster kidney cells as described by (Gruenberg et al. 1989) (data not shown). Similarly, when the internalization of α2β1 integrin was followed in the presence of Lysotracker that accumulates in acidic compartments, there was no apparent colocalization of α2β1 integrin with Lysotracker (I, Fig. S3B).

Labeling of different endosomal markers together with α2β1 integrin or EV1 was also performed. No significant colocalization of EV1 with the EE marker EEA1, the late endosomal and lysosomal marker CD63, or the lysosomal marker LAMP-1 was observed (I, Fig. S3A, III, Fig. 3A). Nor did EV1 enter LEs enriched with lysobisphosphatidic acid (LBPA), a lipid commonly associated with acidic LE (II, Fig. 2D). Further, the EV1 receptor α2β1 integrin did not colocalize with endocytosed DilLDL, which is known to follow the acidic pathway (II, Fig. 2E), lysosomal marker cation-independent mannose-6-phosphate receptor (CI-MPR) (III, Fig. S1B; I, Fig. S3A) or Rab7 (III, Fig. 3B). Interestingly, α2-MVBs slowly accumulated Cav1 and also SV40 (I, Figs. 1A-C), which has been shown to traffic through Cav1 positive compartments, sometimes dubbed as caveosomes (Pelkmans et al. 2001). Although the initial entry of EV1 seemed to be Cav1 independent, caveolin was important at later stages, because over-expression of a Cav3 KSY mutant in SAOS-α2β1 cells inhibited the infection completely (I, Fig. 3D).

Altogether, these results show that α2β1 integrin internalizes into neutral endosomes which lack the typical markers of lysosomes and classical, acidic endosomes, but which accumulate Cav1.

5.2.2 α2-MVB formation is dependent on ESCRTs

The maturation of vesicular structures in endosomal pathways involves the accumulation of internal vesicles into the lumen of vacuoles, late endosomes having a clear multivesicular appearance. Ultrastuctural studies of the EV1 internalization pathway revealed that clustered α2β1 integrin and EV1 were found in similar multivesicular structures after 2 h of internalization (I, Fig 4B; II, Fig. 3E). α2 positive endosomes gradually accumulated internal vesicles from 15 to 180 min after internalization, indicating maturation of the structures (I, Fig. 4B). Despite the morphological similarity to acidic endosomes, the earlier colocalization data suggested that these structures are separate (II, Figs. 2D, 2E; I, Fig. S3B; I, Fig. S3A; III, Fig. 3A; III, Fig. 3B). To study whether α2-MVBs are still formed by the same mechanism as classical endosomes, the importance and association of endosomal sorting complex of transport proteins (ESCRTs) in α2-MVB formation was tested. ESCRTs function in multivesicular body formation and they also assist the budding of the eneveloped viruses in cell egress by promoting or stabilizing negative curvature of the membranes & the machinery consists of four protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and many associating proteins, like VPS4 (see Review of the literature 2.3.4 and (Roxrud et al.).

First, SAOS-α2β1 cells were transfected with a DN Vps4-GFP fusion protein (VPS4-E235Q-GFP) that has been shown to prevent the formation of internal vesicles (Sachse et al. 2004, Wollert et al. 2009). Then the same cells were infected with EV1. Infection was drastically inhibited in VPS4-E235Q-GFP cells compared to mock transfected cells (II, Figs. 3A, 3B). A closer look at the cells expressing VPS4-E235Q-GFP revealed that clustered α2β1 integrin remained at the limiting membrane of the endosomes (II, Figs. 3C). Knock-down of selected proteins from ESCRT-I and –III complexes, namely TSG101, VPS37A and VPS24, by siRNA, further showed the importance of a functional ESCRT system in the EV1 entry pathway, as VPS37A and VPS24 knock-down significantly inhibited infection (II, Figs. 4A-C). The association of ESCRTs with α2-MVBs was verified by live microscopy and immunofluorescence studies. Internalization of α2β1 integrin after antibody clustering in GFP-Hrs (ESCRT-0 component) transfected SAOS-α2β1 cells was followed live with confocal microscopy. After ~10 min the integrin started to colocalize with GFP-Hrs and colocalization increased up to ~30 min and was still strong ~90 min after internalization (II, Fig. 5A, Video S1). Immunofluorescent labeling of endogenous Hrs, VPS37A and VPS24 after α2 clustering showed colocalization of internalized α2β1 integrin with ESCRTs (II, Figs. 5B, 5C, S4). Further confirmation of association of ESCRTs with α2-MVBs was acquired by labeling Hrs and VPS24 from cryo-EM sections. Electron micrographs showed ESCRT labeling on the limiting membrane of α2-MVBs (II, Figs. 6A-C).

5.2.3 Comparison to the EGFR internalization and degradation pathway

Most of the current knowledge on thte multivesicular body pathway and its regulators has been gained by studying the internalization and intracellular trafficking of growth factor receptors, especially EGFR. EGFR endocytosis has become a canonical model for transmembrane protein internalization and other plasma membrane receptors are thought to follow similar pathway. Interestingly, integrins and growth factor receptors seem to have a close relationship & they are known to regulate each other's function (for review, see (Ivaska et al. 2010). Due to the close association of these receptors and the morphological similarity between the multivesicular endosomes of the EGFR and α2-MVB pathway, it was reasonable to study whether these two pathways overlap after internalization. First, the internalization of α2β1 integrin or EV1 together with biotinylated-EGF was followed in SAOS-α2β1 cells. The confocal images showed no apparent colocalization between biotin-EGF and α2β1/EV1 after 15 min and 2 h, although the structures seemed to reside sometimes very close to each other (II, Figs. 7A, 7B). As similar experiment using high (100 ng / ml) and low (1 ng / ml) doses of EGF was performed and endogenous EGFR was labeled and screened for colocalization with α2β1 integrin, but as with biotin-EGF, no apparent overlap was detected (II, Figs. 7B, 7C). The experiment was repeated in a different cell line, A549, with similar results (II, Fig. S4). A visual observation suggested that thte EGFR signal was diminishing between 15

min and 2 h, while the α2β1 integrin signal was increasing due to the accumulation of integrin in endosomes (II, Figs. 7A, 7B). This was verified by quantifying the amount of fluorescence in 30 cells (II, Figs. 7D, 7E).

Next it was tested whether the EGF stimulation can enhance the internalization of α2β1 integrin or the number of α2-MVBs produced in the cytoplasm. The cells were stimulated with a high dose of EGF simultaneously with α2β1 integrin clustering and the amount of internalized integrin was then calculated from confocal images by segmenting integrin positive vesicles. The results showed that EGF stimulation had no effect on α2β1 integrin internalization or EV1 infection (II, Fig. 7F and 7G). These results indicate that EGFR and α2β1 integrin internalize and traffic through separate pathways. While EGFR is targeted for down-regulation in lysosomes, α2β1 integrin is accumulate in cytoplasmic vesicles during the 2 h observation period.

5.3 Ubiquitin

5.3.1 Ubiquitin is needed to facilitate internalization of clustered integrin

The signal directing proteins for degradation is ubiquitin, a small 8.5 kDa protein. Specific ubiquitin ligases covalently link ubiquitin moieties to lysine residues in the target protein. Ubiquitin can also be attached to other ubiquitins, thus forming poly-ubiquitin chains. Mono-ubiquitination and multi mono-ubiquitination targets proteins to MVBs, whereas poly-ubiquitinated proteins are degraded in proteasomes. Under some circumstances, selective autophagy can possibly target both mono-ubiquitinated and poly-ubiquitinated proteins to lysosomes. Some receptors need ubiquitination for internalization, but in most cases ubiquitination merely enhances internalization and functions as a targeting signal. Mono-ubiquitin is recognized by ESCRT proteins that then direct the ubiquitinated protein to intraluminal vesicles of MVB. Since α2β1 integrin clustering was associated with the ESCRT-dependent formation of MVBs, the role of ubiquitination in EV1 driven α2β1 integrin internalization and α2-MVB formation was investigated.

