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Nina Rintanen

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Editors
Varpu Marjomäki
Department of Biological and Environmental Science, University of Jyväskylä
Pekka Olsbo, Ville Korkiakangas
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ABSTRACT

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The small non-enveloped RNA virus and a human pathogen, echovirus 1 (EV1) enters into the cell by clustering its cellular receptors, α2β1 integrins, on the plasma membrane. The clustering induces a macropinocytosis-like uptake process. The aims of the thesis were to define the role of cholesterol in the integrin internalization and EV1 infection and moreover, to characterize the intracellular route and the properties and the maturation of α2 integrin containing structures. In addition to EV1, integrin antibodies and collagen-I were used as inducers of integrin internalization. Normal cholesterol content on the plasma membrane was essential for the uptake of EV1, integrin and collagen as was verified with cholesterol sequestering drugs and by preventing cholesterol synthesis. Furthermore, cholesterol was needed in the maturation of internalized structures and EV1 infection, especially virus uncoating was dependent on intact cholesterol domains. EV1 uncoating started at 30 min post infection inside the α2 integrin structures and virus increased the permeability of the structures clearly after 2 to 3 h. The biogenesis of α2 integrin structures involved the formation of intraluminal vesicles that was dependent on the endosomal sorting complexes required for transport (ESCRTs). Despite the similar appearance of α2 integrin containing multivesicular bodies (α2-MVBs) with the conventional late endosomes, these structures remained separate since they lacked the common markers of the late endosomes and they were not markedly acidified. The clustered integrins avoided recycling, but instead their turnover was enhanced. The degradation was promoted by neutral calpain proteases that were activated and associated with the α2-MVBs, whereas it was independent of the lysosomal hydrolases. The kinetics of the clustered integrin turnover, with half-life under 6 h, was clearly more rapid than that of the unclustered integrins, but still considerably slower compared to the turnover of activated epidermal growth factor receptor.

Keywords: Calpain; cholesterol; degradation; echovirus 1; integrin; multivesicular body.

Nina Rintanen, University of Jyväskylä, Department of Biological and Environmental Science/Nanoscience Center, P.O. Box 35, FI-40014 University of Jyväskylä, Finland.

Author's address Nina Rintanen
Department of Biological and Environmental Science/
Nanoscience center
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
nina.rintanen@jyu.fi

Supervisors Docent Varpu Marjomäki, Ph.D.
Department of Biological and Environmental Science/
Nanoscience center
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Reviewers Docent Tero Ahola, Ph.D.
Institute of Biotechnology
P.O.Box 56
00014 University of Helsinki
Finland

Professor Pekka Lappalainen, PhD
Institute of Biotechnology
P.O.Box 56
00014 University of Helsinki
Finland

Opponent Docent Vesa Olkkonen, Ph.D.
Minerva Institute for Medical Research
Biomedicum Helsinki 2U
Tukholmankatu 8
00290 Helsinki
Finland

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LIST OF ORIGINAL PUBLICATIONS

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

I 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. *Cellular Microbiology*, 13: 1975-1995.

II Rintanen N., Karjalainen M., Alanko J., Paavolainen L., Mäki A., Nissinen L., Lehkonen M., Kallio K., Cheng R.H., Upla P., Ivaska J., Marjomäki V. 2012. Calpains promote α2β1 integrin turnover in nonrecycling integrin pathway. *Molecular Biology of the Cell*, 23:448-463.

III Siljamäki E. *, Rintanen N. *, Kirsi M., Upla P., Wang W., Karjalainen M., Ikonen E. and Marjomäki V. 2012. Cholesterol dependence of collagen and virus uptake and trafficking along the novel α2β1 integrin internalization pathway. Submitted manuscript.

*Equal contribution

RESPONSIBILITIES OF NINA RINTANEN IN THE ARTICLES OF THIS THESIS

Article I: I performed the thin-frozen section labelings and participated in imaging of the samples. I was responsible for the LBPA and LDL experiments. I participated in finalizing the article together with Mikko Karjalainen and Varpu Marjomäki.

Article II: I was responsible for HRP-based degradation assays, [^{35}S]-based integrin degradation assays, proteasome inhibition experiments, colocalization assays and EM studies. I performed autophagosome studies with the exception of the starvation study with EV1, which was done by Moona Lehkonen. Immunoisolation and -blotting experiments for calpain labelings were done together with Paula Upla and Liisa Nissinen. I prepared the samples for t-BOC assay. Anti-Alexa488 based recycling assays were done together with Anita Mäki and bleaching experiments with Varpu Marjomäki. Fluorescence based degradation assays and analyses were done together with Mikko Karjalainen and Anita Mäki. I performed pH measurements together with Varpu Marjomäki and Katri Kallio and conducted analyses together with Katri Kallio. I wrote of the article together with Varpu Marjomäki and processed all the figures.

Article III: I was responsible for collagen internalization assays and EM-imaging. I performed EV1 infection assays with U18666A, Fumonisin B_1 and mβCD-cholesterol treated cells. I prepared and imaged the filipin and dsRNA-labelled samples. I wrote the article together with Varpu Marjomäki and Elina Siljamäki and prepared the figures together with Elina Siljamäki.

All studies in this thesis were performed under the supervision of Docent Varpu Marjomäki.

ABBREVIATIONS

α2-MVB	α2 integrin containing multivesicular body
Alix	ALG-2-interacting protein X
Arf	ADP-ribosylation factor
Atg	autophagy
β_2-AR	β_2-adrenergic receptor
BSA	bovine serum albumin
CAV9	coxsackievirus A9
CI-MPR	cation-independent mannose-6-phosphate receptor
CD63	CD63 antigen
DENV	dengue virus
DMEM	Dulbecco's modified Eagle's medium
DN	dominant-negative
DRM	detergent resistant membranes
DUBs	deubiquitinating enzymes
ECM	extracellular matrix
EE	early endosome
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EIPA	5-(N-ethyl-N-isopropyl)amiloride
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESCRT	endosomal sorting complex required for transport
EV1	echovirus 1
FA	focal adhesion
Fab	antigen binding fragment
FAK	focal adhesion kinase
FMDV	foot-and-mouse disease virus
FYVE	domain conserved in Fab1, YOTB, Vac1 and EEA1
GEFs	guanine-nucleotide-exchange factors
GPI-AP	glycophosphatidylinositol anchored protein
GTPase	guanosine triphosphate hydrolyzing enzyme
HCMV	human cytomegalovirus
HPEV1	human parechovirus 1
HPV-16	human papillomavirus type-16
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
ILVs	intraluminal vesicles
JEV	Japanese encephalitis virus
Lamp	lysosome-associated membrane protein
LBPA	lysobisphosphatidic acid
LDLR	low-density lipoprotein receptor
LE	late endosome

LPDS	lipoprotein-deficient serum
LY	lysosome
mβCD	methyl-β-cyclodextrin
MHC	major histocompatibility complex
MMPs	matrix metalloproteinases
MPR	mannose-6-phosphate receptor
MT1-MMP	membrane-type 1 matrix metalloproteinase
MVB	multivesicular body
NOX2	NADPH oxidase 2
NPC	Niemann-Pick type C
ORP	oxysterol-binding protein related proteins
OSBP	oxysterol binding protein
p.i.	post infection
PI3K	phosphatidyinositol 3-kinase
PI3P	phosphatidylinositol 3-phosphate
$PI(3,5)P_2$	phosphatidylinositol 3,5-bisphosphate
PKC	protein kinase C
PTD	phosphotyrosine like domain
Rab	Ras-related in brain
Rac	Ras-related C3 botulinum toxin substrate
RE	recycling endosome
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
t-BOC	7-amino-4-chloromethylcoumarin,t-BOC-l-leucyl-l-methionine amide
Tf	transferrin
TfR	transferrin receptor
TGN	trans-Golgi network
TRPML1	Transient receptor potential mucolipin 1
TSG101	tumor susceptibility gene 101 protein
U18666A	3-β-[2-(diethylamino)ethoxy] androst-5-en-17-one
uPARAP	urokinase-type plasminogen activator receptor associated protein
V-ATPase	vacuolar H^+ ATPase
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2
VP	viral protein
VPS	vacuolar protein sorting-associated protein
VSV	vesicular stomatitis virus
VTA1	vacuolar protein sorting -associated Vta1 homolog protein
WNV	West Nile virus

1 INTRODUCTION

Cell biology has gone through extensive progress in knowledge during the last decades, which has been interrelated to the advances and availability of new methods and techniques. For example microscopic techniques have improved greatly due to the development of more elegant microscopes and dyes, but also because of efficient image analysis. Microscopy is a central tool in investigating cellular pathways such as endocytosis. Clathrin-mediated endocytosis was long thought to be the only mechanism for cells to internalize material. It was only in the 1990s, when it finally became proven and accepted that endocytosis can proceed without clathrin. Nowadays several different endocytic mechanisms have been found, although many details of the pathways are still unclear. Viruses have been important tools in studying endocytosis since they exploit the existing cellular pathways for their entry into the cells. On the other hand, many viruses are pathogens that can cause severe infections in humans as well as in animals and plants. Integrins are key mediators of cell adhesion and they are also linked to several pathological conditions as in cancer or thrombosis. Therefore the understanding of endocytic pathways of viruses and integrins is important for the development of new therapies and it simultaneously helps us to understand the cellular functions in general.

The aim of this thesis was to increase the knowledge of the endocytic pathway of echovirus 1 (EV1) and its receptor α2β1 integrin. The study provided new aspects about the importance of lipids in this pathway and in EV1 infection. The clustering-triggered integrin endocytosis route revealed an endocytic structure with unique characteristics which is essential for EV1 infection. Furthermore, the study shed light on the integrin trafficking and turnover mechanisms within the cell in general.

2 REVIEW OF THE LITERATURE

2.1 Integrins

Integrins are transmembrane receptors on the plasma membrane that mediate cell adhesion to the extracellular matrix (ECM) or to other cells. In addition, integrins can recruit various proteins at their intracellular tails for establishing connections to the cytoskeleton, and thus mediate signals through the plasma membrane in both directions (van der Flier & Sonnenberg 2001, Hynes 2002). This property of cells to interact with their environment bidirectionally makes integrins essential molecules for example in development, immune responses, wound healing and in many diseases like cancer (Hynes 2002). Integrins are heterodimers consisting of α and β subunits with no covalent linkages between them (Hynes 2002). In mammals, 18 α and 8 β subunits can form 24 different integrin receptors that can bind various ligands. Most integrins bind to components of the ECM like collagen, laminin, fibronectin and vitronectin but some integrins bind to their counter receptors on other cells (e.g. intercellular or vascular cell adhesion molecule 1), plasma proteins (e.g. fibrinogen, von Willebrandt factor) or complement factors (e.g. inactivated complement 3b) (van der Flier & Sonnenberg 2001). In addition to physiological ligands, many viruses use integrins as their receptors (Stewart & Nemerow 2007).

2.1.1 Structure

Integrin α and β chains consist of large ectodomains, single helical transmembrane domains and small intracellular domains (Fig. 1). Crystal structures of the ectodomains of αVβ3 (Xiong et al. 2001), αIIbβ3 (Zhu et al. 2008) and αxβ2 integrin (Xie et al. 2010) have been determined. The ectodomain of α subunit consist of four or five domains: β-propeller, tight domain as well as two calf domains, and nine of the integrins contain also the fifth domain called I domain (Luo et al. 2007). For integrins containing this domain, the αI domain is the ligand binding site. It has a metal-ion dependent adhesion site that is essential for ligand binding (Lee et al. 1995a). The β subunit contains also its

own I domain that is inserted in the hybrid domain. These domains are followed by the plexin-semaphorin-integrin (PSI) domain and by four epidermal growth factor (EGF) domains as well as the β tail domain (Luo et al. 2007, Campbell & Humphries 2011). In integrins lacking the αI domain, ligand binds to the interface between the βI domain and the β-propeller. These domains also contain metal ion binding sites that have an influence on ligand binding.

In addition to domain-based division, ectodomains are often divided according to the shape of the molecule to the head part and to the upper as well as the lower legs (Fig. 1). Between the upper and lower legs there are flexible linkages (knees or genu) that allow conformational changes of integrin between bent and extended modes (Luo et al. 2007, Campbell & Humphries 2011). These conformational states are suggested to be essential for regulating the affinity for the ligands (Takagi et al. 2002). In addition to overall conformational changes of integrin subunits, conformational changes occurs also within the I domains. The αI domain exist naturally in two conformations: closed with low-affinity and open with high-affinity (Lee et al. 1995b). In addition to closed and open conformations, a transition conformation with ligand-bound and low-affinity, between these states has been described with βI domain (Takagi et al. 2002, Xiao et al. 2004).

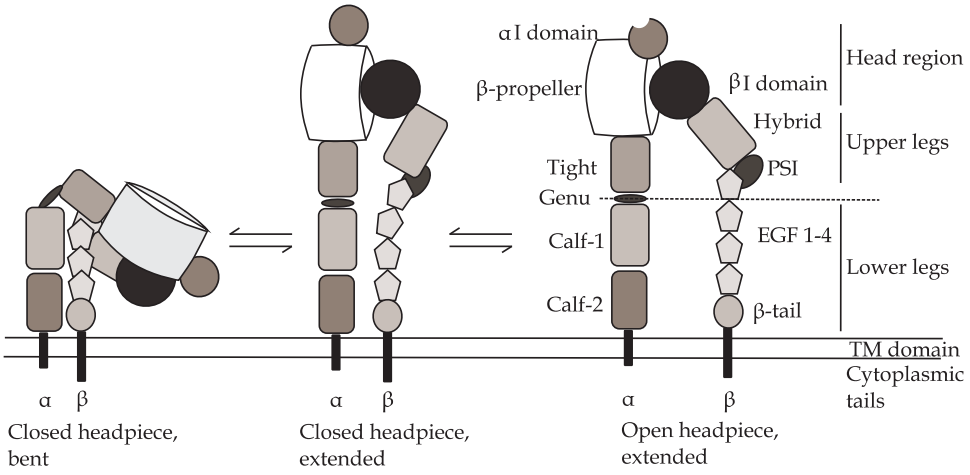

FIGURE 1 Integrin structure and conformational stages. Inactivated integrin is in a bent conformation with a low-affinity for ligand. When activated, integrin becomes extended, the legs are separated and the ligand binding site is in a high-affinity state. An intermediate conformation that shows an extended conformation, but low-affinity for a ligand is also shown. Modified from Luo et al. 2007 and Campbell & Humphries 2011.