First, to study the ubiquitin dependence of α2β1 integrin internalization, cells were treated with a specific E1 ubiquitin ligase inhibitor PYR-41 that inhibits the activation of E2 and E3 and thereby the function of the ubiquitination machinery (Yang et al. 2007). Strikingly, pre-incubation of the cells for 30 min with PYR-41 blocked internalization completely, while in untreated control cells, internalization took place normally, suggesting that ubiquitination is needed for the internalization of α2β1-integrin (Fig. 8A).

Because ubiquitin-related processes seemed to regulate α2β1 integrin internalization, the importance of a functional ubiquitination system on EV1 infection in different stages of the infection cycle was examined. PYR-41 was added to cells at different time points during infection and the cells were fixed 6

h p.i. Pre-incubation with PYR-41 for 15 min before infection inhibited the infection completely, the result being in line with the integrin internalization data. But interestingly, E1 ligase activity seemed to be important even after 30 minutes of infection (Fig. 8B).

To study the role of ubiquitination in α2 internalization in more detail, myc-tagged ubiquitin was over-expressed in SAOS-α2β1 cells and the cells were screened for colocalization of ubiquitin and α2-MVBs by immunofluorescent methods. α2β1 integrin was clustered by antibodies and integrin clusters were allowed to internalize for different periods of time. Confocal images showed what seemed to be the accumulation of ubiquitin at the vicinity of α2-MVBs, as is seen in images taken after 30 min of internalization (Fig. 8C). The effect of PYR-41 was also examined by EM. To visualize the integrins, protein A (PA) gold was bound to a secondary antibody prior to internalization. Internalization was allowed to proceed for 30 min before PYR-41 was added and cells were further incubated at 37°C for 90 min together with PYR-41 (2 h of total internalization time). In the PYR-41 treated cells most of the integrin was in early structures suggesting that further maturation of α2-MVBs was inhibited (Fig. 8D). When the mean size of the α2 positive structures was measured from EM images, vesicles were found to be significantly smaller in PYR-41 treated cells compared to untreated control cells (Fig. 8E).

To further confirm the role of ubiquitination in α2β1 internalization, α2β1 integrin was clustered by EV1 or antibodies & after 5 & 30 min of clustering, cells were lysed and α2 integrin was immunoprecipitated with protein A sepharose bound a rabbit anti human integrin antibody. Stimulation of ubiquitination could be seen in western blots, when α2β1 integrin was clustered either with antibodies or EV1, compared to an unclustered control treated only with secondary antibody (Fig. 8F). Ubiquitination seemed to increase between 5 and 30 min, suggesting that ubiquitination continued also after internalization. To further verify that ubiquitination takes place already at the plasma membrane, paramagnetic Dynabeads coated either with α2 antibody, EV1, or control IgG were used. Due to the large size of the beads (Ø 4.5 μm) they remain at the plasma membrane (data not shown). The beads were incubated on SAOS-α2β1 cells for 5, 15 and 30 min, after which cells were lysed and the beads were collected by magnetic separation. Ubiquitination was analyzed by western blotting and a clear stimulation of ubiquitination was observed with both the α2 antibody and EV1 coated beads already after 5 min & the signal increased after 15 and 30 min (Fig. 8G).

Altogether, these results indicate that ubiquitin is needed for α2β1 internalization and continued ubiquitination is necessary for proper α2-MVB formation.

5.3.2 α2 integrin is not a target for ubiquitination

Ubiquitination of EGFR is known to act as a trigger for its targeting to the ILVs inside MVBs, which then leads to its lysosomal degradation. Electron microscopic observations of clustered α2 integrin had already suggested that α2β1 integrin is not found exclusively in the ILVs. Instead, it is often found lining the limiting membrane. This raised a question if α2 integrin itself or some other integrin associated proteins on the plasma membrane might be ubiquitinated. Therefore, α2 integrin ubiquitination upon clustering was investigated. The cytosolic C-terminus of the α2 integrin contains four lysine residues that may serve as putative ubiquitination sites. For this reason α2 c-terminal lysines were mutated to arginines (see Materials and methods). First it was tested if the mutation of lysine residues had any effect on the internalization of α2 integrin after clustering. SAOS wt cells that do not endogenously express α2 integrin were transiently transfected with the mutant or wt α2 and integrin was then clustered and allowed to internalize for 2 h. The previously published internalization labeling method to distinguish internalized α2 integrin from the plasma membrane derived integrin was used (II). Shortly, first the integrin was clustered by sequential treatment with primary antibody and secondary antibody conjugated to a fluorescent dye (e.g. Alexa 555). After 2 h of internalization, cells were fixed and the plasma membrane pool of integrins was labeled again, this time with a different dye. The labeling showed that mutating the lysines did not block integrin internalization as after 2 h of internalization amounts of cytoplasmic α2-MVBs were normal (Fig. 9A). We also evaluated the cells with the mutated α2 by EV1 infection. The results showed that cells with mutant α2 were also infected normally by EV1, further indicating that the lysine residues of α2 integrin do not act as a target for ubiquitination (Fig. 9B). These data are in line with the ubiquitination data (Fig. 8G) showing no apparent signal in the location of α2 integrin (≈ 150 kDa).

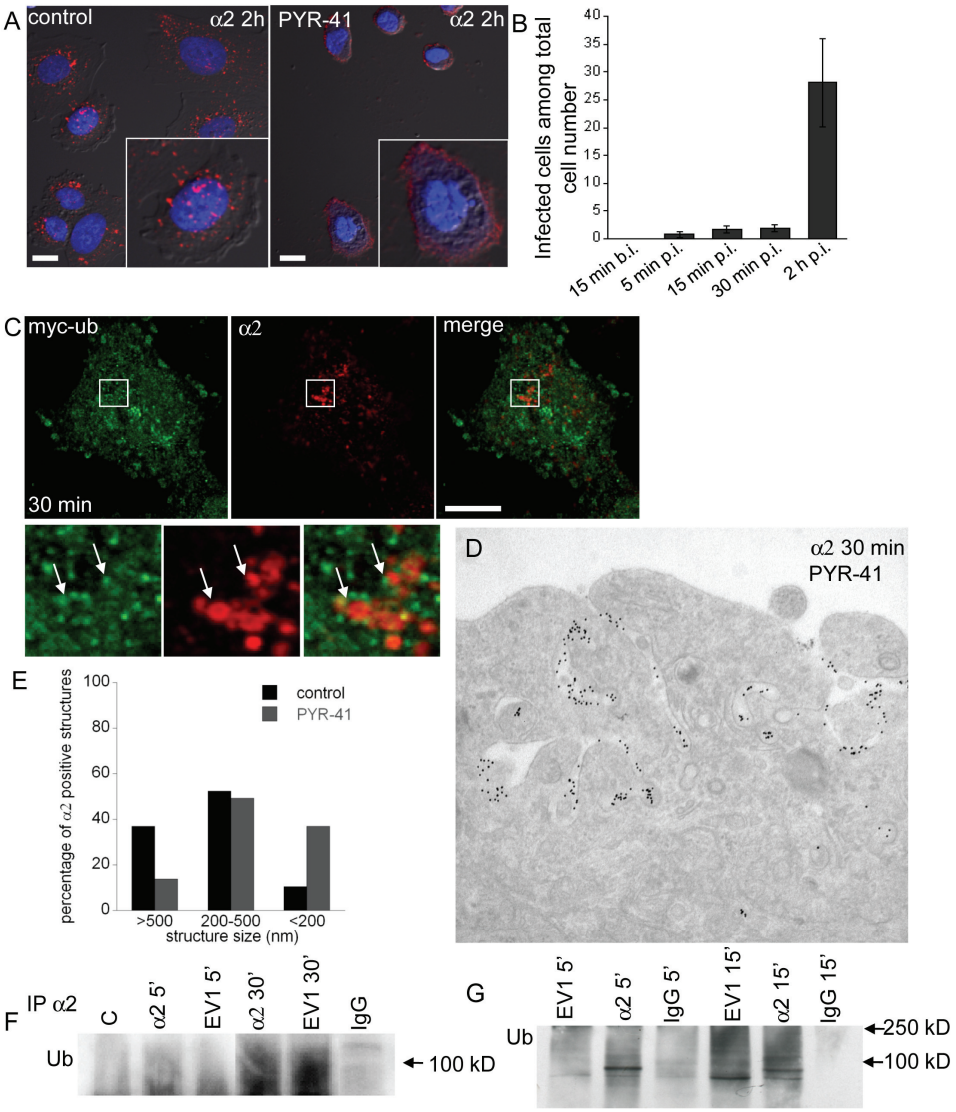

FIGURE 8 A functional ubiquitination system is essential for α2β1 internalization and EV1 infection. (A) Incubating cells in the presence of the E1 ligase inhibitor PYR-41 at a concentration of 50 μM effectively inhibited the internalization of α2β1 integrin (red). (B) PYR-41 was added to the cells at different time points during infection to assess the importance of ubiquitination at different stages of infection. Cells were fixed 6 h p.i. (C) Confocal images of myc-ubiquitin transfected SAOS-α2 cells. Myc-ubiquitin was expressed for 24 h and α2β1 was clustered and allowed to internalize for different periods of time. Myc and α2β1 were then labeled with anti-myc and anti-α2, respectively. Accumulations of myc-ubiquitin (green) in α2-MVBs (red) is indicated with arrows. (D) EM images of PYR-41 exposed cells show that integrin mostly remains on early tubular structures near the plasma membrane. (E) The mean size of the α2 positive structures was measured from EM images. (F) α2β1 integrin was clustered by EV1 or antibodies & after 5 & 30 min of clustering, cells were lysed and α2 integrin was immunoprecipitated with protein A sepharose bound rabbit anti human integrin antibody. Samples were then western blotted and labeled for ubiquitin. (G) Dynabeads coated either with α2 antibody, EV1, or control IgG were used to examine ubiquitination at the plasma membrane. The beads were incubated on SAOS-α2β1 cells for 5, 15 and 30 min, after which cells were lysed and the beads were collected by magnetic separation. Ubiquitination was analyzed by western blotting.

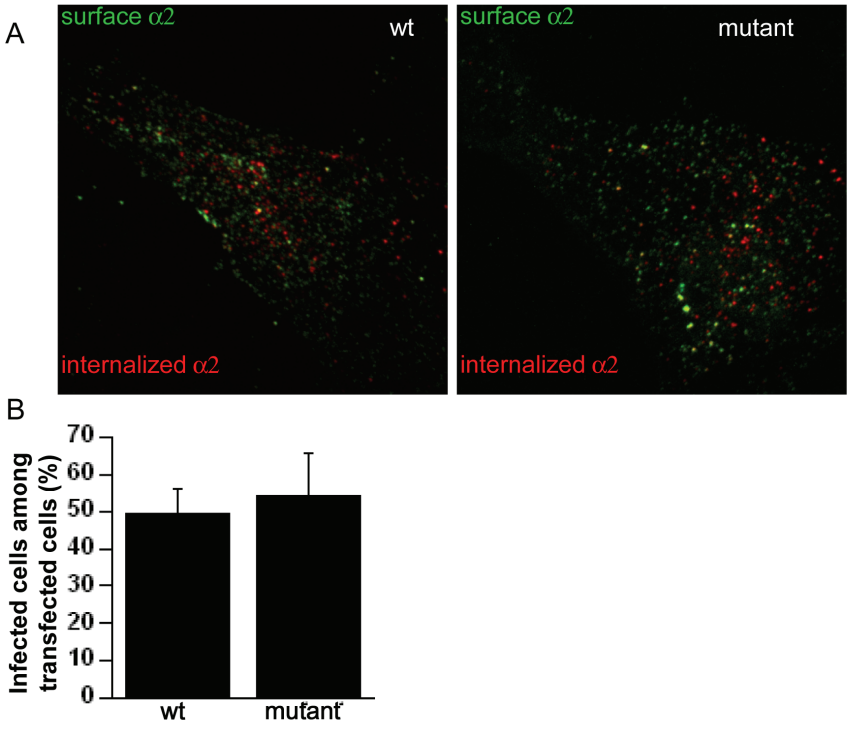

FIGURE 9 Mutating possible ubiquitin binding sites on the integrin α2 tail does not affect the internalization or targeting of integrin α2β1. α2 c-terminal lysines were changed to arginines to study the role of the α2 tail on targeting of the integrin to MVBs. Mutated α2 integrin was transfected into SAOS-2 cells that lack endogenous α2 integrin. Internalization was induced and surface integrin was differentially labeled as described by Upla et al (2004) (A). (B) The effect of mutated α2 on EV1 infection. SAOS-2 cells were transfected either with mutated or wt α2 for 48 h and then infected with EV1 for 6 h. The result is a mean value of three independent experiments (±SE).

5.4 Clustering of α2β1 integrin leads to enhanced integrin turnover that is dependent on calpains but independent of lysosomes and proteasome

5.4.1 α2-MVBs are degradative structures

Normally, integrins are constitutively endocytosed and recycled back to the plasma membrane in migrating cells (Bretscher 1989, Bretscher 1992). In some situations integrins are also degraded, for example to allow proper migration (Lobert et al. 2010). In SAOS-α2β1 cells, under normal growth conditions, α2β1 integrin is mainly found diffusely on the plasma membrane, with some integrin also seen in cytoplasmic vesicles (II, Fig 1C; III, Fig 1A). Rapid changes in the distribution of integrins are seen upon EV1 binding or antibody clustering, with a large portion of α2β1 integrin internalizing into cytoplasmic vesicles (see e.g. I, Fig, 1A; II, Fig.1A; III, Figs. 1B, 1C). Interestingly, immunofluorescent labeling showed that a notable proportion of the integrin signal seemed to be lost

between 2 to 6 h after virus induced clustering (III, Fig. 1B). To verify this observation, the loss of integrin signal after 2, 6 and 24 h of clustering in the presence or absence EV1 was quantified. The results showed that after clustering with antibodies, 60% of the fluorescent signal was lost after 6 h & after 24 h the signal was almost completely lost (III, Fig. 1C). A similar trend was evident when clustering was done in the presence of EV1 (III, Fig. 1B). Metabolic labeling showed that the half-life of α2β1 integrin in unstimulated conditions in SAOS-α2β1cells was roughly 36 h (III, Fig. 1D). Since there was a possibility that the loss of the integrin signal measured from fluorescent images was due to degradation of IgG or its fluorescent conjugate used in the clustering protocol, we evaluated the α2 signal by labeling the integrin twice in the same assay: first during clustering and then after fixation, using a different antibody. Analysis of the fluorescent intensity showed a clear reduction in the signal from 2 h to 24 h for both labels & confocal images showed strong colocalization of the different antibodies, indicating that they recognized the same pool of α2β1 integrin (III, Fig. 1E).

The loss of fluorescence from integrin positive endosomes could partially originate from integrin recycling back to the plasma membrane. Several different approaches showed that there was no significant recycling of α2β1 integrin after clustering the receptor with antibodies or EV1: First, there was no evidence of recycling in a live assay based on anti-Alexa. The outer surface of the cell was exposed to anti-Alexa that quenches the fluorescence of Alexa dyes after internalization and thus, if any extensive recycling was to happen, the overall fluorescence of labeled integrin would decrease in the cell & this was not seen (III, Figs. 2A and 2B). In contrast, when the anti-Alexa treatment was performed under non-clustering conditions, the data showed that after 1 h of internalization 70% of the α2 integrin pool was sensitive to the treatment suggesting that the majority of α2 integrin was still on the plasma membrane (III, Fig. 2B). Subsequent anti-Alexa treatments caused a further decrease in the signal, while in cells that were not further treated with anti-Alexa, the signal remained higher (III, Fig. 2B). Second, photobleaching studies where the fluorescence was bleached from the cell outside of the perinuclear vesicles & the appearance of fluorescence to cell edges was monitored in 1 h intervals, further indicated fthat α2-MVBs were stable structures after 2 h of internalization and there was no apparent increase in the fluorescent signal at cell boundaries during the >6 h imaging period (III, Fig. 2C). Third, EM studies revealed that there was no apparent enrichment of PA-gold labeled integrin patches at the plasma membrane between 2 and 24 h after clustering; rather the integrin label was located at the same perinuclear areas at both time points (III, Fig. 2D).