The ectodomains are connected to the transmembrane helices through flexible linkers (Campbell & Humphries 2011). Transmembrane domain packing (Partridge et al. 2005) and disulfide bonds at the interface (Luo et al. 2004) are suggested to stabilize the integrin to a low-affinity state. The interactions

between the cytoplasmic tails are predicted to be weak in the resting state (Vinogradova et al. 2002, Weljie et al. 2002). Salt bridges are suggested to keep the β3 integrin tails in low-affinity state (Hughes et al. 1996), whereas they were not essential for β1 integrin (Czuchra et al. 2006). However, there is a consensus that integrin activation causes separation of integrin legs and cytoplasmic tails (Vinogradova et al. 2002, Kim et al. 2003, Luo et al. 2004).

2.1.2 Activation

Integrins can be found in an active or inactive conformation, which reflects their affinity for a ligand. During the development of organisms, cell migration is an essential and highly regulated process and integrins play a central role in it. Active integrins are needed in the leading edge of a migrating cell to make new contacts with the ECM while integrins are inactivated at the rear to break the old connections (Moser et al. 2009). Regulation of integrin activation is also required for proper functions of blood cells. For example, a common platelet integrin, αIIbβ3, meets constantly its ligand, fibrinogen (Hynes 2002, Moser et al. 2009). Normally, αIIbβ3 integrin is in an inactive conformation but injury leads to its activation that causes ligand binding and aggregation of platelets to stop the bleeding. Incorrect activation of integrins would thus lead to pathological thrombosis.

Integrin receptors are bidirectional which means that the receptor can be activated and transfer signals from both of its ends (Hynes 2002). Ligand binding to the ectodomain or protein binding to the cytoplasmic tail causes leg separation and conformational change of the integrin molecule that mediates signal transfer across the plasma membrane. Regulation of integrin affinity for their ligands by intracellular signals is called inside-out signaling. Integrins can be activated by talin (Calderwood et al. 1999) as it binds to the β tail (Horwitz et al. 1986, Pfaff et al. 1998, Calderwood et al. 1999). In addition to talin, kindlin also can function as an integrin activator (Moser et al. 2009). Besides integrin activators, negative regulators are also needed. For example phosphorylation of integrin β tail increases the affinity of docking protein 1 to integrin and competes with talin binding (Oxley et al. 2008). Phosphorylation can also reduce kindlin binding and thus reduce activation of integrins (Bledzka et al. 2010). Filamin can also regulate integrin activation negatively since filamin and talin have overlapping binding sites on integrin (Harburger & Calderwood 2009).

Outside-in signaling starts with ligand binding to the integrin ectodomain (Ginsberg et al. 2005, Legate et al. 2009, Shattil et al. 2010). The property of integrins to form clusters is significant for integrin function, especially for triggering outside-in signaling, integrin recycling and mechanotransduction (Legate et al. 2009, Shattil et al. 2010). Whether clustering is induced with conformational changes or if multivalent ligand binding starts the clustering leading to conformational changes is still unclear. Since integrins do not have enzymatic functions of their own, recruitment of enzymes like the focal adhesion kinase (FAK) and proto-oncogene tyrosine-protein kinase Src are

needed to activate signaling cascades (Ginsberg et al. 2005, Mitra & Schlaepfer 2006, Legate et al. 2009). Also other adaptor proteins e.g. paxillin, vinculin and talin are assembled as mediators to connect the ECM to the cytoskeleton and for formation of focal adhesions (FAs). Extracellular ligation can cause various signaling cascades leading e.g. to cell adhesion, proliferation, survival, apoptosis or motility.

2.1.3 Trafficking

Cell migration, proliferation and apoptosis are examples of situations when adhesion contacts need to be re-formed in cells. Already earlier studies showed that many integrins (α5β1, α6β4, Mac-1) but not all (α3β1, α4β1, LFA-1) are continuously endocytosed and recycled back to the plasma membrane (Bretscher 1989, Bretscher 1992). This led to a model where integrins are recycled from the rear to the front of the motile cell, and thereby support cell movement by providing fresh adhesion receptors to the leading edge of the cell (Fig. 2) (Pellinen & Ivaska 2006).

Integrin internalization can occur both by clathrin-mediated and clathrin-independent mechanisms (Caswell et al. 2009). For example α5β1 integrin is internalized in a caveolin-1 dependent manner in a route leading to fibronectin turnover (Shi & Sottile 2008), and the leucocyte specific integrin, αLβ2, is endocytosed via clathrin-independent pathway from detergent resistant membranes (DRMs) (Fabbri et al. 2005). αVβ3 is associated with membrane-type 1 matrix metalloproteinase (MT1-MMP) and caveolin-1 in endothelial cells (Galvez et al. 2004), but in Hela cells it is internalized in a clathrin-dependent manner (Nishimura & Kaibuchi 2007).

A recent study showed that freely diffusing β1 integrins that are not associated to FAs, associate with clathrin coated pits and are endocytosed with disabled homolog 2 (Teckchandani et al. 2009). This kind of constant endocytosis could thus provide cells an internal storage of integrins for new adhesion sites. However, β1 integrin internalization has also been shown to be coupled to FA disassembly (Ezratty et al. 2005, Chao et al. 2010, Wang et al. 2011). Disassembly is induced by microtubules and mediated by FAK and dynamin-2 (Ezratty et al. 2005). Also another kinase, type I phosphatidylinositol phosphate kinase β is detected as a regulator of disassembly (Chao et al. 2010). Other known regulators of FA disassembly are calpains. Proteolysis of talin, FAK and paxillin by calpain is critical for FA disassembly (Franco et al. 2004, Chan et al. 2010, Cortesio et al. 2011). Internalization and vesicular trafficking of integrins along microtubules is highly regulated by the Rab (Ras-related in brain subfamily) and Arf family GTPases, guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins in addition to several kinases. β1 integrin endocytosis is described to be regulated with protein kinase C (PKC) α that associates with the cytoplasmic β tail (Ng et al. 1999). Other regulators, Rab5 and Rab21 associate with the α2 tail in MDA-MB-231 cancer cells and allow integrin internalization with the help of Rab5/21 GEFs (Pellinen et al. 2006). Futhermore, ADP-ribosylation factor 6 (Arf6) can also function as a

regulator of β1 integrin internalization and recycling from the endosomes. BRAG2, a GEF of Arf6, was shown to activate Arf6-GDP upon β1 internalization (Dunphy et al. 2006). Arf6-GTP hydrolysis is later needed for recycling of integrin from endosomes back to the plasma membrane (Brown et al. 2001).

After internalization by clathrin-dependent or independent mechanism, integrins end up in the early endosomes (EEs). Receptors destined for degradation are guided to multivesicular bodies and recycling receptors are transported back to the plasma membrane directly from Rab4-positive structures (short loop) or through the perinuclear Rab11-positive recycling endosomes (RE, long loop) (Caswell & Norman 2006, Pellinen & Ivaska 2006). αVβ3 and α5β1 are transported through Rab4 containing endosomes to perinuclear Rab11-positive recycling endosomes from where they are recycled back to plasma membrane in 3T3 fibroblasts (Roberts et al. 2001). Recycling of αVβ3 and α5β1 from Rab11 containing endosomes is regulated by serine/threonine kinase Akt (Roberts et al. 2004). Also other β1 integrins are shown to accumulate in perinuclear, Rab11- positive recycling compartments in starved Hela and MDA-MB-231 cells and recycle back to plasma membrane in Arf6-dependent manner (Powelka et al. 2004). Afr6 is stimulated with GTPase-activating protein, ACAP1 that is further activated by Akt phoshopylation (Li et al. 2005). PKCε has also been reported to regulate β1 integrin exit from endosomes (Ivaska et al. 2002) by phosphorylating the intermediate filament binding protein vimentin in fibroblasts (Ivaska et al. 2005).

Less is known about the short loop recycling of integrins, which may be associated with growth factor activation. Platelet-derived growth factor stimulus addresses αVβ3 to rapid, Rab4-mediated recycling, but does not have an effect on α5β1 recycling rate (Roberts et al. 2001). In addition to Rab4, this short loop recycling is dependent on the PKC effector protein kinase D1, and promotes αVβ3 recycling to the newly made FAs (Woods et al. 2004). αVβ3 also controls α5β1 trafficking since inhibition of αVβ3 integrin increases Rab11 dependent α5β1 integrin recycling rate and leads to random cell migration (White et al. 2007). In addition, inhibition of αVβ3 integrin function stimulates epidermal growth factor receptor (EGFR) recycling, and promotes cell migration (Caswell et al. 2008). It also enhances tumor angiogenesis since it normally regulates negatively vascular endothelial growth factor (VEGF) - induced angiogenesis by controlling vascular endothelial growth factor receptor-2 (VEGFR-2) and neuropilin-1 association (Robinson et al. 2009). These thus indicate that αVβ3 integrin has an important role in suppressing other integrins and receptor tyrosine kinases that promote cell migration.

2.1.4 Degradation

Although integrin internalization and recycling are widely studied, little is known about the degradation of integrins. Integrins seem to be quite stabile molecules with varying half-lives from 8 h to 32 h depending on the cell type (De Strooper et al. 1991, Dalton et al. 1992, Delcommenne & Streuli 1995, Moro

et al. 2004, Lobert et al. 2010). Remodelling of the ECM is required especially during organogenesis, angiogenesis and in invasion of cancer cells. The ECM can be degraded extracellularly by matrix metalloproteinases (MMPs) and urokinase plasminogen activator system (Yue et al. 2012) or after endocytosis intracellularly (Panetti et al. 1995, Engelholm et al. 2003, Kjoller et al. 2004, Shi & Sottile 2008). Recent studies have revealed that MT1-MMP can have a regulative role for ligand and receptor endocytosis (Lee et al. 2007, Messaritou et al. 2009, Shi & Sottile 2011). For example, MT1-MMP can regulate α5β1 integrin endocytosis by its proteolytic activity on fibronectin fibrils (Shi & Sottile 2011). Furthermore, plasma membrane receptors can regulate ECM endocytosis too, such as integrins and urokinase-type plasminogen activator receptor associated protein (uPARAP) (Panetti & McKeown-Longo 1993, Engelholm et al. 2003). For example, integrins are able to regulate the ECM endocytosis like α5β1 with fibrillar fibronectin (Shi & Sottile 2008), β1 integrin with fibrillar collagen (Shi et al. 2010) and αVβ5 with vitronectin (Panetti & McKeown-Longo 1993, Panetti et al. 1995). In addition, phagocytic uptake of collagen beads is β1 integrin-mediated (Arora et al. 2003). In contrast, endocytosis of soluble collagen is mediated by uPARAP through a non-phagocytic route and this uptake is not dependent on β1 integrin (Shi et al. 2010, Madsen et al. 2011). Currently only one article proposes that the integrin itself can be ubiquitinated and targeted to lysosomal degradation when induced with soluble fibronectin (Lobert et al. 2010), while another study showed that soluble fibronectin stimulates Rab25-mediated recycling of α5 integrin in another cell line (Caswell et al. 2007).

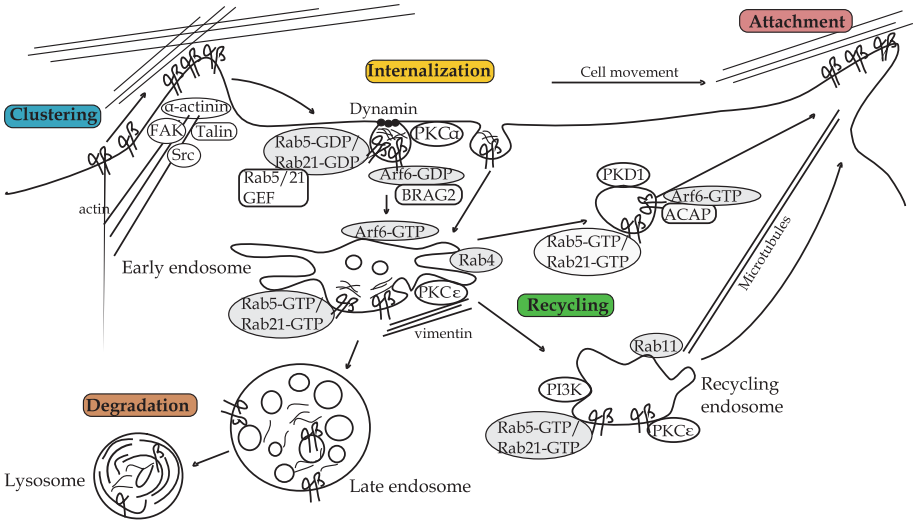

FIGURE 2 Integrin trafficking in migrating cells. Clustering of integrins induces the activation and attachment to ECM and formation of FAs. Disassembly of FAs is coupled to integrin internalization. Endocytosed integrin is either recycled via short or long loop or targeted for degradation. PKD1, protein kinase D1; Src, proto-oncogene tyrosine-protein kinase. Modified from Pellinen & Ivaska 2006.

2.1.5 Integrins as virus receptors

Many viruses have learned to use integrins as their primary attachment receptors or as co-receptors for cell entry. It is no wonder, because integrins are abundant in many cell types and they can mediate signaling events to promote virus entry. Viruses have been also used as tools to study integrins since viruses are thought to use existing cellular pathways rather than create their own.

Both enveloped and non-enveloped viruses are able to exploit integrins for internalization. As an example, enveloped human cytomegalovirus can use α2β1, α6β1 and αVβ3 integrins as entry receptors (Feire et al. 2004, Isaacson & Compton 2009) after initial binding by heparin sulfate (Compton et al. 1993). Also the non-enveloped adenoviruses (serotype Ad2 and Ad3) use αV integrins as their co-receptors (Meier & Greber 2004). In addition, many picornaviruses, including EV1, utilize integrins in their entry to the cell. It is suggested that EV9 binds only αVβ3 integrin (Nelsen-Salz et al. 1999), whereas foot-and-mouse disease virus (FMDV) is able to interact with several integrins (αVβ1, αVβ3, αVβ6, αVβ8) (Berinstein et al. 1995, Jackson et al. 2002, Duque et al. 2004, Jackson et al. 2004). FMDV capsid contains the arginine-glycine-aspartic acid sequence that is essential for integrin binding (Fox et al. 1989). It is this sequence that causes FMDV as well as integrin to be endocytosed in a clathrin-mediated way and traffick to EEs and to some extent to REs (Berryman et al. 2005, O'Donnell et al. 2005, Johns et al. 2009). Furthermore, Coxsackievirus A9 (CAV9) and human parechovirus 1 (HPEV1) use αVβ3 and αVβ6 integrins as its receptors (Roivainen et al. 1994, Triantafilou et al. 1999, Triantafilou et al. 2000, Triantafilou et al. 2001, Williams et al. 2004, Heikkilä et al. 2009, Seitsonen et al. 2010), nevertheless CAV9 also needs an additional receptor, β2-microglobulin for entry (Triantafilou et al. 1999, Heikkilä et al. 2010).