Collectively these results suggest that, when internalized into α2-MVBs upon EV1 or antibody stimulation, α2β1 integrin, is not recycled back to the plasma membrane and that the loss of the integrin signal is likely due to the degradation of the receptor.

5.4.2 α2β1 integrin degradation is independent of lysosomes or the proteasome

Cells have two well-documented compartments for the degradation of material: lysosomes and proteosomes. As shown above, α2-MVBs do not accumulate markers of classical endosomes, i.e. the endosomes associated with the pathway leading from the plasma membrane to lysosomes. The exclusion of α2-MVBs from classical endosomes was further studied by preventing the acidification of endosomes and inhibiting the function of selected lysosomal enzymes.

To directly investigate the role of lysosomes in α2β1 degradation, the protease inhibitor leupeptin was allowed to accumulate in lysosomes, thus inhibiting lysosomal proteases (Petiot et al. 2003). Then α2β1 integrins were clustered with antibodies and allowed to internalize for 0 to 24 h. Antibodies were conjugated either with an Alexa dye or horse radish peroxidase (HRP) and the degradation of the conjugate was measured. In both cases leupeptin had only a small inhibitory effect on degradation (III, Figs. 6A and 6B). Other protease inhibitors tested, namely antipain, trypsin inhibitor, aprotinin and elastatinal had no effect on degradation (III, Fig. S1D). Interestingly, treating cells with bafilomycin, a vacuolar H+ATPase inhibitor, inhibited EV1 infection to some extent when added 15-60 min p.i (III, Fig. 4B). However, after 2 h, when α2-MVBs are fully matured, bafilomycin did not interfere with infection. When the pH of α2-MVBs was measured after 4 and 6 h p.i., there was a minor elevation of the pH of drug treated cells. Bafilomycin also seemed to slightly decrease the degradation of α2β1 integrin compared to control cells, as confirmed by fluorescence intensity measurements (III, Fig. 4C). Together these results suggest that lysosomes do not have a major role in the degradation of clustered α2β1 integrin.

For the degradation of cytosolic material i.e. proteins or organelles, cells use a pathway called autophagy, where the cargo is first enclosed by double membrane, forming an autophagosome that then delivers the cargo to lysosomes. Since viruses closely related to EV1, Coxsackieviruses B3 and B4 have been shown to use autophagosomes for replication (Wong et al. 2008, Yoon et al. 2008) it was reasonable to test the possible connections of α2β1 integrin and EV1 with autophagosomes. This was done in SAOS-α2β1 cells transiently expressing the autophagosomal marker LC3 fused to GFP (LC3-GFP). Infection assays showed that over-expression of LC3-GFP had an inhibitory effect on EV1 infection (III, Fig. 5C. Interestingly, the amount of LC3 positive structures seemed to decrease in infected cells (III Fig. 5D). Colocalization studies with α2β1 integrin and LC3-GFP further indicated that the α2-MVB pathway is not connected to autophagosomes, because there was no significant overlap 2, 6 and 24 h after internalization (III, Fig. 5E).

Although the proteasome is mainly used for the degradation of intracellular proteins, there are indications that the degradation of some plasma membrane proteins is dependent on the proteasome (Longva et al. 2002, Kaabeche et al. 2005, Bruns et al.). However, in this study, the proteasomal

inhibitors lactacystin and Bortezomib did not have any effect on EV1 infection or the degradation of α2 conjugated HRP (III, Figs. 5A, 5B).

Thus taken together, these results suggest that α2β1 integrin is not degraded extensively in lysosomes or proteosomes. However, because of the minor effect bafilomycin had on degradation, a marginal lysosomal involvement cannot be excluded.

5.4.3 Calpains promote integrin turnover in the α2-MVB pathway

An interesting group of proteins with degradative functions are the calpains, which are calcium-dependent cysteine proteases that degrade cytoskeletal and cytoplasmic proteins. Although their exact physiological role is still unclear, calpains have been shown to be involved in various cellular processes, such as migration and cell cycle progression and they have various substrates, including transcription factors, transmembrane receptors, cytoskeletal proteins as well as focal adhesion and signaling molecules (Suzuki et al. 1998, Goll et al. 2003). It has been shown previously that EV1 infection is dependent on calpains that also seem to localize in EV1 positive vesicles (Upla et al. 2008). This raised the question whether calpains could also be responsible for α2β1 integrin degradation. To examine this, various tests were performed.

First, the association of calpains with α2β1 integrin was confirmed by performing a series of immunoprecipitations (IP). IP of α2 integrin with a polyclonal antibody (pAb) showed a significant fraction of calpain-1 in the immunoisolates, even when α2β1 integrin was left unclustered (III, Fig. 7A). A control lysate showed that calpain-1 is an abundant protein in cells and mostly found in its mature 80 kDa form in non-treated cells. In contrast α2 integrin seemed to associate preferably with more processed forms of calpain-1, possibly the autolysed 78 kDa form. Next, an IP was performed by collecting the clustering antibodies bound to α2 integrin before internalization; calpain-1 was also enriched in these precipitates after 15 min and 2 h, which further suggest that calpain was present in internalized α2-MVBs. Then, immunoprecipitation was also performed via β1 integrin using two different antibodies. First, a β1 integrin antibody was bound on the plasma membrane and then incubated for 2 h at 37°C with or without EV1 induced integrin clustering. In line with the α2 IP, calpain-1 seemed to precipitate also with β1 integrin. Interestingly, EV1 induced clustering caused a disappearance of the higher 80 kDa form and was mostly associated with the smaller processed form (III, Fig. 7A). Altogether these results show that calpain-1 co-precipitates with both clustered and unclustered α2β1 integrin and it seems that after clustering, integrin associated calpain-1 is mainly in the processed, possibly active form.

Next, calpain activity in α2-MVBs was tested. This was done using a cell-permeable fluorogenic calpain substrate 7-amino-4-chloromethylcoumarin, *t*-BOC-l-leucyl-l-methionine amide (t-BOC), a peptidase substrate that becomes fluorescent when cleaved by calpains. A clear increase in calpain activity was seen in cells with antibody (±EV1) clustered integrin compared to an

unclustered control 6 h post internalization (III, Fig. 7B). Fluorescent images showed that high calpain activity was often found in integrin positive structures and a colocalization analysis revealed that 80% of the α2 positive structures were colocalizing with t-BOC (III, Fig. 7C), strongly suggesting that calpains are indeed active and present in α2-MVBs.

Since calpains seemed to associate with internalizing integrin and virus their role in α2β1 integrin degradation was also investigated. Leupeptin, which was used to inhibit lysosomal proteases, has been shown to have some effect also on calpains. Indeed, integrin degradation was slightly affected by leupeptin but this could have been also due to the inhibition of lysosomal enzymes, as mentioned above (III, Figs. 6A, 6B). Therefore, a more specific inhibitor, calpeptin was used & as a result, the degradation of the integrin bound cargo, namely Alexa-dye or HRP was almost completely prevented even 24 h after internalization of the integrin (III, Fig. 6A, 6B). Similar results were obtained using specific calpain-1 and -2 inhibitors (III, Fig. S1C), suggesting a clear role for calpains in the disappearance of the fluorescence signal. In vitro studies showed that calpains were able to degrade both the integrin itself and the HRP bound to it via antibodies (III, Fig. 6C).

To conclude, these results show that calpains are present in α2-MVBs and the degradation of α2β1 integrin or its associated cargo is dependent on calpain activity.

6 DISCUSSION

6.1 EV1 entry by macropinocytosis

Most animal viruses depend on the endocytic activities of the host cell to reach the cellular machinery needed for their replication. Among those internalization pathways are clathrin-mediated endocytosis, caveolar/lipid raft-mediated endocytosis and macropinocytosis. The route taken by a virus seems to be determined by particle size, receptor, the mode of transmission and cell tropism (Marsh et al. 2006). Previous work has shown that EV1 accumulates in Cav1 positive cytoplasmic structures, but the initial entry seems to be caveolin-independent (Marjomäki et al. 2002, Pietiäinen et al. 2004). This work further confirmed that EV1 and its receptor α2β1 integrin do not markedly colocalize with caveolin at the plasma membrane, rather colocalization emerges after internalization (I). Recent evidence suggests that Cav1 can be trafficked through MVBs on their way to degradation in lysosomes (Hayer et al. 2010) so it is possible that caveolar trafficking intersects with the EV1 internalization pathway at some point. Our unpublished results show a minor portion of the EV1 in caveolar invaginations. This might be a naturally occurring secondary, less efficient pathway that EV1 is using. Also, under some conditions, such as when clustering with certain antibodies or upon EGF stimulation, α2 integrin can internalize through caveolae (Upla et al. 2004, Ning et al. 2007).