2.2 Echovirus 1

Echoviruses belong to the family *Picornaviridae* and further to *Enterovirus* genus (Knowles et al. 2011). Genera are divided to species, and EV1 is one of the 60 serotypes among the *human enterovirus B species*. Enteroviruses cause many illnesses to humans with varying symptoms (Muir et al. 1998, Sawyer 2002). Mostly infections are mild causing fever, skin or mucosal membrane symptoms as in hand-foot-mouth disease or in herpangina. However, sometimes enteroviral infections can cause severe illnesses, such as with coxsackievirus and poliovirus being responsible for acute human myocarditis (Martin et al. 1994, Dennert et al. 2008, Andreoletti et al. 2009). Additionally, enteroviruses are described to be involved also in chronic cardiac muscle diseases (Klingel et al. 1992, Kandolf et al. 1999, Andreoletti et al. 2000) and in the genesis of type 1 diabetes (Ylipaasto et al. 2004, Dotta et al. 2007). Sometimes enteroviruses, like poliovirus and coxsackie- or echoviruses, are able to pass the central nervous

system barrier and cause severe infections as paramyelitis and meningitis, respectively (Rhoades et al. 2011).

2.2.1 Structure and life cycle

The fine structure of many picornaviruses, including poliovirus 1 (Hogle et al. 1985, Rossmann et al. 1985), human rhinovirus 14 (Rossmann et al. 1985), FMDV (Acharya et al. 1989) and EV1 (Filman et al. 1998) has been defined by crystallographic methods, and they all share similarities in their capsid structure. Viral protein (VP) 1, VP2 and VP3 form the outer surface of the icosahedral capsid and VP4 is buried inside the capsid. These four proteins form the protomers, which are assembled as pentamers around the 5-fold axis so that VP1 is connected to 5-fold axis and VP2 and VP3 alternate around the 3-fold axes (Fig. 3). Together 12 pentamers are needed to make the intact capsid with an approximate of 30 nm diameter. VP1, VP2 and VP3 form similar kind of structures that contain eight stranded beta barrel fold with two helices (Hogle et al. 1985, Rossmann et al. 1985). They differ mainly in loops connecting the strands and at the amino and carboxyl ends of the proteins. N-terminus of VP4 is myristylated in many picornaviruses (Chow et al. 1987), and it has been suggested that this modification might be needed for capsid assembly (Marc et al. 1989). Most picornaviruses have a canyon between VP1 and VP3 that is used as a binding site of a receptor, and the base of the canyon can be occupied with a cell-derived fatty acid (a pocket factor) that is thought to stabilize the virus (Rossmann et al. 2002).

Picornaviruses have single-stranded (+) RNA genomes with a length of 7-8.5 kb (Bedard & Semler 2004, Tuthill et al. 2010). Both ends of the genome have noncoding sequences and the 5'-end is linked to a small basic protein, VPg, and the 3'-end contains a polyA-tail. The genome contains a single open reading frame that encodes a polyprotein. The polyprotein is processed into precursor proteins that are further cleaved into capsid proteins, non-structural proteins and enzymes needed for replication. The genomic RNA can be directly used as an mRNA for translation after uncoating and release of the genome to the cytoplasm. In the replication process, the genome is first copied to a complementary (-) RNA strand that is used as a template for producing viral mRNA. Viral mRNA can be used in translation of new viral proteins and as genomic RNA for new virions. The produced viruses are finally released by cell lysis.

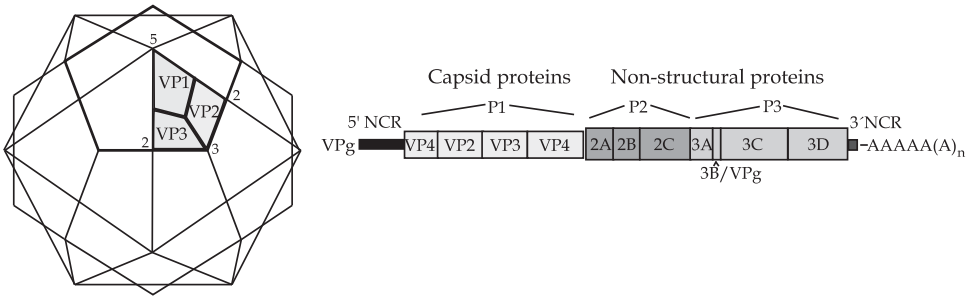

FIGURE 3 Structure of picornaviral capsid and genome. The icosahedral capsid consists of 60 copies of protomers that contain four structural proteins (VP1-4). VP4 is buried inside the capsid. Symmetry axes are marked with numbers. In the genome, the single open reading frame is flanked by noncoding regions (NCR) at both ends. VPg is a protein primer needed for RNA synthesis. P1-3 are precursor proteins that are further processed to capsid and non-structural proteins. Non-structural proteins (2A-3D) are required for the replication. Modified from Hogle et al. 1985 and Bedard & Semler 2004.

2.2.2 α2β1 integrin as the EV1 receptor

EV1 uses α2β1 integrin (also called as VLA-2) as its receptor (Bergelson et al. 1992). α2β1 integrin is a transmembrane receptor on the plasma membrane and it mediates cell attachment to the ECM through collagen and laminin (Elices & Hemler 1989, Emsley et al. 1997). The EV1 binding site is shown to be different from the ECM ligands, since different monoclonal antibodies inhibit virus and collagen binding (Bergelson et al. 1993). Furthermore, virus binding is cation independent (Bergelson et al. 1993, King et al. 1997) and binding is not increased with PKC activator, phorbol 12-myristate 13-acetate or β1 antibody (TS2/16) treatment as seen with collagen (Bergelson et al. 1993). EV1 and echovirus 8 were shown to bind specifically to α2 subunit of the receptor (Bergelson et al. 1993, King et al. 1995). EV1 interacts with the I domain of the α2 subunit and mutation studies revealed that amino acids 199-201 and 212-216 are involved in virus attachment (King et al. 1997). Defining the crystal structure of α2β1 integrin I domain revealed that these residues are located at a different site than the collagen binding site (Emsley et al. 1997). Despite the fact that EV1 and collagen bind to different sites of the I domain, they cannot bind α2 integrin simultaneously based on a cryo-electron microscopy model (Xing et al. 2004). In fact, it was shown that EV1 has a much higher affinity to the receptor than collagen type 1 has, and thus the receptor favors EV1 binding (Xing et al. 2004). Recently it was also suggested that EV1 favors the closed conformation of integrin in its binding whereas collagen binds to activated integrins (Jokinen et al. 2010).

2.2.3 Entry

EV1 binding to α2β1 integrin causes a lateral movement of integrins on the plasma membrane and results in clustering of the receptors (Fig. 4) (Upla et al.

2004). A molecular model generated has suggested that the 5-fold axis of the virus particle is able to bind up to 5 integrin heterodimers at the same time without steric hindrance, thereby enabling the clustering (Xing et al. 2004). Clustering leads to internalization of the virus and its receptor, and requires PKCα activation (Upla et al. 2004, Jokinen et al. 2010). Uptake of EV1 resembles macropinocytosis since EV1 is also internalized in vesicles that accumulate with fluid phase markers. Entry can be blocked by inhibiting the function of C-terminal-binding protein-1/brefeldinA-ADP ribosylated substrate (Liberali et al. 2008), p21-activated kinase 1, Ras-related C3 botulinum toxin substrate (Rac) 1, phospholipase C and amiloride sensitive Na+/H+ exchanger with 5-(N-ethyl-N-isopropyl)amiloride (EIPA), all of which are regulators of macropinocytosis (Karjalainen et al. 2008). EV1 internalization is independent of flotillin-1, glycophosphatidylinositol-anchored proteins (GPI-APs), clathrin and caveolin-1 routes (Pietiäinen et al. 2004, Upla et al. 2004, Karjalainen et al. 2008), however caveolin is shown to accumulate gradually to EV1 structures, but only after internalization (Pietiäinen et al. 2004, Karjalainen et al. 2008).

After the first minutes of uptake (5-15 min), EV1 is found in tubulo-vesicular structures that mature quickly into multivesicular bodies (MVBs) from 15 min to 2 h post infection (p.i.) (Karjalainen et al. 2008). Virus uncoating starts at 30 min p.i. inside these structures (Marjomäki et al. 2002, Pietiäinen et al. 2004), but the release of the genome is thought to occur later since replication begins only after 3 h p.i. (Upla et al. 2008).

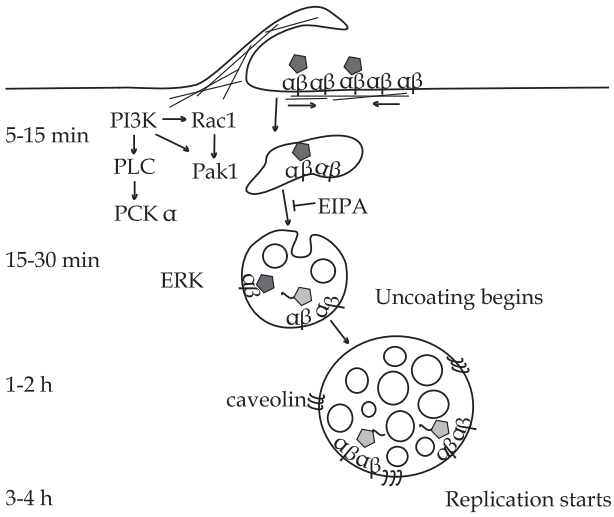

FIGURE 4 EV1 entry pathway. Virus binding induces the clustering of integrins at the plasma membrane and internalization in a process that involves activation of the regulators of macropinocytosis. Internalized structures mature into α2 integrin containing multivesicular bodies (α2-MVB) that contain both the virus and the receptor. ERK, extracellular signal-regulated kinase. Pak1, p21-activated kinase 1; PI3K, phosphatidyinositol 3-kinase; PLC, phospholipase C. Modified from Karjalainen et al. 2008.

2.3 Fate of endocytosed material

The plasma membrane forms a border between the extracellular and intracellular environments. However, these two worlds are not separate but communicate with each other. The function of the cell is guided by the signals that originate from extracellular events, but also the other way around exist in that intracellular signals can modify the cell in relationship with its environment. This communication between the two environments often involves endocytosis. Endocytosis means the uptake of extracellular fluid, solutes and molecules as well as plasma membrane components and particles inside the cell. In the processs, invaginations of the plasma membrane are formed, which are then budded into cytosolic vesicles (Huotari & Helenius 2011). Endocytosis can be continuous or it can be triggered by binding of a specific ligand (Mayor & Pagano 2007). With endocytosis, the cell can control e.g. the amount of transmembrane receptor for a certain ligand, regulate cell migration, cytokinesis or take nutrients from the outside.

There are numerous different pathways that deliver material inside the cell (Fig. 5) (Mayor & Pagano 2007, Mercer et al. 2010). They can be divided to either phagocytosis, which mediates large particle uptake, or pinocytosis mediating the uptake of fluid, solutes and smaller molecules. Pinocytic pathways can be further divided to macro- and micropinocytosis. In macropinocytosis large amounts of extracellular fluid is taken inside the cell and the formed vesicles are much larger than in micropinocytic pathways. Best characterized mechanisms are clathrin- and caveolin-mediated endocytosis and macropinocytosis, but there are many other less well-characterized routes such as Arf6, flotillin and interleukine-2 pathways. Pathways can be classified in many ways on the basis for requirement of different cellular factors like lipid composition, actin involvement, regulators or coat proteins (Mayor & Pagano 2007, Mercer et al. 2010).

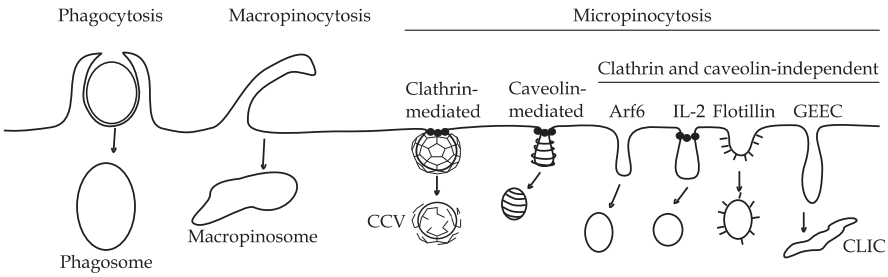

FIGURE 5 Cellular uptake mechanisms. Phagocytosis mediates internalization of larger particles whereas macropinocytosis is involved in the uptake of larger amounts of fluid. Micropinocytic uptake is divided into several pathways in the basis of coat or regulatory proteins needed. CCV, clahtrin coated vesicle; CLIC, clathrin independent carrier; GEEC, GPI-anchored protein enriched early endosomal compartment; IL-2, interleukin-2. Modified from Mayor & Pagano 2007 and Mercer et al. 2010.

2.3.1 Endocytic structures

Despite the endocytotic pathway chosen on the plasma membrane, all internalized vesicles are believed to fuse with the early endosomal compartment, which is a sorting station for endocytosed material (Mellman 1996, Gruenberg 2001, Huotari & Helenius 2011). However, according to recent studies there seem to be different subpopulations of EEs (Miaczynska et al. 2004, Lakadamyali et al. 2006), and sorting for recycling could start already at the plasma membrane (Lakadamyali et al. 2006). Most of the endocytosed material is recycled back to the plasma membrane, but some of the material is sorted to the Golgi or to late endosomes (LE) that targets material mainly for degradation (Fig. 6) (Huotari & Helenius 2011). In fibroblasts for example, 50% of their surface area is internalized and 5-10% of their volume is trafficked through by pinocytic activity per hour (Steinman et al. 1983). This means that most of the membrane and proteins have to be recycled back to plasma membrane to avoid cell shrinkage. Indeed, many plasma membrane receptors have a long lifetime compared to their ligands and they avoid degradation. Quantifications with the low-density lipoprotein receptor (LDLR) have shown that the half-life of the receptor is about 25 h, but the half-life of the ligand is only 5 min (Steinman et al. 1983).