Although Cav1 had a negligible role in the internalization of EV1, it seemed to be important at later stages of infection, as shown the by over-expression of DN caveolin mutants (I). Whether this effect is direct or indirect, e.g. via altered cholesterol trafficking remains to be studied. There are indications that caveolins might have an important role in regulating cholesterol balance which in turn has implications for signaling processes, such as Ras and PDGFR signaling, originating from putative lipid rafts (Roy et al. 1999, Carozzi et al. 2002, Fielding et al. 2003, Fielding et al. 2004), providing possible explanation for the caveolin dependency of EV1 infection.

Three aspects suggested that a pathway other than caveolae could be used by EV1. 1) typically, caveolae mediated uptake has slow kinetics (Thomsen et al. 2002), compared to the rapid internalization of EV1, 2) the initial entry of EV1 is independent of caveolin and 3) the internalized EV1 vesicles are larger than caveolae. Eventually, most evidence points towards macropinocytosis-like internalization, which is dependent on CtBP1/BARS, as a mechanism of entry and infection (Liberali et al. 2008). Macropinocytosis is normally triggered by external stimuli, usually growth factors that then activate receptor tyrosine kinases, launching a signaling cascade that leads to changes in the actin cytoskeleton and causes plasma membrane ruffling. In addition to natural ligands, several viruses have been shown to induce macropinocytosis; among those are vaccinia virus, adenovirus 3, Coxsackievirus B, herpes simplex virus and human immunodeficiency virus (Mercer et al. 2009). Interestingly, EV1 induced macropinosomes seemed to be smaller than those induced by EGF (Liberali et al. 2008). Although this difference could merely arise from different the cell types used, it is tempting to think that different ligands involve different signaling pathways.

Macropinocytosis is still an incompletely characterized process and it is possible that different forms of macropinocytosis occur in cells. It has been suggested that the plasma membrane protrusions involved in macropinocytosis can take various forms with minor differences - depending on the cell type and the nature of ligand, planar lamellipodia, circular ruffles or blebs can be induced (Buccione et al. 2004, Charras et al. 2006, Mercer et al. 2009). An interesting observation is that PDGF stimulated macropinocytosis requires dynamin-2, a GTPase usually associated with micropinocytic membrane scissions (Liu et al. 2008b). It has been speculated that dynamin might be involved in macropinosome closure originating from circular ruffles, similar to its role in formation of the phagocytic cups (Mercer et al. 2009). In contrast, the membrane scission of lamellipodial derived macropinosomes is independent of dynamin (Cao et al. 2007), instead, this role seem to be taken by CtBP1/BARS (Liberali et al. 2008). Previous studies have shown that EV1 infection is dependent on dynamin in CV-1 cells (Pietiäinen et al. 2004), which is in contrast to findings in this thesis showing that EV1 infection is independent of dynamin-2 in SAOS-α2β1 cells. The induction of different kind of macropinocytosis with slightly different regulators can offer one possible explanation for this divergence.

There are probably also other differences between cell lines. Recent unpublished results (Lehkonen et al. unpublished) suggest that there are more EV1 particles in the caveolae of A549 than in those SAOS cells. As discussed above, EV1 may take the caveolar pathway as a secondary entry route. This way EV1 still reaches the α2-MVBs, though not as efficiently as via macropinocytosis. The ability to use more than one endocytosis pathway for entry is not unique to EV1. For example SV40 has been reported to internalize through caveolae but also non-caveolar pathways (Gilbert et al. 2000, Damm et al. 2005) and herpes simplex virus is known to use at least three different

pathways (Milne et al. 2005). Moreover, the entry of dengue virus can occur by a non-classical endocytic pathway independent of clathrin, caveolae and lipid rafts, but dependent on dynamin in some cells, while in the majority of the cell lines studied dengue virus entry is clathrin-dependent (Acosta et al. 2009).

6.2 α2β1 integrin is degraded in novel multivesicular structures

6.2.1 Novel, neutral MVB

In the present work it was shown that, after internalization, EV1 and α2β1 integrin quickly started to accumulate in endosomes with a multivesicular appearance. Already 15 min p.i., integrin-positive structures showed some internal vesicles and after 2 h, most of the internalized receptor was localized inside MVBs (I). MVBs are usually determined as intermediate carriers of transmembrane receptors (e.g. growth factor receptors) destined for degradation in lysosomes (Gruenberg et al. 2004). Sequestering receptors to ILVs prevents signaling that would be otherwise possible if the receptor remained at the limiting membrane.

The multivesicular bodies that are formed after α2β1 integrin clustering by EV1 proved to be crucial structures that promote efficient infection (I, II). Despite the striking similarities in morphology, several experiments showed that α2-MVBs are distinct from the well-known multivesicular late endosomes (I, II, III). First, the intra endosomal pH measurement showed that during the first 3 h of infection, the time needed for EV1 to escape the endosomes and start replicating, α2-MVBs do not markedly acidify. This is in line with previously published and present results, showing that α2-MVBs are devoid of markers commonly associated with late endosomes or lysosomes, namely CD63, CI-MPR, LAMP-1, Dil-LDL and Rab7 (I, II, III). Second, co-internalization studies with EGFR, which is the best characterized receptor to use multivesicular bodies and is sorted for lysosomal degradation, provided evidence that despite the reported close relationship between α2β1 integrin and the EGFR receptor on the plasma membrane (Yu et al. 2000, Moro et al. 2002), they do not accumulate into the same endosomes after internalization (III). These results show that not all MVB pathways lead to degradation in lysosomes. Increasing evidence points towards an impression of different types of subpopulations of MVBs in cells, with slightly varying lipid and protein composition. There are plenty of examples: EGFR may traffic through multivesicular bodies that lack the lipid LBPA commonly found on late endosomes (White et al. 2006), class II MHC and tetraspanins accumulate in MVBs and form a specialized class II MHC compartment (MIIC) (van Niel et al. 2006). Further, not all MVBs have degradative functions: examples of non-degradative MVBs, associated with exosomal pathways, include the alpha granules of platelets and the Weibel-Palade bodies of endothelial cells (Heijnen et al. 1999, Kobayashi et al. 2000) &

the azurophilic granules of neutrophils lack LAMP-1 and -2, but have CD63 enriched ILVs (Cham et al. 1994, Cieutat et al. 1998). Another non-degradative MVB is the melanosome (Theos et al. 2006). There are indications that the exit of EV1 genome from the α2-MVBs relies on specific morphological changes that are associated with the destabilization of endosomal membranes (Soonsawad et al. unpublished). Ending up at lysosomes would most likely be harmful for EV1 and prevent infection, so the formation of MVB that avoid contact with lysosomal hydrolases is crucial for EV1 to successfully infect cells. The underlying signaling mechanism is yet to be described.

The EV1 induced MVBs seem to also differ from conventional macropinosomes. After detachment the conventional macropinosomes move deeper into the cytoplasm, undergo acidification and are involved in homo- and heterotypic fusion events with other macropinosomes and lysosomes (Racoosin et al. 1993, Hewlett et al. 1994). They also seem to be sensitive to the cytoplasmic pH (West et al. 1989). This might be explained by a recent finding by Koivusalo and colleagues (2010), providing evidence that lowering the submembraneous pH prevents membrane ruffling and thus macropinocytosis by inhibiting the activation of RhoGTPases. In macrophages, macropinosomes behave like classical endosomes, since during their maturation process they gain and lose the classic early and late endosomal markers and then fuse with lysosomes (Racoosin et al. 1993). However, in human carcinoma A431 cells, a large portion of macropinosomes recycle back to the cell surface and release their contents into the extracellular space (Hewlett et al. 1994). It seems that the trafficking and characteristics of macropinosomes depend on the cell type and the source of stimulation, as demonstrated also in this thesis.