2.3.1.1 Early endosomes and recycling endosomes

Typical early endosomal structures are tubulovesicular structures found in the cell periphery (Yamashiro & Maxfield 1987, Griffiths et al. 1989, Tooze & Hollinshead 1991, Gruenberg 2001, Sachse et al. 2002). Long and narrow tubules emerge from the vesicular body, which can already contain a few intraluminal vesicles (ILVs). Depending on the cell type, internalized cargo reaches the EE within 1-5 min (Yamashiro & Maxfield 1987, Griffiths et al. 1989, Schmid et al. 1989, Tooze & Hollinshead 1991) where the pH is slightly acidic (6.1-6.2) (Sipe & Murphy 1987, Yamashiro & Maxfield 1987). Common marker proteins present on early endosomal membranes are early endosomal antigen 1 (EEA1) and Rab5 (Mu et al. 1995, Simonsen et al. 1998).

Recycling molecules are sorted to the tubular parts of EEs and transferred directly back to the plasma membrane, or firstly to the REs (Maxfield & McGraw 2004). Fast recycling happens through the Rab4 domains whereas slowly recycled molecules are targeted to the Rab11-positive structures. REs have a slightly acidic pH (~6.1-6.2) environment (Sipe & Murphy 1987, Yamashiro & Maxfield 1987) and are highly tubular (Maxfield & McGraw 2004). Transferrin (Tf) and its receptor (TfR), commonly used markers for recycling compartments, are recycled together via both Rab4 and Rab11 pathways (Van Der Sluijs et al. 1991, Trischler et al. 1999, Sheff et al. 2002). Also the membrane marker, lipid analog C6-NBD-SM, recycles through the same structures and has the similar kinetics as Tf and LDLR (Mayor et al. 1993, Hao & Maxfield 2000). In addition to this kind of bulk recycling, also sequence specific recycling is known. For example β_2-adrenergic receptor (β_2-AR) is sorted to a special subset

of tubules in EEs that are distinct from TfR (Puthenveedu et al. 2010). β_2-AR contains a PDZ domain, which is needed for recycling and links the receptor to actin (Cao et al. 1999). This domain functions as a sorting sequence that guides receptor to actin stabilized tubules whereas TfR tubules are more dynamic (Puthenveedu et al. 2010). Recycling from the recycling compartment varies depending on the molecule. Many GPI-APs recycle via the recycling compartment but they return to the plasma membrane three times slower than Tf (Mayor et al. 1998). However, in cholesterol depleted cells GPI-APs recycle at the same rate, indicating that lipid environment can influence the recycling.

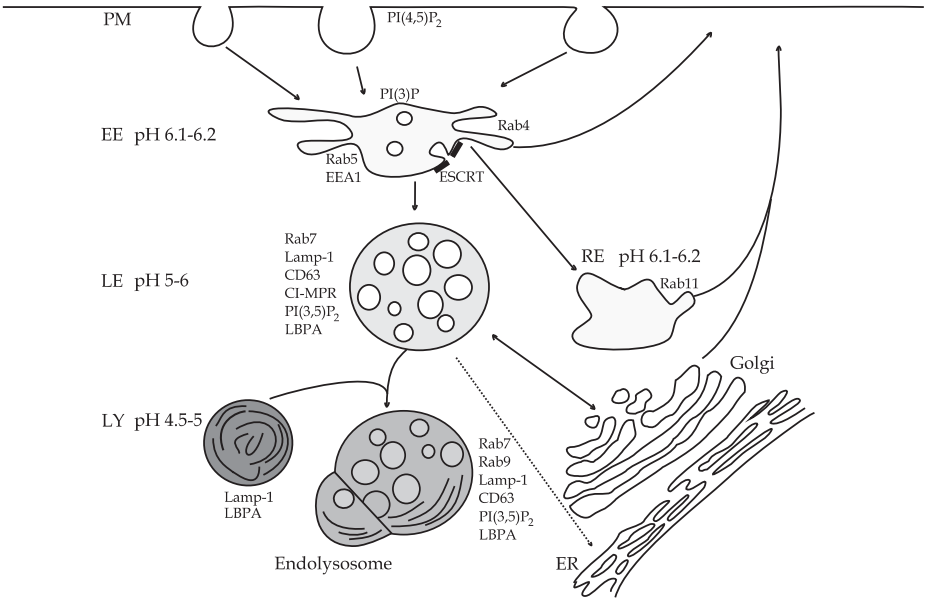

FIGURE 6 Endocytic structures and routes. Internalized material is guided to slightly acidic early endosomal structures. Recycled material is targeted directly in a Rab4-mediated way or through perinuclear recycling endosomes to the plasma membrane. Early endosomes mature into late ensosomes that contain more acidic pH and different lipid and protein composition. Material for degradation ends up into lysosomes or hybrid organelle where lysosomal hydrolases break down the proteins. $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane. Modified from Mercer et al. 2010.

2.3.1.2 Multivesicular bodies

Receptors destined to degradation are sorted into the vesicular part of the EEs and sorting to ILVs starts already at this stage. LEs are round or oval structures containing numerous ILVs (Griffiths et al. 1989, Gruenberg 2001) and have a pH below 6 (Merion et al. 1983, Tycko et al. 1983, Murphy et al. 1984, Yamashiro & Maxfield 1987). Also transitional structures exist between early and LEs, which gradually loose characteristic early endosomal constituents and receive secretory components from trans-Golgi network (TGN) in a process called endosome maturation (Rink et al. 2005, Huotari & Helenius 2011, van Weering et al. 2012). In addition, separate carrier vesicles between early and LEs are

suggested to deliver cargo for lysosomal degradation (Gruenberg et al. 1989, Vonderheit & Helenius 2005) and it is possible that both mechanisms are used depending on the cargo or the cell type. In both cases a conversion of Rab-GTPases is detected from the early endosomal Rab5-positive structures to the late endosomal Rab7 containing structures (Rink et al. 2005, Vonderheit & Helenius 2005). Recently, the fission of tubular elements from EEs was shown to be necessary for further maturation of the endosomes (Mesaki et al. 2011). Inhibition of fission events with dynamin inhibitor prevented the endosomal movement along microtubules, further acidification, and caused impaired degradation.

Although MVBs usually sort cargo for degradation, not all material that is sorted to MVBs is targeted to conventional lysosomes (LYs). For example melanosomes are LY-related organelles that synthetize and store melanin (Raposo & Marks 2007). They originate from the early endosomal structures and the multivesicular intermediates, but they are separate from the conventional endosomes and LYs. They can contain conventional endosomal markers but they do not function as degrative organelles, instead they produce and store melanin (Raposo et al. 2001, Theos et al. 2006, Raposo & Marks 2007, van Niel et al. 2011). In dendritic cells, specialized MVBs called major histocompatibility complex (MHC) class II compartments, degrade internalized antigens to peptides which are bound to MCH class II molecule and which are finally transported in tubules to the plasma membrane for antigen presentation (van Niel et al. 2008). MVBs can also fuse with the plasma membrane and release ILVs into the extracellular space. This kind of exosomal release is detected in e.g. B-cells, T-cells and in platelets (Heijnen et al. 1998, Heijnen et al. 1999, Fevrier & Raposo 2004).

2.3.1.3 Lysosomes

LYs are defined as a terminal degradation station for endocytosed proteins (Luzio et al. 2007). Also phagosomes and autophagosomes are able to fuse and deliver their content to LYs (Luzio et al. 2007). Classical LYs are dense membrane bound bodies with diameter from 250 to 500 nm (Novikoff et al. 1956). They contain acid phosphatases (Essner & Novikoff 1961) and have a pH between 4.5 to 5 (Ohkuma & Poole 1978, Tycko et al. 1983, Murphy et al. 1984). However, drawing a line between the LEs and the LYs is not straightforward. Despite their morphological differences, LEs and LYs have many similarities: they both contain lysobisphosphatidic acid (LBPA), lysosome-associated membrane proteins (Lamps) and acid hydrolases, but LYs seem to lack mannose-6-phosphate receptors (MPRs) (Pillay et al. 2002). More complexity is brought about with the fact that degradation can start already in the LEs (Renfrew & Hubbard 1991, Tjelle et al. 1996, Humphries et al. 2010). This might be due to the fact that as multivesicular structures are fully mature, they are able to fuse with LYs (Futter et al. 1996, Bright et al. 1997, Mullock et al. 1998). Fusion event can be a transient, 'kiss and run'-type or full fusion of structures that leads to the formation of a hybrid organelle (endolysosome), thus containing properties of both structures (Luzio et al. 2007, Pryor & Luzio 2009).

Dense LYs are suggested to be more like storage organelles of hydrolytic enzymes since proteolysis starts already in these hybrid organelles (Tjelle et al. 1996, Bright et al. 1997). Hydrolytic enzymes are produced in the endoplasmic reticulum (ER) and further modified at the Golgi from where they are transported to the endosomes (van Meel & Klumperman 2008). Most of the lysosomal hydrolases are tagged with mannose-6-phosphate and are transported to the endosomes by cation-independent or –dependent MPRs. However there are also hydrolases that do not have the mannose-6-phosphate tag and their transport is not dependent on MPRs (van Meel & Klumperman 2008).

Fusion of two organelles needs tethering and formation of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Tethering between the LEs and the LYs has been detected by electron microscopy (Futter et al. 1996, Bright et al. 1997), but the exact protein components are not known. Rab7 and the homotypic fusion and vacuole protein sorting complex are suggested to be involved in the process (Luzio et al. 2007, Huotari & Helenius 2011). Vesicle associated membrane protein 7, Syntaxin-7, vesicle transport through interaction with t-SNARE homolog 1B and Syntaxin-8 are needed in the assembly of the SNARE complex that brings the membrane in close contact and mediates the fusion (Pryor et al. 2004). In addition to the SNAREs Ca^{2+} and calmodulin are also required for fusion (Pryor et al. 2000).

2.3.2 Sorting of endocytosed material

In the degradative pathway, endosomes go through massive changes when maturing from the EEs to the LEs. This includes e.g. increased acidification and ILV biogenesis, changes in the composition of lipids and Rabs, as well as a gain of lysosomal hydrolases and membrane proteins. The endosomal movement along the microtubules to the perinuclear area is also connected to this maturation (Bayer et al. 1998, Baravalle et al. 2005, Mesaki et al. 2011). Here, some of the key aspects that are essential for protein sorting to the degradative pathway are reviewed.

2.3.2.1 Acidity

Acidity of the structures is essential in many ways in the endosomal pathway. Mildly acidic pH of the EEs helps to dissociate ligands from several recycling receptors (Schwartz et al. 1982, Davis et al. 1987), and it controls endosomal trafficking towards LEs (Bayer et al. 1998, Gu & Gruenberg 2000, Baravalle et al. 2005). In addition, the lysosomal hydrolases are activated at a low pH (Mindell 2012). Furthermore, many pathogens take advantage of the acidic environment in their entry route (Mercer et al. 2010). Acidification of the intracellular structures is caused mainly by the vacuolar H^+ ATPase (V-ATPase). V-ATPase is an ATP-dependent proton pump that uses energy released from the ATP hydrolysis for transport of protons to intracellular compartments (Jefferies et al. 2008). V-ATPase contains two structural domains, a membrane bound V_0

subdomain for proton translocation and a soluble V_1 domain that is responsible for the ATP hydrolysis (Jefferies et al. 2008, Marshansky & Futai 2008). Organelle acidity can be regulated for example by altering the V-ATPase density on the membrane, by reversible dissociation of the subunits, changing the conductance of the organelle by other ion channels or pumps or by differential targeting of V-ATPase isoforms (Jefferies et al. 2008, Marshansky & Futai 2008). For example in baby hamster kidney cells the ratio of V_1/V_o subunits varies between different endosomes so that ratio is higher in LEs than in EEs (Lafourcade et al. 2008), and thereby explains the higher acidity of the LEs. V-ATPases are shown to locate in the DRMs of endosomes, and by modifying late endosomal cholesterol content with 3-β-[2-(diethylamino)ethoxy] androst-5-en-17-one (U18666A) the pH could be changed (Lafourcade et al. 2008). This thus suggests that cholesterol could also have a role in the regulation of the V-ATPase function (Lafourcade et al. 2008).

A common inhibitor to block the function of the V-ATPase is bafilomycin A1. It neutralizes endosomal structures and inhibits lysosomal degradation (Yoshimori et al. 1991). In addition to the neutralization effect, it can cause other changes in the endosomal pathway. Internalization and recycling of the cargo is not inhibited by bafilomycin A1 (Bayer et al. 1998, Baravalle et al. 2005), but it can slow down the recycling rate of the cargo (van Weert et al. 1995), and EEs can become more tubular (Clague et al. 1994). However, bafilomycin A1 can inhibit transport from the EEs to the LEs in Hela cells (Bayer et al. 1998, Baravalle et al. 2005) or inhibit the fusion of the LEs with the LYs in HepG2 cells (van Weert et al. 1995). Lately, it was suggested that V-ATPase could itself work as a pH-sensor that could regulate vesicular trafficking in the degradative pathway (Hurtado-Lorenzo et al. 2006). The α2 isoform of the V-ATPase, located on the early endosomal membrane, was shown to bind to cytosolic Arf6 GEF (ARNO) and Afr6 only when the endosomal lumen was acidic (Hurtado-Lorenzo et al. 2006). It has been also shown that acidic pH is needed in the Arf1-dependent association of endosomal coat protein I to the early endosomal membrane and budding of the endosomal carrier vesicle (Aniento et al. 1996, Gu & Gruenberg 2000). These results imply the V-ATPase to be a pH-sensor, which could regulate the Arf6- and Arf1-mediated vesicular trafficking towards degradative compartments and explain the block in transport caused by bafilomycin A1.

2.3.2.2 Lipids

Lipid composition of the cellular membranes is unique for each organelle and is important for their proper function (van Meer et al. 2008). Lipids do not simply form membrane barriers between different environments but they have many other functions too. Lipids can e.g. function as signaling molecules, form signalling platforms, enhance membrane curvature, enable fusion and fission events or recruit proteins to membranes. Certain lipids, like phosphatidylinositol 3-phosphate (PI3P) and cholesterol, have been found to be especially important in regulating endosomal trafficking. Therefore their role in endosomal sorting is discussed more precisely.

PI3P is derived from phosphatidylinositols by PI3K phosphorylation. It is located mainly on the limiting membrane of the EEs, but it can be also found in the ILVs of the MVBs (Gillooly et al. 2000). PI3P is able to recruit several proteins to the cellular membranes that are involved in cell signaling and trafficking. It has been shown to bind to proteins containing a FYVE domain (domain conserved in Fab1, YOTB, Vac1 and EEA1) like EEA1 and hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), (Gaullier et al. 1998, Stenmark & Aasland 1999, Gaullier et al. 2000) and also some proteins containing phox homology domain such as sorting nexins (Ellson et al. 2002, Seet & Hong 2006). PI3P can thus recruit Rab5 together with its FYVE containing effectors EEA1 (Simonsen et al. 1998) as well as rabenosyn-5 (Nielsen et al. 2000) and mediate the EE fusions. PI3P is essential also in the MVB formation. The PI3K inhibitor, Wortmannin, causes enlarged endosomes containing only few ILVs (Fernandez-Borja et al. 1999, Futter et al. 2001). The PI3P binding protein Hrs is also involved in the formation of ILVs since Hrs knockdown reduced ILV formation and increased the size of LEs (Bache et al. 2003).

The PI3P is also a precursor for phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) created by the PIKfyve kinase (Ho et al. 2012). $PI(3,5)P_2$ is located in the late endosomal membrane and its amount increases during endosome maturation (De Matteis & Godi 2004). Inhibition of the function of PIKfyve induced accumulation of swollen, lucent endosomes (Ikonomov et al. 2001). However, PIKfyve was not needed for sorting of Ub-containing cargo to degradation (Ikonomov et al. 2003, Rutherford et al. 2006). The role of $PI(3,5)P_2$ in the endosome maturation is still unknown, but it is suggested to regulate Ca^{2+} efflux from LE. Transient receptor potential mucolipin 1 (TRPML1), localized at LEs and LYs, is an cation channel that is activated by the presence and production of $PI(3,5)P_2$ (Dong et al. 2010). Enlarged LE phenotype observed in $PI(3,5)P_2$-deficient mouse fibroblasts can be suppressed by overexpression of TRPML1 (Dong et al. 2010). These results suggest that $PI(3,5)P_2$ could mediate fusions between LEs and LYs that is regulated with Ca^{2+} (Peters & Mayer 1998, Pryor et al. 2000).

Another specific lipid found in the LEs is LBPA (also called as bis-monoglyserol phosphate). Firstly it was found to be present in LYs (Wherrett & Huterer 1972, Poorthuis & Hostetler 1978), but later it was discovered to be also abundant in the ILVs of the LEs (Kobayashi et al. 1998). However, LBPA is not found in all types of MVBs. Activated EGFR is trafficked to LYs via different subpopulation of MVBs that do not colocalize with LBPA in several cell lines (White et al. 2006). In antigen presenting cells, MHC class II is sorted to specific MVBs, exosomes, containing ILVs that are highly enriched with cholesterol whereas LBPA structures contain only little cholesterol (Möbius et al. 2003). The amount of PI3P is also low in the LEs, however, PI3P and LBPA-positive ILVs can be found inside the same endosome, but these ILVs are distinct (Gillooly et al. 2000).

ILV formation that targets proteins to degradation is observed primarily in the EEs and in non-lamellar MVBs where LBPA is absent (White et al. 2006).

However, LBPA can also promote ILV formation within acidic liposomes and these structures are regulated by ALG-2-interacting protein X (Alix) (Matsuo et al. 2004). Similarly, Alix controls budding of ILVs into LEs (Falguieres et al. 2008). It is also suggested that the LBPA containing ILVs could have a role in the backfusion of ILVs with the limiting membranes. For example vesicular stomatitis virus (VSV) capsid (Le Blanc et al. 2005) and anthrax toxin (Abrami et al. 2004) are suggested to be released to the cytoplasm from the LEs by backfusion. Actually, LBPA-rich membranes are shown to promote the VSV G protein-mediated membrane fusion *in vitro* (Roth & Whittaker 2011). However, contradictory results are reported, suggesting that VSV fusion occurs already in the EEs (Johannsdottir et al. 2009).

Lipid rafts are dynamic lipid microdomains on cellular membranes that consist of cholesterol, sphingolipids and associated proteins, espescially GPI-APs, and they are resistant for the classical treatment with cold non-ionic detergents (Lingwood & Simons 2010). There has been a great deal of debate about the size, formation and stability of the rafts, since imaging of these structures *in vivo* has been challenging due to the limits in the resolution of the microscopes (Simons & Gerl 2010). Although lipid rafts were originally proposed to locate at the plasma membrane, also endosomal structures, including REs (Mayor et al. 1998, Gagescu et al. 2000, Chatterjee et al. 2001) as well as LEs (Balbis et al. 2007, Sobo et al. 2007, Nada et al. 2009) are reported to have lipid microdomains. Currently, little is known about their intracellular role, but they have been reported to be important in protein sorting (Helms & Zurzolo 2004) and as signaling platforms (Balbis et al. 2007).

Cholesterol is highly enriched in the RE in many cells (Mukherjee et al. 1998, Hao et al. 2002, Möbius et al. 2003) and also the ILVs of non-lamellar MVBs are rich in cholesterol (Möbius et al. 2003). Cholesterol levels are lower in the LEs and LYs but, at the same time, the LBPA concentration is increased (Möbius et al. 2003). Membrane cholesterol is derived either from the endocytosed LDL or from the *de novo* biosynthesis at the ER (Mesmin & Maxfield 2009). LDL is endocytosed together with its receptor, LDLR, via the clathrin-mediated pathway. LDLR itself is sorted back to the plasma membrane, but LDL is targeted to the LEs and LYs where it is hydrolysed, and free cholesterol is transported to the plasma membrane and the ER (Ikonen 2006). This cholesterol exflux is dependent on the Niemann-Pick type C1 and C2 (NPC1 and NPC2) proteins that are located in the LEs and LYs (Mukherjee & Maxfield 2004, Chang et al. 2005). Non-functional NPC1 leads to impaired cholesterol traffic causing the NPC disease (Mukherjee & Maxfield 2004). In NPC-type fibroblasts cholesterol accumulates in the LEs containing LBPA and Rab7 whereas in normal cells such accumulation is not detected (Kobayashi et al. 1999). Also MPR, which normally recycles between the TGN and the LEs, is trapped on the LEs (Kobayashi et al. 1999). LBPA antibody (Kobayashi et al. 1999) and U18666A (Liscum & Faust 1989) treatments were also able to induce similar effects. LBPA-rich membranes thus seem to regulate cholesterol efflux. In addition oxysterol binding proteins (OSBPs) and OSBP related proteins (ORPs) are connected to the cholesterol intracellular traffic (Vihervaara et al.

2011). The exact mechanism of how ORPs mediate sterol transfer between membranes is not known, but it is suggested that they can bring ER and other organelle membranes in close contact by binding to the PIs. It is also proposed that they can themselves deliver sterols between membranes or function as a sterol sensor that recruits other proteins to sterol-rich membranes (Vihervaara et al. 2011). Recently it was reported that NPC1 and ORP5 cooperate in the cholesterol removal from the LEs/LYs. Knockdown of ORP5, which was found on the ER membranes, caused cholesterol accumulation to the LEs/LYs as in the NPC disease (Du et al. 2011).

2.3.2.3 Ubiquitin as a sorting signal

The default pathway of the endocytosed cargo is recycling. Thus the proteins targeted to the ILVs need to be separated from the cargo sorted to recycling or to the TGN. The common label of proteins to be sorted for MVBs is ubiquitin, although ubiquitination is not necessary for all ILV-targeted proteins such as melanosome protein Pme117 or yeast protein Sna3 (Piper & Katzmann 2007, Davies et al. 2009). In addition to the degradation through the MVBs, ubiquitin is attached to cytosolic proteins targeted to proteasomal degradation. Proteasomal degradation involves the attachment of mainly polyubiquitin chains to proteins through the ubiquitin lysine-48. However, it has been recently discovered that also other lysines can be ubiquitinated and protein can still be degraded in proteasomes (Finley 2009). Plasma membrane proteins sorted to the MVBs are normally mono- or multimonoubiquitinated and lysine-63 is the primary ubiquitin chain type involved in endosomal sorting (Clague & Urbe 2010). However recent studies have revealed that polyubiquitin is needed for targeting the MHC class I protein and the tropomyosin-regulated kinase A to the MVBs (Duncan et al. 2006, Geetha & Wooten 2008). Ubiquitin can also to be a signal for autophagysince ubiquitinated aggregates, peroxisomes, ribosomes and mitochondria are targeted to LYs via selective autophagy (Kirkin et al. 2009).

Ubiquitination is a three-step process that requires activating, conjugating and ligating enzymes also called as E1, E2 and E3. First E1 activates ubiquitin in an ATP-dependent process. Activated Ub is transferred to a ubiquitin carrier protein, E2 and E3 then makes an isopeptide bond between the C-terminus of the ubiquitin and the lysine residue of the target protein (Hershko & Ciechanover 1998). Normally there is only one E1 enzyme, but several E2 and E3 giving specificity for different cargoes (Hershko & Ciechanover 1998). Ubiquitinated proteins are usually deubiquitinated before degradation by deubiquitinating enzymes (DUBs), which thus maintains high cellular pools of ubiquitin (Komander et al. 2009, Reyes-Turcu et al. 2009). DUBs have also many other roles including processing free polyubiquitin chains to free monoubiquitin, rescuing of proteins from degradation, removing non-degradative ubiquitin signals or modifying ubiquitin chains on proteins (Komander et al. 2009, Reyes-Turcu et al. 2009).

2.3.2.4 ESCRTs and formation of ILVs

Endosomal sorting complex required for transport (ESCRT) is a protein machinery that is required for formation of the ILVs of multivesicular endosomes (Hurley 2008, Raiborg & Stenmark 2009). However, also ESCRT independent ILV formation has been reported (Trajkovic et al. 2008, Stuffers et al. 2009, van Niel et al. 2011). In addition to MVB biogenesis, ESCRTs are also mediating fission during cytokinesis (Carlton & Martin-Serrano 2007, Morita et al. 2007), and many enveloped viruses like human immunodeficiency virus type 1 exploit this machinery for budding out of cells (Bieniasz 2006, McDonald & Martin-Serrano 2009). Common for all these processes is the same topology of membrane budding away from cytosol.

ESCRT machinery in the biogenesis of the MVBs contains ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III complexes and other accessory components like vacuolar protein sorting-associated protein (VPS) 4 and VPS-VTA1 homolog protein (VTA1) (Fig. 7) (Nickerson et al. 2007, Hurley 2008, Raiborg & Stenmark 2009). ESCRT-0 complex initiates the MVB pathway. It localizes to the early endosomal membrane by binding to PI3P via Hrs FYVE domain. It binds to the ubiquitinated cargo with several ubiquitin binding domains present in Hrs and signal-transducing adaptor molecule and thus concentrating cargo targeted to ILVs. Flat clathrin lattices that are recruited to endosomal membranes via Hrs clathrin box domain support concentration of the cargo. The ESCRT-0 complex also recruits ESCRT-I to the endosomal membrane via direct binding. The rod shaped ESCRT-I binds with one end to ESCRT-0 and with the other end to the ESCRT-II and has also an ubiquitin binding site. ESCRT-II can attach to ubiquitinated cargo, but also to the membrane via PI3P binding domain. In the conventional model, ubiquitinated cargo is transferred from one ESCRT complex to the next (Hurley & Emr 2006, Piper & Katzmann 2007), or they could all bind with different cargoes and thus form a membrane domain concentrated with cargo to be sorted to the ILVs (Nickerson et al. 2007, Hurley 2008). The final step is recruitment of ESCRT-III components and their assembly on the membrane. Moreover deubiquitination of cargo is done at this stage. ESCRT-II can activate VPS20 protein, which functions as a nucleator for ESCRT-III polymerization. Assembly of ESCRT-III drives membrane budding and finally also the membrane scission process (Wollert et al. 2009). ESCRT-III components are able to oligomerize into filaments and take a spiral form on membranes (Ghazi-Tabatabai et al. 2008, Hanson et al. 2008, Lata et al. 2008), which is suggested to drive the membrane scission (Guizetti et al. 2011). When the assembly is ready, VPS4-VTA1 complex catalyses disassembly of ESCRT-III filaments in an ATP-dependent process and recycles ESCRT-III components (Hurley 2008, Wollert et al. 2009).