6.2.2 Case of the caveosome

Recently, shadow has been cast over the existence of the cellular compartment known as the caveosome (Hayer et al., Parton et al. 2010). The caveosome was first identified as an intermediate organelle in the SV40 entry pathway, characterized by a high caveolin content, neutral pH, as well as a lack of markers for endosomes, lysosomes, endoplasmic reticulum (ER) and Golgi (Pelkmans et al. 2001). Moreover, it did not accumulate ligands of the clathrin-mediated pathway. In the present study SV40 was used as a marker of the caveosome and a population of SV40 positive compartments also accumulated α2β1 integrin, clustered by antibodies (I). In line with findings by Pelkmans et al., the EV1 induced α2-MVB is non-acidic, accumulates Cav1 and lacks the markers of classical endosomes as well as other cellular compartments, although the morphology of the "original" caveosome and the α2-MVB are markedly different. This led to the conclusion that EV1 is internalized into caveosomes (I). However, Hayer et al. suggest that the non-acidic caveosomes described by Pelkmans et al. actually arise from the over-expression of Cav1 in transfected cells. Further, findings that SV40 actually traffics through and is dependent on acidic endosomes on infection (Engel et al. 2011) hint that

caveosomes might have originally been an artifact. In light of this information, the statement that EV1 enters caveosomes needs to be re-evaluated. The colocalization of SV40 and EV1 seen in co-infection studies (I, (Pietiainen et al. 2004) may be due to EV1-induced signaling events that could direct also some of SV40 to non-acidic structures, in analogy to EV1 rerouting dextran uptake (I). Cav1 may also be influenced by integrin clustering-associated signaling and lead to the redistribution of caveolae to the cytoplasm. Cav1 may accumulate in α2-MVBs also because of slower caveolae mediated uptake of virus, as discussed above. This would also explain the relatively slow appearance of Cav1 to α2-MVBs. Finally, the morphological differences between the α2-MVB and the caveosome are so obvious that calling the EV1 positive endosome a caveosome was premature.

6.2.3 Role of ESCRT in α2-MVB biogenesis

In spite of the apparent lack of markers associated with the lysosomal pathway in α2-MVBs, they seem to be constructed by the same mechanisms as classical, acidic MVBs. ESCRT proteins that catalyze budding events away from the cytoplasm and are responsible for ILV formation in growth factor induced MVBs (see for example (Raiborg et al. 2009), were shown to be important also for α2-MVB formation. In the present study the involvement of ESCRTs in the α2-MVB pathway was confirmed by several experiments: First, the expression of a dominant negative form of VPS4, second, siRNAs against selected ESCRT proteins & third, by colocalization studies between ESCRTs and α2-MVBs (II). The most striking piece of evidence supporting the importance of ESCRTs came from studies with a DN VPS4-GFP fusion protein (VPS4-E235Q-GFP). *In vivo* and *in vitro* studies with VPS4 by others suggest that it has a role in recycling ESCRT-III proteins thus allowing continued inward vesiculation (Babst et al. 1998, Wollert et al. 2009). In this study, the expression of DN VPS4 caused significant inhibition of EV1 infection and the accumulation of α2β1 integrin in tubulovesicular and small structures or enlargened, morphologically immature endosomes with few or no ILVs, as shown by EM and confocal microscopy. The apparent small size of a large portion of α2 positive vesicles in DN VPS4 transfected cells suggests that the maturation of the endosomes is halted. Over-expression of this mutant might lead to disruption of the entire ESCRT machinery, thus preventing the sorting and consequently the inclusion of more membrane to forming α2-MVBs, leaving the α2-positive endosomes small and immatured. Although the majority of α2 positive endosomes in DN VPS4 expressing cells seemed to remain immature, a portion of the α2-positive endosomes were large, "empty" endosomes without ILVs. Abnormal, giant MVBs in VPS4-E235Q expressing cells have been described also by others (Fujita et al. 2003, Sachse et al. 2004).

The formation of ILVs seems to be somehow essential for EV1 infection. Further evidence for the importance of multivesicularity on EV1 infection comes from studies with the amiloride analog EIPA that caused a block in α2-

MVB maturation and the accumulation of peripheral tubulovesicular structures with no intraluminal vesicles (I). EV1 was enriched in these structures but they could not promote infection. Recent data may offer a possible explanation for this: EV1 induced integrin clustering is accompanied by a size increase and size variation of the ILVs as well as an increase in the total volume α2-MVBs, compared to the MVBs induced by the mere antibody clustering of integrins (P. Soonsawad et al. unpublished). There was also clear rupturing and destabilization of the limiting membrane visible in the largest ILVs of the α2-MVB before the viral replication started in the cytoplasm. These changes may be the key to why the development of MVBs is important for EV1 infection. It is possible that EV1 might have the ability to impact on ILV and MVB size. It has been shown that the regulation of VPS4 activity affects the size and rate of formation of ILVs (Nickerson et al. 2010). According to recent findings also ESCRT-II regulated assembly of ESCRT-III and especially the length of Snf7 filaments determine the size of the ILVs (Teis et al. 2010). Whether EV1 is able to regulate ESCRTs or VPS4 and consequently affect MVB and ILV morphology remains to be studied.

Little is known about the utilization of ESCRTs by non-enveloped viruses. However, several enveloped viruses are known to rely on ESCRT proteins, primarily to escape from cells by budding. Among those viruses are RNA viruses such as retroviruses (Garrus et al. 2001), herpesviruses (Pawliczek et al. 2009, Tandon et al. 2009), rhabdoviruses (Jayakar et al. 2004), filoviruses (Hartlieb et al. 2006), arenaviruses (Urata et al. 2006) & possibly ortho- and paramyxoviruses (Takimoto et al. 2004). In macrophages, under some circumstances, HIV-1 has been shown to replicate in multivesicular bodies positive for some late endosomal markers (Joshi et al. 2009). In contrast to the viruses mentioned above, the non-enveloped virus EV1 needs ESCRTs to develop a mature α2-MVB that is needed for the efficient initiation of infection & it seems that EV1 is the first non-enveloped mammalian virus shown to be dependent on ESCRTs and MVBs for successful replication.

An interesting finding was the association of the ESCRT-0 protein Hrs with the α2-MVB pathway. It has been shown that Hrs is essential for the lysosomal targeting of EGFR (Pons et al. 2008). However, in this study, despite the fact that the α2 positive structures are not targeted to lysosomes, visualization by light microscopy and EM revealed that Hrs association with α2β1 integrin increased over time, when receptors were clustered by antibody treatment (II). These results suggest that Hrs may also have functions other than lysosomal targeting, or additional adaptor molecules are needed for lysosomal targeting. Ubiquitin has an important role in sorting to ILVs during the EGFR internalization process. EGFR is multi-mono-ubiquitinated and Hrs/STAM complex (ESCRT-0) interacts with the ubiquitinated receptor via its ubiquitin binding domains and recruits ESCRT-I and ESCRT-II to the cargo (Bache et al. 2003a, Bache et al. 2003b, Slagsvold et al. 2005, Kostelansky et al. 2007). The recruitment of Hrs to internalizing integrin suggests that ubiquitination may be involved in sorting to α2-MVBs.

6.3 Role of ubiquitin in sorting to α2-MVBs

The involvement of ESCRTs in the biogenesis of α2-MVBs led to experiments addressing whether the ubiquitination of integrin or its associated proteins was needed for this process. Strikingly, inhibition of E1 ligase and thus the whole ubiquitination system with PYR-41 revealed that ubiquitination is essential for the internalization of clustered α2β1 integrin and EV1. This is different compared to EGFR and α5β1 integrin as their internalization has been shown to be independent of ubiquitin (Huang et al. 2007, Lobert et al. 2010). In these cases ubiquitin is generally needed for further sorting. However, maturation of the α2-MVBs seems to be tightly controlled by the ubiquitination system. The introduction of the E1 ligase inhibitor PYR-41 prevented the formation of α2-MVBs even when added 30 min after clustering of the integrins. The data also suggested that the ubiquitination levels of proteins co-precipitating with α2 integrin gradually increased at least up to 30 min after clustering or infection. Also the immunofluorescence data did not show apparent ubiquitination until 30 min after internalization. This may be explained by the findings of others indicating that the ubiquitination of receptors continues after internalization and this continued ubiquitination may enhance the sorting of the receptor (Longva et al. 2002, Umebayashi et al. 2008). Also the deubiquitinating enzyme UBPY has shown to be ubiquitinated when activated and recruited to MVBs (Mizuno et al. 2005). UBPY can protect ESCRT-0 from degradation (Niendorf et al. 2007) and help to maintain the bulk flow of cargo (Berlin et al. 2010). There also seemed to be increased ubiquitination of proteins in a cell if α2β1 integrin was clustered with EV1, compared to the clustering with antibodies. This might be connected to the morphological differences described above: increasing ubiquitination may help to boost the infection potency of MVB.

Mutating the putative ubiquitination sites in the α2 tail by changing lysines to arginines did not have any effect on the internalization of integrin or EV1 infection, indicating that α2 integrin is not the target for ubiquitin. Immunoprecipitations also suggested that neither the α2 nor the β1 units were ubiqutinated since there was no visible ubiquitin smear around the expected molecular weight. Although it seems that neither the α2 nor the β1 subunit is the target for ubiquitination, sometimes the ubiquitination of accessory proteins is important for receptor internalization. For example Hrs, EPS15, epsin1 & epsin2 proteins are mono-ubiquitinated in response to EGF treatment, possibly amplifying the ubiquitination network (Klapisz et al. 2002, Polo et al. 2002). Also it seems that an active ubiquitin-system but not the ubiquitination of the growth hormone receptor (GHR) itself is required for uptake, hinting that there might be a yet unidentified protein that needs to be ubiquitinated in order to allow GHR endocytosis (Sachse et al. 2001). Similarly, beta 2-adrenergic internalization is regulated by the ubiquitination of beta-arrestin (Shenoy et al. 2001). In the present study, immunoprecipitation of α2 integrin and subsequent western blotting showed extensive ubiquitination of several co-precipitated

proteins. It can be speculated that binding of EV1 to α2β1 integrin recruits the internalization machinery to the lipid rafts and the subsequent ubiquitination of the components of the machinery regulates the internalization. The exact targets of ubiquitin are currently under investigation.

6.4 α2β1 integrin degradation

In the present work it was shown that internalized α2β1 integrin was lost from cells, independent of lysosomes or the proteasome and there was no apparent recycling either (III). This is strikingly dissimilar to integrin dynamics in migrating cells, where integrins are constitutively recycled via rab-regulated recycling pathways (Caswell et al. 2006, Pellinen et al. 2006b). Recently it was shown that a fraction of integrin is also degraded together with fibronectin in lysosomes to ensure productive adhesion sites (Lobert et al. 2010). Degradation of clustered integrin seemed to be a slow process compared to EGFR down-regulation. While EGFR is almost completely degraded after 2 h upon EGF stimulation as shown previously (Burke et al. 2001, Sigismund et al. 2008) and in the present study (II), there was still some α2β1 signal left 6 h after clustering and it took 24 h to totally dissipate internalized integrin as shown by light microscopy studies (III). Calpain proteases seemed to be centrally involved in the degradation of α2β1 integrin in α2-MVBs (III). Calpains are calcium-dependent cysteine proteases that degrade cytoskeletal and cytoplasmic proteins and need a pH neutral environment to be active (Suzuki et al. 1998, Goll et al. 2003). Calpains are known to regulate various processes in cells, most importantly cell migration, although their exact physiological role has remained obscure.

Several experiments pointed towards a role of calpains in α2β1 integrin degradation: calpains were able to degrade α2β1 integrin *in vitro*, there was an increase in calpain activity upon clustering of integrin and calpains were also co-precipitated with α2 integrin. Finally, studies with calpain inhibitors revealed a clear dependency of calpains on integrin dissipation (III). Interestingly, the luminal domain of α2β1 integrin in α2-MVBs was also prone to degradation. This means that the calpains have to somehow penetrate the limiting membrane of the endosome because everything bound to the C-terminus of the integrin will end up inside the intraluminal vesicles. Unpublished data (Soonsawad et al.) indicate that there are discontinuities in the limiting membrane of α2-MVBs upon EV1 infection. This may serve a possible explanation for how calpains are able to attack also the N-terminus of integrins. It has been shown previously that calpains can contribute to the down-regulation of β1 integrin in normal but not prostate cancer cells, in addition to lysosomal degradation (Moro et al. 2004). This suggests that the calpain-associated degradation pathway may exist without EV1-induced clustering. Yet the physiological role of the calpain mediated degradation

pathway presented in the current study can be only speculated on, but the neutrality of the α2-MVBs is probably the key to calpain function. Recent data also suggest that the formation of ILVs is essential for the degradation of clustered integrin (Karjalainen et al. 2011, unpublished). This neutral calpain-mediated alternative to enhanced integrin turn-over may provide the cell with more possibilities to regulate integrin turn-over (III, Fig. 8).

7 CONCLUSIONS

The main conclusions of this thesis are:

1. EV1 entry into the host cell depends on factors associated with macropinocytosis, namely PLC, PI3K, Rac1, Pak1 & is sensitive to the macropinocytosis inhibitor EIPA.

2. EV1 and clustered α2β1 integrin internalize to tubulovesicular structures that quickly mature into non-lysosomal multivesicular bodies. The maturation of these structures is dependent on ESCRT proteins and a functional ubiquitination system.

3. EV1 induced, α2β1 integrin positive multivesicular structures are important for EV1 infection and promote the degradation of integrins in a pH neutral manner. Calpain proteases are present in MVBs and are at least partially responsible for the dissipation of integrins after EV1 induced internalization.

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YHTEENVETO (RESUMÉ IN FINNISH)

Echovirus 1 infektoi isäntäsolun uudenlaisten monirakkulaisten solurakenteiden kautta.

Maapallolla elävien eliöiden ja virusten kehitys on läheisessä suhteessa toisiinsa. Itse asiassa niin läheisessä, että vieläkään ei olla varmoja kumpi oli ensin, virus vai solu? Joka tapauksessa, virukset voivat infektoida kaikkia tunnettuja eliöitä. Virukset ovat pieniä, kymmenistä muutamaan sataan nanometrin kokoluokkaa olevia partikkeleita. Niiden rakenne voi vaihdella suunnattomasti, aina yksinkertaisista symmetrisistä monikulmioista faagien todella monimutkaisiin ulkoasuihin. Kuitenkin kaikilla viruksilla proteiineista muodostuvan kuoren sisään on pakattuna perimäaines, joka sisältää tiedon uusien viruksien rakentamisesta. Virukset eivät kuitenkaan pysty lisääntymään itsenäisesti, vaan tarvitsevat siihen isäntäsolun, joka voi olla mikä tahansa aina bakteerista ihmisen ohutsuolen seinämän soluun. Solussa on luontainen este, solukalvo, joka päästää vain tarkoin säädellysti materiaalia lävitseen. Päästäkseen hyödyntämään solun resursseja uusien virusten valmistamiseen, viruksen täytyy siis jotenkin ohittaa tämä seinämä. Virukset ovatkin oppineet hyödyntämään soluissa luontaisesti esiintyviä mekanismeja, joiden avulla esimerkiksi hormonit ja ravintoaineet pääsevät solun sisään.