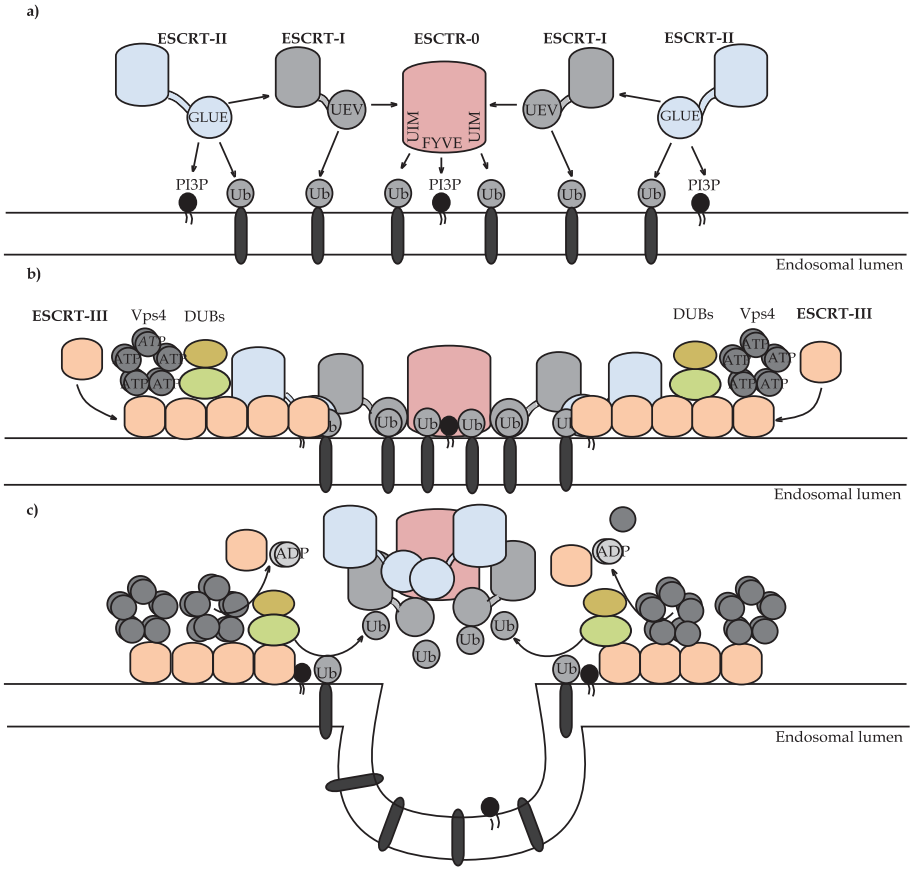

FIGURE 7 Concentric model of the ESCRT machinery. a) ESCRT-0, I and -II are recruited to membrane via PI3P and Ub-recognizing domains and they consentrate the cargo on the membrane. b) ESCRT-III oligomerizes around the concentrated cargo that is targeted to ILVs and drives the budding. c) Deubiquitination of the cargo by DUBs and dissassembly of ESCRT-III filaments by VPS4 release the components for reuse. GLUE, GRAM-like Ub binding in Eap45; Ub, ubiquitin; UIM, Ub-interacting motif; UEV, Ub E2 variant. Modified from Nickerson et al. 2007.

2.4 Other cellular degradation systems

2.4.1 Proteasomes

While endocytosed plasma membrane proteins are degraded in LYs, many cytosolic and nuclear proteins are regulated by proteasomal degradation. Proteins that control cell cycle (cyclins) and transcription factors are typical proteins that are proteolysed through proteasomes. Likewise abnormal/misfolded proteins are degraded by the proteasomal system. The proteasome is a large 26S protease that consists of two subcomplexes: a 19S

regulatory part and a 20S catalytic part (Pickart & Cohen 2004, Ciechanover 2012). Together they form a cylindrical structure, which has a lid at the entry site and catalytic activity inside the chamber. The regulatory part recognizes polyubiqitinated proteins, marked for degradation, and unfolds proteins so that they can fit into the narrow proteasomal cavity. Translocation of proteins into the 20S chamber is an ATP-dependent process and arriving proteins are then cut to short peptides by the catalytic unit. Ubiquitin is released from proteins for reuse before degradation by the regulatory part.

Proteins targeted for proteasomes are ubiquitinated in a three-step process as described above. In the case of polyubiquitination, multiple cycles of ubiquitination is performed (Glickman & Ciechanover 2002, Sriram et al. 2011). Polyubiquitination targets proteins commonly to proteasomes, but ubiquitin tags can also function as a signal for internalization, lysosomal degradation or nuclear targeting (Ciechanover 2012). Furthermore, proteasomal degradation and lysosomal proteolysis can be also coupled as seen in the case of e.g. EGFR and VEGFR-2. Proteasomal inhibition has been shown to inhibit EGFR targeting to ILVs and thus its degradation (Longva et al. 2002). In contrast, it also has been reported that proteasome activity is needed for deubiqitination of a receptor prior to lysosomal degradation (Alwan et al. 2003). VEGF-A stimulated VEGFR-2 activation and proteolysis is not affected by inhibition of proteasomes (Bruns et al. 2010). However, the inhibition of proteasome was shown to prevent the proteolysis of VEGFR-2 cytoplasmic tail, which caused prolonged signaling and increased cell migration. This thus suggested that proteasomal degradation of the cytoplasmic tail is executed before lysosomal degradation, and proteosome-mediated proteolysis attenuates VEGFR-2 signalling after growth factor stimulus (Bruns et al. 2010).

2.4.2 Autophagy

Autophagy is a LY dependent degradation process that enables degradation of cytoplasmic material and organelles (Eskelinen & Saftig 2009, Yang & Klionsky 2010). Regulated degradation by autophagy is important for cells in many ways such as during development and differentiation when massive remodelling is happening (Meijer & Codogno 2004). Autophagy is also required to keep up the cellular homeostasis e.g. during starvation when the released amino acids are needed for cell survival or when damaged or undesired organelles and molecules are removed.

Autophagy can be divided into three subclasses: macroautophagy, microautophagy and chaperone-mediated autophagy (Eskelinen & Saftig 2009). In macroautophagy, often called as autophagy, cytosolic material is taken inside into a forming autophagosome that is later fused with LYs. In microautophagy instead, lysosomal membrane takes small amounts of cytosolic material inside the LY by forming invaginations and internalizing them (Cuervo 2011). In chaperone-mediated autophagy, selected cargo is transported through lysosomal membrane from the cytosol with the help of a molecule, like heat-shock cognate 70 chaperone (Cuervo 2011). Macroautophagy starts by

formation of a phagophore, a double membrane cisterna which gradually grows and forms a cup around the material targeted for degradation (Eskelinen & Saftig 2009, Yang & Klionsky 2010). When material is completely surrounded by a double membrane it is called the autophagosome. This organelle fuses first with an endosome or directly with LYs and produces an amphisome or autolysosome, respectively. The origin of a phagophore membrane in mammalian cells is still unresolved and it has been suggested to be formed either by *de novo* or from other organelles. Many recent publications have shown that autophagosomes might arise from the ER (Axe et al. 2008, Yla-Anttila et al. 2009, Geng & Klionsky 2010, Tooze & Yoshimori 2010), the Golgi (Young et al. 2006, Guo et al. 2012) or mitochondrial membranes (Hailey et al. 2010). Currently over 30 autophagy-related (Atg) genes have been found, but the function of many Atg proteins is still unclear (Weidberg et al. 2011). Starvation-induced inactivation of mammalian target of rapamycin leads to activation of the serine and threonine kinase ULK1/ULK2-complex and class III PI3K (Yang & Klionsky 2010, Weidberg et al. 2011). Formation of PI3P on the phagophore membrane recruits PI3P binding Atgs that are required for formation of the autophagosome. The phagophore contains also the mAtg9 that shuttles between TGN and phagophore and it is suggested to deliver membrane or proteins components. LC3-II and Atg12-Atg5-TG16l complexes are thought to be involved in elongation of the phagophore.

2.4.3 Calpains

Calpains are calcium-dependent, cytosolic cysteine proteases. There are two major isozymes of calpains, calpain-1 and calpain-2, also called as μ- and m-calpains, respectively, based on their requirement of calcium. In addition, also tissue-specific calpains are known (Suzuki & Ohno 1990, Suzuki & Sorimachi 1998, Goll et al. 2003). Calpain proteolysis is not specific to certain amino acid sequences but it is thought to cleave proteins based on more global structural elements (Tompa et al. 2004). Calpains have over 100 substrates that include transcription factors, transmembrane receptors, cytoskeletal proteins as well as FA and signaling molecules (Goll et al. 2003, Franco & Huttenlocher 2005). Cytoplasmic domains of beta integrins, including β1 integrin, have also been shown to be cleaved by calpains (Pfaff et al. 1999). In addition, numerous other calpain substrates are involved in cell migration and adhesion including talin, spectrin and FAK. Calpains have been reported to affect cell migration by affecting adhesion sites, cell spreading and formation of protrusions (Franco & Huttenlocher 2005, Lebart & Benyamin 2006). For example, calpain inhibition has been shown to reduce both β1 and β3 integrin-mediated cell migration by stabilizing cytoskeletal linkages and reducing detachment of adhesions at the cell rear (Huttenlocher et al. 1997, Palecek et al. 1998). On the other hand, calpain has been shown to induce the formation of early integrin clusters which contained Rac binding protein(s), calpains and calpain cleaved β3 integrins (Bialkowska et al. 2000).

Calpain molecules consist of a 80 kDa large catalytic subunit and a 30 kDa small regulatory subunit. Calpain activation is regulated in many ways that are not completely understood. In addition to calcium requirement, calpains have a natural inhibitor, calpastatin that regulates their activity. Also autolysis of the catalytic domain can increase the activity by lowering the need of calcium, however the autolysis is not always obligatory for calpain activation (Suzuki & Sorimachi 1998, Goll et al. 2003, Franco & Huttenlocher 2005, Hanna et al. 2008). Phospholipid binding has also been shown to lower calcium need and increase activation (Saido et al. 1992, Sprague et al. 2008). Moreover, direct phosphorylation by ERK in EGF-induced fibroblasts has been shown to activate calpain-2 without an increase of cytosolic calcium levels (Glading et al. 2004).

Calpains are reported to be essential for infection of several viruses. Previous studies with EV1 showed that calpains are crucial for the replication start-up (Upla et al. 2004). Other EV1-related enteroviruses, coxsackieviruses B3 and B4 use autophagosomes in their replication and therefore need calpains for their infection (Wong et al. 2008, Yoon et al. 2008). Calpains can also mediate the apoptotic responses of cells. In a reovirus infection for example, calpain activation is needed for virus-induced apoptosis (Debiasi et al. 1999). Instead, viral proteins of hepatitis C virus activate calpains, which leads to calpain-mediated proteolysis of specific proteins that reduce apoptotic response (Simonin et al. 2009).

3 AIM OF THE STUDY

Viruses have evolved to exploit various endocytic routes to infect cells. To fight against these pathogens, detailed information of their life cycles is needed. Viruses can also be used as tools to study cellular pathways in general. Both of these aspects have been combined in this thesis to reveal more detailed information about EV1 infection and integrin endocytic pathways. The aims of this doctoral thesis were:

1. To define the properties and biogenesis of multivesicular structures triggered by EV1
2. To characterize the fate of integrin and its cargo via the α2β1 integrin clustering-triggered pathway to α2 integrin containing multivesicular bodies (α2-MVBs)
3. To describe the role of cholesterol in EV1 infection, α2 integrin internalization and formation of MVBs in α2β1 integrin clustering-induced route

4 SUMMARY OF MATERIALS AND METHODS

The materials and methods used in these studies are listed in Table 1. Detailed descriptions can be found in the original papers indicated with Roman numerals. The permeability test of α2-MVBs is described in detail since it is not included in these publications.

TABLE 1 Summary of materials and methods used in the thesis. The Roman numerals refer to the original articles where the detailed descriptions can be found.

Material/Method	Publication
Virus production and purification	I, II, III
EV1 infection and integrin clustering	I, II, III
Immunoprecipitation and immunoblotting	II
Recycling assays	II
Surface biotinylation	II
t-BOC assay	II
Metabolic labeling	II
Intra-endosomal pH measurement	I, II
Drug treatments	I, II, III
Immunofluorescence labeling	I, II, III
Confocal microscopy	I, II, III
Data analysis with microscopy data	I, II, III
Electron microscopy	I, II, III
Cryo-immunoelectron microscopy	I, II
Collagen coating assay	I, II, III
Internalization assay	I, II, III
EVI uncoating assays	I, II, III
Transfection	I
Small interfering RNA experiments	I, II, III

4.1 *In vitro* permeability test of α2-MVBs

EV1 (Farouk strain, American Type Culture Collection) was bound to stabile transfected human osteosarcoma cells (SAOS-α2β1 cells, clone 45) (Ivaska et al. 1999) on ice for 1 h and unbound virus was removed by extensive washes with 3% bovine serum albumin (BSA) in PBS. Cells were further treated on ice with rabbit antibodies against EV1 (Marjomäki et al. 2002) in 1% DMEM for 45 min and washed, after which the viruses with antibodies were allowed to internalize in 10% Dulbecco's modified Eagle's medium (DMEM) at +37 °C. After 1, 2 or 3 h internalization period cells were placed on ice again and surface-derived EV1 was labeled with goat anti-rabbit antibody conjugated with Alexa Fluor 633 (Invitrogen). Cells were washed with 3% BSA-PBS three times and once with PBS before permeabilization according the protocol originally described by Robinson and Kreiss (Robinson & Kreis 1992). Wells were aspirated empty so that only residual buffer was left on coverslip, after which cells were rapidly permeabilized by placing the coverslips on a metal plate at -80 °C for 20 s. This technique allows the permeabilization of the plasma membrane, controlled with Trypan blue assay, but retains intact intracellular structures. After permeabilization cells were immediately incubated with 30 µl drop of monovalent antigen binding fragments (Fabs) of goat anti-rabbit IgG conjugated with DyLight 488 (Jackson ImmunoResearch Laboratories) in 1% DMEM on ice for 30 min. Control cells were treated with fluorescent Fabs without permeabilization. Total EV1 signal was monitored after permeabilization of control cells with 0.2% Triton X-100 for 5 min and labeling them similarly with Fabs after fixation. Fixation was performed with 3% paraformaldehyde for 15 min on ice. Permeability of α2-MVBs was observed from confocal images as the intensity of the Fab DyLight 488 signal subtracted with the control surface Fab DyLight 488 labeling from non-permeabilized cells.

In order to monitor the permeability of the mock-infected samples, anti-α2 integrin antibody (A211E10, a gift from Fedor Berditchevski, Institute of Cancer Studies, Birmingham, United Kingdom) and rabbit anti-mouse antibodies (Sigma-Aldrich) were sequentially used instead of EV1. The integrins were internalized for 1, 2 and 3 h at +37 °C and placed on ice. As described above, Fab DyLight 488 of goat anti-rabbit IgG was bound onto the cells with or without permeabilization and then the cells were fixed. As a control, αV integrin was clustered with sequential treatments with αV integrin (L230, ATCC) and rabbit anti-mouse antibodies on ice followed by 1 h internalization. The permeability for the intracellular αV integrin-positive endosomes was tested as above.