Tässä työssä tutkittiin ihmiselle muun muassa aivokalvontulehdusta aiheuttavaa echovirus 1:tä (EV1). Se on pieni, vain 30 nm halkaisijaltaan oleva RNA-virus ja kuuluu pikornavirusteen perheeseen, jonka pahamaineisin edustaja on hyvin tunnettu poliovirus. Tämän väitöskirjan ensimmäisessä osatyössä selvitettiin EV1infektion varhaisia vaiheita – kuinka virus tunkeutuu solukalvon lävitse ja mihin virus kulkeutuu heti solukalvon ohittamisen jälkeen. Soluilla on useita hieman toisistaan eroavia tapoja ottaa pala solukalvoa ja siihen sitoutunutta materiaalia sisäänsä. Näistä parhaiten tunnettuja ovat klatriini- ja kaveolivälitteinen sisäänotto sekä makropinosytoosi. Lisäksi on useita vähemmän tunnettuja mekanismeja. Tässä työssä EV1:n todettiin käyttävän makropinosytoosia soluun tunkeutumiseen. Tämä todettiin, kun muille reiteille tyypilliset säätelijämolekyylit eivät vaikuttaneet EV1:n infektioon. Myöskään kaveoliini- ja klatriinivälitteisille sisäänottomekanismeille tyypillisiä merkkimolekyylejä ei havaittu infektioreitin varrella. Sen sijaan makropinosytoosille ominaiset säätelijät olivat merkittävässä roolissa EV1:n soluun menossa. Lisäksi makropinosytoosin estäminen lääkeaineilla esti myös viruksen sisäänmenon, antaen lisäviitteitä EV1:n käyttämästä reitistä.

Makropinosytoosi liittyy nimensä mukaisesti suurien, jopa 5 µm:n kokoisten partikkelien sisäänottoon. Kun taas useimmat muut, kuten yllämainitut kaveoliini- ja klatriinivälitteiset reitit kykenevät kuljettamaan suurimmillaan vain 100 nm kokoista lastia. Välittömästi EV1:n päästyä solukalvon ohi solulimaan, huomattiin kalvosta kuroutuneiden rakkuloiden olevan suurempia kuin tyypilliset kaveoliini- ja klatriinireittien rakkulat, mikä myöskin viittaa makropinosytoosiin. Mielenkiintoista kyllä, EV1 näytti kuitenkin lopulta, noin kahden tun-

nin jälkeen päätyvän monirakkulaisiin rakenteisiin, jotka muistuttivat hyvin paljon myös klatriini- ja kaveoliinireitiltä löytyviä solurakenteita. Niille on tyypillistä muun muassa hapan pH ja tietynlaiset merkkimolekyylit sekä lipidikoostumus. Toisessa ja kolmannessa osatyössä kuitenkin osoitettiin, että EV1:n aiheuttamat monirakkulaiset rakenteet pysyivät neutraaleina, eikä niihin kertynyt muille reiteille tyypillisiä merkkimolekyylejä. Tämä viittasi siihen, että EV1 kykenee synnyttämään solussa uudenlaisen solurakenteen. Rakenteen muodostuminen myös osoittautui elintärkeäksi viruksen infektiolle – kun rakenteen synty estettiin, myöskin viruksen infektio epäonnistui.

Vaikka EV1:n aiheuttamat monirakkulaiset rakenteet eivät sisältäneet klatriinireitin merkkimolekyylejä, niin samankaltainen ulkonäkö sai pohtimaan, onko perimmäinen solubiologinen koneisto niiden synnyn takana kuitenkin samanlainen? Tätä tutkittiin toisessa osatyössä. Osoittautui, että todellakin perustyökalut ovat yhteisiä. Monirakkulaisten rakenteiden muodostamisesta vastaavat useimmiten ESCRT-proteiinikompleksit, joita on neljä kappaletta. ESCRT:it ovat tulleet tunnetuksi epidermaalisen kasvutekijäreseptorin (EGFR) tutkimuksesta. Kun solukalvolla olevaan EGFR:iin sitoutuu epidermaalista kasvutekijää, reseptori aktivoituu ja alkaa signaloida, aiheuttaen solussa vasteen, joka voi esimerkiksi kiihdyttää solun kasvua. Jatkuvaa signalointia ei kuitenkaan ole tarkoituksenmukaista pitää yllä, joten solu ottaa aktiivisen reseptorin sisäänsä ja suuntaa sen hajotettavaksi happamiin lysosomeihin. Reitti lysosomeihin johtaa välirakenteiden kautta, joita kutsutaan etenemisjärjestyksessä varhaisiksi (EE) ja myöhäisiksi endosomeiksi (LE). EE:t ovat lievästi happamia (pH 6,5 – 6,0), kun taas LE:t ovat astetta happamampia (pH 6,0-5,0) ja lysosomeissa pH laskee jopa 4,5:een. Rakenteiden monirakkulaisuus lisääntyy LE:ja kohti siten, että rakenteisiin muodostuu sisärakkuloita kuroutumalla endosomin kalvolta rakenteen sisään, pois päin solulimasta. Tätä prosessia katalysoivat ESCRT-proteiinit, jotka osallistuvat myös solukalvolta hajotettavaksi suunnatun reseptorin tunnistamiseen ja ohjaamiseen monirakkulaisen rakenteen sisälle. Osatyössä II osoitetaan, että ESCRT:it osallistuvat myös EV1:n aikaansaamien neutraalien monirakkulaisten rakenteiden muodostamiseen, ja että EV1 on ensimmäinen nisäkkäillä tunnettu vaipaton virus, jonka näytetään olevan riippuvainen ESCRT:eista ja niiden synnyttämästä monirakkulaisesta rakenteesta. Työssä käy myös ilmi, että EV1:n aiheuttama rakenne on erillinen EGFR:n käyttämästä reitistä.

Solukalvon proteiinit, jotka suunnataan hajotukseen merkitään liittämällä niiden soluliman puoleiseen osaan pieni proteiini nimeltään ubikitiini. ESCRT-proteiinit tunnistavat ubikitiinin ja osaavat siten ohjata proteiinin oikealle reitille, joka siis johtaa monirakkulaisten rakenteiden kautta lopulta lysosomeihin. Tässä työssä kuitenkin osoitettiin, että EV1:n reseptori $\alpha 2\beta 1$ integriini ei itsessään ubikitinoidu, vaikka yleinen ubikitinaation taso solussa nousee viruksen vaikutuksesta. Joten luultavasti jokin integriiniin liittyvä proteiini merkataan ubikitiinilla. Tätä ei kuitenkaan selvitetty tässä työssä, vaan asia vaatii lisätutkimuksia. Mielenkiintoista kyllä, toimiva ubikitinaatiokoneisto oli tärkeä myös EV1:n käyttämän monirakkulaisen rakenteen synnylle. Lisäksi tulokset osoitti-

vat, että ESCRT:it voivat ohjata ubikitinoituja reseptoreja myös muualle kuin hajotukseen.

Kolmannessa osatyössä jatkettiin EV1:n aiheuttaman monirakkulaisen rakenteen tutkimusta. Erityisesti perehdyttiin viruksen käyttämän reseptorin, α2β1-integriinin kohtaloon. Paljastui, että suuri osa integriinistä hajoaa sisäänmenon jälkeen jo noin kuudessa tunnissa ja vuorokaudessa α2β1 on hävinnyt jo lähes kokonaan solusta. Integriinin häviäminen ei johtunut kierrättämisestä takaisin solukalvolle tai hajotuksesta lysosomeissa tai proteosomeissa, jotka ovat solun kaksi yleisintä tapaa tuhota tarpeettomat tai vioittuneet proteiinit. Sen sijaan kalpaiini-proteaaseilla näytti olevan suuri rooli integriinin häviämisessä. Kalpaiinien fysiologinen rooli ei ole täysin selvä, mutta niiden tiedetään osallistuvan soluvaelluksen ja solusyklin säätelyyn ja hajottavan useita eri proteiineja. Kalpaiineja löytyi integriinin kanssa samoista rakenteista ja niiden osoitettiin olevan yhteydessä integriineihin myös biologisesti. Lisäksi osoitettiin kokeellisesti, että kalpaiini pystyy hajottamaan integriiniä ja kalpaiinit ovat aktiivisia rakenteissa, joihin integriini joutuu soluun sisäänmenon jälkeen.

EV1 käyttää siis makropinosytoosia infektoidakseen solun ja aiheuttaa solussa uudenlaisen rakenteen synnyn, joka on tärkeä viruksen infektion onnistumisen kannalta. Mielenkiintoinen uusi tieto on myös, että tämä rakenne osallistuu kalpaaiinivälitteiseen α2β1 ingeriinin hajotukseen.

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