5 REVIEW OF THE RESULTS

5.1 EV1 is targeted into non-acidic multivesicular structures

5.1.1 EV1-induced multivesicular structures are separate from the classical late endosomes

Previous studies with EV1 have suggested that α2-MVBs, which are formed after EV1 internalization, are separate from acidic LEs (Marjomäki et al. 2002, Pietiäinen et al. 2004, Karjalainen et al. 2008). However, the appearance of α2-MVBs at the level of electron microscopy greatly resembles the structure of the LEs (Sachse et al. 2002, Möbius et al. 2003, Russell et al. 2006, Karjalainen et al. 2008). Therefore, the biogenesis and characteristics of α2-MVBs needed to be studied more carefully. At 15 min many of the α2 integrin structures are still tubular, but already after 30 min multivesicular structures are common, and after 2 and 3 h most of the structures contain ILVs (72% and 96 %, respectively, II, Fig. 3A-C) (Karjalainen et al. 2008). Tubular elements were no longer detected after 6 h and structures were still multivesicular. At 6 h, some of the structures contained ILVs that were indistinct and also the limiting membrane of these structures was less conspicuous (II, Fig. 3A).

During the maturation of endosomes, major changes are known to take place, including acidification of the endosomal lumen and changes of protein composition of membranes (Mercer et al. 2010). To analyse the pH of the α2-MVBs more precisely, α2 integrin was clustered with equal amounts of secondary antibodies conjugated either to pH-sensitive FITC or insensitive Alexa 555 dyes (I, Fig 2A, II, Fig. 5A). When examining the EV1 structures, virus was bound on the plasma membrane first before the antibody clustering. The ratio of intensity between the two conjugates was determined from 5 min to 6 h from confocal images taken from live experiments. The ratios of intensities were compared to the standard curve acquired from the same experiment by treating the cells with defined pH buffers supplemented with nigericin (I, Fig 2B, II, Fig. 5A). The pH measurements showed that the α2-MVBs were not markedly acidified during 3 h (I, Fig.2C) and only a subtle drop in pH was detected after

that (II, Fig 5A). As a comparison, the pH of the EGFR structures was clearly acidic already after 1 h and acidification was inhibited with bafilomycin A1 (II, Fig 5A). Also the experiments with Lysotracker Green supported the results of the pH measurements, since the EV1 structures were not positive for the Lysotracker when cells were traced for 4 h (I, Suppl.Fig. 2A).

To characterize the α2-MVBs further, several typical late endosomal markers were labeled together with EV1 or α2β1 integrin. In the early time points (5 or 15 min) no clear association of α2 integrin structures with early endosomal marker EEA1 was seen (II Fig. 4A, II Fig. S1C). Endocytosed fluid phase material targeted for degradation is normally concentrated in LEs and LYs already after 30 min (Griffiths et al. 1989, Humphries et al. 2011). However, when colocalization of integrin with CD63 antigen (CD63), Lamp-1, Rab7 or cation-independent mannose-6-phosphate receptor (CI-MPR) was analysed from 30 min up to 6 h, no significant accumulation of these proteins was detected (II, Fig. 4A-C, II, Fig. S1C-D). Also no clear colocalization between LBPA and EV1 was seen at 1 or 2 h p.i. (I, Fig 2D) and neither was EV1 or integrin colocalizing with fluorescent LDL (Dil-LDL) that was fed to cells overnight to label LEs and LYs (I, Fig 2D, II Fig. S1C).

As the degradative pathway of EGFR is well defined and it is shown to follow a route from EEs to LYs after EGF stimulus (Sorkin & Goh 2008), the EGFR and α2 integrin pathways were studied in parallel. For this purpose, integrins were clustered with EV1 or antibodies on ice and EGFR was stimulated with biotin-EGF simultaneously. When internalization and intracellular pathways were monitored by confocal microscopy, no clear colocalization between the biotin-EGF and the α2-MVBs was detected at 5 min or 2 h. However, these structures were often seen in close contact with each other (I, Fig. 8A). Similar results were also observed when non-biotinylated EGF was used and EGFR was labeled post fixation (I, Fig. 8B). Quantification of images verified that colocalization between α2 integrin and EGFR was low and the amount of EGF (1 ng/ml or 100 ng/ml) did not have any effect on colocalization (I, Fig. 8C). Also a lung carcinoma cell line, A549, expressing higher amounts of EGFR, gave similar results (I, Fig. S5A).

Altogether these results showed that although α2-MVBs have similar appearance as LEs, they are devoid of classical endosomal or lysosomal markers and are not markedly acidified during their biogenesis.

5.1.2 ESCRTs promote multivesicularity of EV1 structures

As biogenesis of MVBs is shown to involve ESCRT complexes and associated proteins (Williams & Urbe 2007), it was studied if the same machinery is also needed for the formation of the α2-MVBs. One crucial and final element in the formation of ILVs is the recruitment of VPS4 on the endosomal membranes that catalyses the dissociation of ESCRT-III components (Hurley 2008, Wollert et al. 2009). Dominant-negative (DN) VPS4 (VPS4-E235Q-GFP) has been shown to reduce the number of ILVs and cause accumulation of clathrin to the endosomal membranes (Sachse et al. 2004). Overexpression of this mutant causes the so

called mammalian class E phenotype of LEs with swollen endosomes containing only few ILVs (Fujita et al. 2003). To test the role of VPS4 in EV1 infection, cells were transfected with the VPS4-E235Q-GFP or with the control GFP plasmid for 48 h before infection. Overexpression of the DN mutant inhibited EV1 infection by 64% compared to the control (I, Fig 3A, 3B). VPS4-E235Q-GFP caused accumulation of EV1 in cytoplasmic structures whereas in control cells the cytoplasm was full of newly produced capsid proteins (I, Fig. 3C).

The DN VPS4 overexpressing cells showed enlarged DN VPS4-GFP structures whereas clustered integrin was found scattered in small cytoplasmic vesicles at 2 h (I, Fig 3D). Some integrin vesicles seemed to be attached around the limiting membrane of the DN VPS4-GFP structures. Detailed analysis of the integrin structures by electron microscopy showed that in control cells 45% of the integrin containing structures were multivesicular after 2 h whereas in DN VPS4 transfected cells only 18% contained ILVs (I, Fig.3E). Typically integrin structures in VPS4-E235Q-GFP transfected cells were small and contained no ILVs (I, Fig 3F). In addition some multivesicular structures with untypical appearance could be found. Compared to control, these structures had fewer ILVs and integrin was found only at the limiting membrane (I, Fig 3F). VPS4-E235Q-GFP and integrin containing structures were also positive for EV1 (I, Fig 4A) and they lacked the late endosomal marker CI-MPR (I, Fig 4B), which ensured that these MVBs are not just an artifact caused by overexpression.

To study the function of ESCRT components in EV1 infection and α2-MVB biogenesis, siRNAs against ESCRT-I components tumor susceptibility gene 101 protein (TSG101) and VPS37A and ESCRT-III protein VPS24 were used. TSG101 siRNA caused modest and statistically insignificant drop in EV1 infectivity whereas VPS37A and VPS24 blocked infection more efficiently (43% and 50% respectively) (I, Fig. 5B). Infection percentages were calculated from cells cotransfected with siGLO transfection indicator (I, Fig 5A). However, RT-qPCR analysis revealed that siRNAs were able to block mRNA only partially (35%, 52% and 28% for TSG101, VPS37 and VPS24). Due to low transfection and siRNA efficiency in SAOS-α2β1 cells, A549 cells were also tested. In A549 cells, higher siRNA efficiency was achieved with TSG101 (82%) and VPS24 (81%) siRNAs but not with VPS37 (I, Fig 5C). TSG101 and VPS24 silencing decreased the infection by 52% and 50%, respectively, compared with control infection, whereas VPS37A siRNA showed only minor block by 22% (I, Fig. 5B). In siRNA-treated cells EV1 remained typically in small cytoplasmic vesicles as was also observed in DN VPS4 cells (I, Fig. 5D).

To support the previous functional studies with ESCRT components, colocalization studies with clustered integrin was performed. Hrs is an ESCRT-0 component that initiates ILV formation by binding ubiquitinated proteins (Nickerson et al. 2007, Hurley 2008, Raiborg & Stenmark 2009). Hrs-GFP showed increasing colocalization with integrin during the 2 h follow-up period (I, Fig 6A). Also immunofluorescence labelings with VPS37A and VPS24 at 30 min after integrin clustering provided evidence that ESCRT components are recruited to α2 integrin structures (I, Fig 6B and C). Localization of Hrs, VPS37A

and VPS24 was also measured by double labeling using cryo-immunoelectron microscopy (I, Table 1 and Fig. 7). Integrin structures showed low but significant labeling for all labeled ESCRT components when compared to the control (I, Table 1.).

5.2 α2β1 integrin is degraded in a calpain-dependent process

5.2.1 α2-MVBs are degradative structures

As α2-MVBs were shown to be distinct from the classical LEs, it was further investigated what happens to integrins after they are targeted to these structures. First, the intensity of the integrin signal was analysed from confocal microscopic images. In unclustered cells, α2 integrin showed a diffuse labeling on plasma membrane with only few cytoplasmic vesicles (II, Fig. 1A). When integrins were clustered with EV1 or by a sequential treatment of primary and secondary antibodies, most of the integrin was internalized into cytoplasmic vesicles (II, Fig. 1B-C). Clustering caused over 60% decrease in the amount of the fluorescence signal (II, Fig. 1B-C), whereas in unclustered control fluorescence remained at the same level between 2 and 6 h (II, Fig. 1A). Half-life of the unclustered α2 integrin, determined from metabolically labelled cells, was over 24 h (II, Fig. 1D). To verify that integrin itself and not only the fluorescent conjugate was diminished after clustering, integrin was clustered with one antibody and the remaining integrin was post-labelled after fixation with an antibody that binds to another site of the α2 integrin. Quantification of the fluorescence intensity of both labels verified that also the amount of integrin was decreased due to clustering (II, Fig. 1E). To verify the integrin degradation results, integrin turnover was determined also by the cell surface biotinylation assay. After biotinylation of the cell surface, integrins were clustered with antibodies and the amount of the biotinylated integrin was detected from immunoprecipitated samples at 2 or 24 h. Clustering caused a significant decrease of both α2 and β1 integrin at 24 h, whereas in unclustered control cells integrin turnover was much slower (II, Fig. 1F).

5.2.2 Clustered and internalized integrin is not recycled

Since integrins are reported to be long-lived proteins that are constantly recycled (Moro et al. 2004, Lobert et al. 2010), it was further studied if a fraction of the clustered α2 integrin could be targeted back to the plasma membrane. Several approaches were used to address this question. First, anti-Alexa 488 was used to quench the cell surface bound integrin signal with 1 h intervals. Fluorescence signal of the clustered integrin decreased the same manner both in anti-Alexa treated cells and in non-treated control cells (II, Fig. 2). When recycling of the unclustered integrin was studied with same method, the results verified that most of the α2 integrins reside on the plasma membrane (II, Fig.

2B). Anti-Alexa 488-treatment did not reduce the integrin signal after the initial drop, indicating that α2 integrin is not heavily recycled in SAOS-α2β1 cells. In addition, a bleaching experiment was performed to study the recycling. For this purpose, cell boundaries were bleached and perinuclear vesicles were left unbleached 2 h after clustering (II, Fig. 2C). Fluorescence recovery was monitored with 1 h intervals from the bleached area but no recovery of fluorescence was detected during 4 h suggesting that integrins are not recycled back to plasma membrane after clustering. Similar results were gained also from electron microscopy calculations (II, Fig. 2D). The amount of integrin conjugated gold patches at the plasma membrane was not increased between 2 and 24 h, and α2-MVBs were located in the same perinuclear area at both time points.

5.2.3 Degradation of integrins is not dependent on lysosomes, autophagosomes or proteasomes

As described above, α2-MVBs seem to avoid extreme acidification that is essential for lysosomal degradation of plasma membrane receptors (Muller et al. 2012). To reveal the machinery involved in integrin degradation, the connection to LYs was studied more carefully. When EGFR activation and integrin clustering were done simultaneously, EGFR signal was decreased between 15 min and 2 h whereas integrin fluorescence increased as the structures became bigger and brighter (I, Fig. 8D-E). In addition, EGF stimulation did not have an effect on the number of α2 integrin structures or EV1 infection, suggesting that EGF stimulation does not enhance or decrease integrin internalization (I, Fig. 8F-G). Furthermore, the effect of the microtubule depolymerizing agent, nocodazole, was tested. Nocodazole is able to block the endosomal trafficking to the perinuclear site and impair lysosomal degradation (Bayer et al. 1998, Baravalle et al. 2005, Mesaki et al. 2011). After nocodazole treatment, EGFR accumulated into peripheral structures after 2 h (I, Fig. 8H) and quantification of fluorescence signal proved that degradation was prevented (I, Fig. 8I). However, despite the nocodazole treatment, localization of EFGR and integrin remained separate (I, Fig. 8I) further verifying that EGFR and integrin use different intracellular pathways and have different kinetics of degradation.

Slightly contradictory to these results, it was observed that bafilomycin was able to slightly increase the pH of α2-MVBs. Therefor it was further checked if bafilomycin could have an effect on EV1 infection or degradation. Interestingly, bafilomycin was able to prevent EV1 infection significantly when added before 2 h p.i. However, if bacilomycin was added after structures had already matured and uncoating had occured, bafilomycin did not have an effect anymore (II, Fig. 5B). The results also showed that bafilomycin had only a small inhibitory effect on integrin degradation (II, Fig. 5C). Collectively, these results suggest that the slight drop in pH can promote EV1 infectivity and degradation.

Moreover, to study the role of lysosomal proteases in integrin degradation, leupeptin was used as an inhibitor to block the function of LYs

(Seglen et al. 1979). Integrin was clustered with a secondary antibody conjugated either to HRP or Alexa dye after which internalization was allowed for 2 or 24 h. Leupeptin treatment had a minor inhibitory effect on degradation when measured by the fluorescence based method (II, Fig. 7A), but no difference to control was detected with the biochemical HRP assay (II, Fig. 7B).

Although LYs did not have a major role in α2 integrin degradation or EV1 infection, autophagosomes are known to be involved in the infection process of some viruses like EV1 related coxsackievirus B3 and B4 (Wong et al. 2008, Yoon et al. 2008). In autophagy, cytosolic material or organelles are surrounded by autophagosomal membrane and finally fused with LYs (Eskelinen & Saftig 2009). First the GFP conjugated autophagosomal membrane protein LC3 was overexpressed in cells. Overexpression of LC3-GFP did not enhance EV1 infection but instead had an inhibitory effect compared to GFP transfected cells (II, Fig. 6C). It was further studied if EV1 infection could increase the amount of LC3-GFP structures in cells, but there was no increase in the number of LC3-GFP structures compared to non-infected cells (II, Fig. 6D). In addition, no apparent colocalization between the LC3-GFP and α2 integrin was detected at 2, 6 or 24 h after clustering (II, Fig. 6E). Furthermore, starvation that is an inducer of autophagy was not able to promote EV1 infection, but was instead inhibiting it (II, Fig. 6F). Although these results cannot completely rule out the possible role of autophagocytosis, these results suggest that EV1 infection or integrin degradation do not essentially need the autophagosomal system.

Because α2 integrin degradation or EV1 infection had no major connection to the lysosomal pathway and degradation, the role of proteasomes in EV1 infection and integrin degradation was studied. While proteasomes are responsible for degradation of cytosolic and misfolded newly synthetic proteins, they are also reported to be involved in degradation of some plasma membrane proteins (Longva et al. 2002, Bruns et al. 2010). Two proteasomal inhibitors, lactacystin and bortezomib, were unable to block the degradation of α2-conjugated HRP (II, Fig. 6A) or EV1 infection (II, Fig. 6B), suggesting that proteasomal activity was not involved in α2-MVB function.

Taking all the results together, they suggest that α2 integrin degradation and EV1 infection are not dependent on lysosomal, autophagosomal or proteosomal degradation. Since bafilomycin and leupeptin had minor effects on degradation and virus infection, some mixing with the lysosomal pathway may still be possible.

5.2.4 Calpains contribute to integrin degradation

Previously, EV1 infection was shown to be dependent on calpains (Upla et al. 2008). When other cellular protease inhibitors, in addition to leupeptin, were tested, only calpain inhibitors were able to prevent integrin degradation (II, Fig. 7A-B, II, Fig. S2C). Calpeptin, an inhibitor of both major calpain isoforms, was able to totally block the degradation of integrin when measured both by fluorescence or HRP based assays (II, Fig. 7A-B). Quantification from confocal images showed that in control cells the fluorescence intensity of α2-MVBs was

abolished almost totally after 24 h (II, Fig. 7A). Leupeptin treated cells showed partial inhibition of degradation whereas calpeptin treatment prevented degradation totally. When integrin degradation was determined by measuring the amount of the HRP conjugate, results showed that after 6 h only 40 % of the HRP was left and it was totally degraded after 24 h in control cells (II, Fig. 7B). Calpeptin was able to prevent HRP degradation totally but, in contrast to the fluorescence results, leupeptin could not block the degradation at all. In addition to calpeptin, also specific calpain-1 and calpain-2 inhibitors were able to block degradation (II, Fig. S2C). Inhibition of degradation was not due to a block in the biogenesis of α2-MVBs since when calpeptin was added on cells 2 h after clustering, when most of the structures are already multivesicular, degradation was still inhibited (II, Fig. S2D).

Since HRP and fluorescence assays are indirect methods, it was further studied if integrins can be proteolysed by calpains *in vitro*. Metabolically labelled and immunoprecipitated integrins were treated with calpain-1 or -2 for 5 and 60 min. Both protease isoforms were able to degrade α2 integrin as detected by the disappearance of the 160 kDa band and by appearance of a new ~27 kDa band (II, Fig. 7C). In addition, degradation increased with prolonged treatments with calpains and calpeptin was able to inhbit the proteolysis. An inhibitory effect of calpeptin was also seen in immunoprecipitated, clustered samples. In calpeptin treated cells more integrin was left after 6 and 24 h compared to control cells, suggesting that calpeptin could inhibit the degradation of α2 integrin (II, Fig. 7D).

Next, it was studied if calpains were present in the α2-MVBs. Integrins were immunopecipitated with protein A-sepharose in various ways: either directly via the secondary antibody introduced already in the clustering, or via the polyclonal α2 or monoclonal β1 integrin antibodies added to the cell lysate. Integrin clustering did not cause changes in the amount of calpain-1 associated with total α2 integrin pool (II, Fig.8), but EV1 seemed to cause disappearance of a higher molecular weight form of calpain-1 that was present in unclustered samples. The disappearance of the 80 kDa form of calpain could be due to calpain activation. When only the pool of clustered integrin was immunoisolated, calpains were associated with the α2 integrins after internalization and were enriched after 2 h of clustering. In addition to immunoprecipitation studies, calpain association with integrin was studied by immunolabeling thin frozen sections. Cryo-immunoelectron microscopy verified that calpain-2 was present in α2-MVBs after 2 and 6 h of clustering. Quantification of the associated calpain-2 label showed significant labelling in both time points in α2-MVBs against control labelling (II, Fig. 9A). Furthermore, calpain activation was studied by using a fluorogenic calpain substrate 7-amino-4-chloromethylcoumarin, t-BOC-l-leucyl-l-methionine amide (t-BOC), which becomes fluorescent upon activation by calpains. Clustering-induced calpain activation was detected after 6 h as an increased total fluorescence caused by proteolysis of t-BOC by calpains (II, Fig.9B), whereas in earlier time points (30 min and 2 h) overall fluorescence signal stayed at the basal level. Calpain activation was inhibited by calpeptin, which was seen as low

fluorescence intensity in cells (II, Fig. S3A). t-BOC label associated with α2-MVBs already after 30 min, although overall fluorescence was still at a low level (II, Fig. S3A). Quantification of colocalization at 2 h showed approximately 80% association between t-BOC and α2 integrin (II, Fig. 9C). Together these results suggest that calpains are associated with α2-MVBs and promote the degradation of integrin and its cargo after clustering and internalization.

5.2.5 Collagen uptake induces α2β1 integrin endocytosis

α2β1 integrin has been shown to bind to collagen fibers in the extracellular matrix, thus fixing the cell to its surroundings (Jokinen et al. 2004). In addition, α2β1 integrin has been shown to act in phagocytic collagen uptake (Arora et al. 2003). In contrast, the uptake of soluble collagen has been shown to involve uPARAP whereas α2β1 integrin is not essential to this uptake (Engelholm et al. 2003, Shi et al. 2010, Madsen et al. 2011). However, as α2β1 integrin can also bind strongly the soluble collagen monomers (Knight et al. 2000, Jokinen et al. 2004), it was studied if the uptake of soluble collagen could represent the physiological pathway that EV1 has learned to utilize. Cells were plated on coverslips coated with soluble collagen and internalization of collagen was allowed for 2, 6 or 24 h. In control cells, collagen was internalized into cytoplasmic vesicles with increasing number from 2 to 24 h (II, Fig. 10A). Interestingly, α2β1 integrin was also found in cytoplasmic vesicles, whereas the integrin label in cells cultivated on plastic at steady state showed only few cytosolic vesicles, most of the integrins being present at the plasma membrane (II, Fig. 10A-B). The number of cytosolic integrin vesicles increased between 2 and 6 h but, strikingly, these vesicles disappered after 24 h (I, Fig. 10A). Double labelings showed that integrin colocalized at least partially with internalized collagen, and after 6 h colocalization was more abundant than at 2 h. When cells were treated with calpeptin, integrins accumulated in the cytoplasmic structures and no decrease in the integrin signal was detected. Furthermore, the integrin surface-labeled with fluorescent monovalent Fabs was followed after plating cells on plastic and collagen. At 2 h after the surface labeling, integrins were detected only in cytoplasmic vesicles, whereas cells grown in plastic showed more plasma membrane labeling (II, Fig. 10B). Moreover, to see if internalized collagen was targeted to lysosomes, Lamp-1 was labelled in addition to collagen (II, Fig. S3B). Similarly as with α2β1 integrin, collagen did not colocalize with Lamp-1 and calpeptin did not cause a change in their colocalization.

These results suggest that collagen uptake stimulates α2β1 integrin internalization and leads to partial colocalization of integrin and collagen in cytoplasmic vesicles. Integrin seems to be targeted to degradation in a similar calpain dependent manner as seen after EV1- or antibody-induced integrin clustering. Altogether, these results propose that collagen uptake pathway might function as a physiological route that EV1 exploits.

5.2.6 Echovirus1 increases the membrane permeability of α2-MVBs

As calpains and their activation were shown to contribute to integrin degradation, a central question to ask is how cytosolic calpains are able to promote the degradation. One possible mechanism is the formation of breakages on the limiting membrane of endosomes, which would allow calpains to enter the endosomal lumen where EV1 and the luminal domain of α2β1 integrin reside. Results of our unpublished transmission electron microscopic studies have shown that EV1-induced α2-MVBs contain ruptures in the limiting membranes and also in the membranes of ILVs after 2 h p.i. (Soonsawad, unpublished). Antibody-clustered α2-MVBs were used as a control to specifically compare to specific virus-induced changes: control clustering showed a low amount of breakages at 2 or 3.5 h but after 24 h these structures also showed ruptures.

To verify the presence of ruptures in EV1-induced structures, a modified permeability assay described previously was used (Robinson & Kreis 1992). A rapid freezing was used to permeabilize the plasma membrane while cytoplasmic structures were left intact. Permeabilization was confirmed by Trypan blue labeling (data not shown). αV integrin structures, induced by antibody clustering, were used as a control to ensure that cytoplasmic structures remained intact (Fig. 8). Triton X-100 was used as a positive control. Permeabilized cells were labelled with fluorescent Fabs against anti-αV integrin antibody that was introduced to cells in the clustering. Results showed no permeability of αV integrin structures.

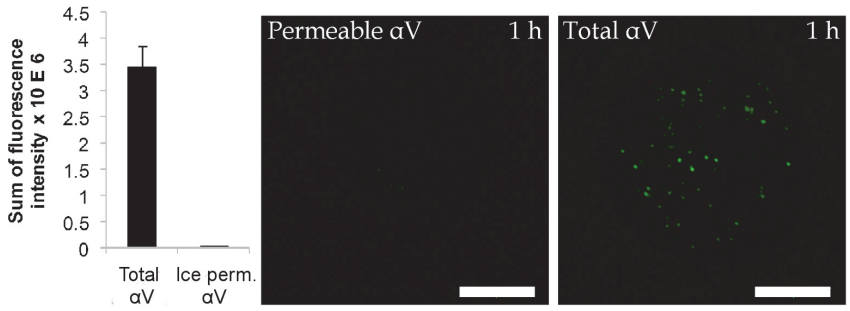

FIGURE 8 Antibody clustering-induced αV integrin structures are intact. 1 h after clustering cells were permeabilized with ice or Triton X-100 (Total αV) and treated with fluorescent Fab antibody fragments. Fluorescence intensity of Fab fragments bound to clustering αV integrin antibody after permeabilization was quantified from confocal z-stacks. Altogether 30 cells from three independent tests were calculatated. Representative images of cells treated with fluorescent Fab fragments bound to clustering αV integrin antibody after ice (left) or Triton X-100 (right) permeabilization are shown. Bars 10 μm.

To evaluate the permeability of EV1-induced structures, ice or Triton X-100 permeabilized cells were labelled with fluorescent Fabs against anti-EV1 antibody that was introduced to cells before EV1 internalization. Results

showed low permeability of α2-MVBs at 1 h p.i., whereas increased permeability was detected after 2 and 3 h p.i. compared to non-permeabilized control cells (Fig. 9a). Altogether 40% of the total EV1 signal was bound by Fab fragments at 3 h p.i. suggesting that almost half of the virus-containing structures showed permeability at 3 h. α2-MVBs induced by antibody clustering showed only a low level of labeling after 1, 2 or 3 h of clustering but a small increase was detected between 2 and 3 h post internalization (Fig. 9b).

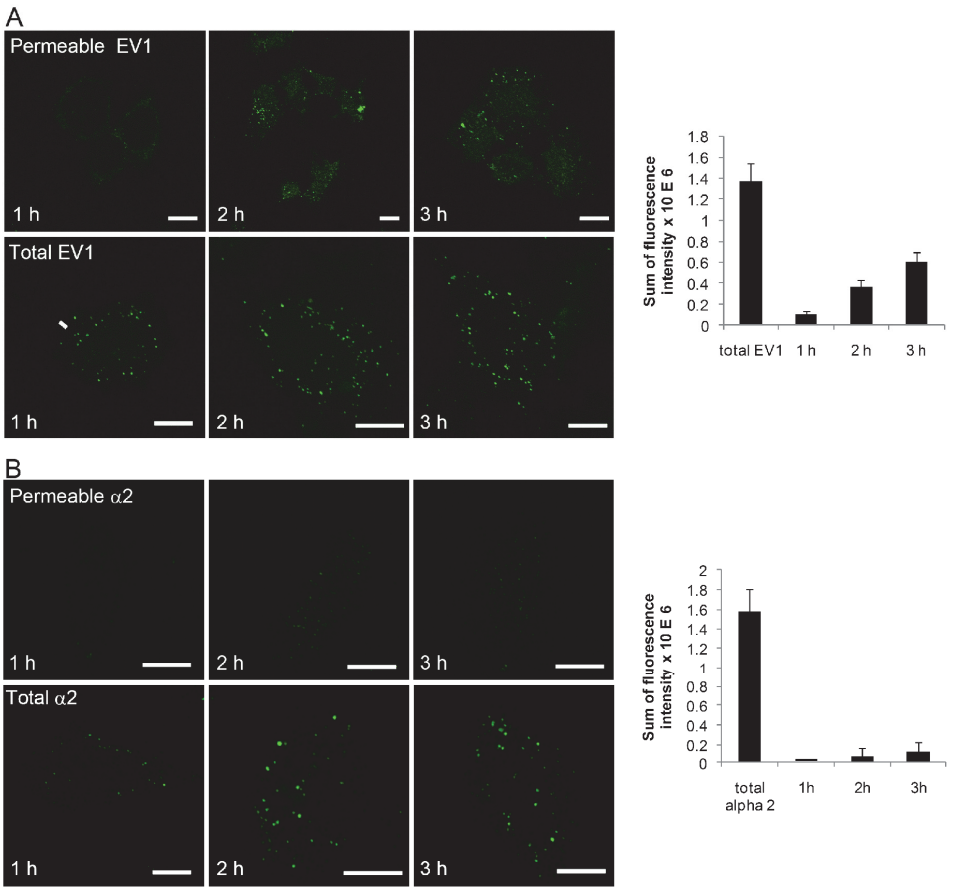

FIGURE 9 Permeability of α2-MVBs increases during EV1 infection. Fluorescence intensity of Fab fragments bound to EV1 (a) or α2-integrin (b) due to permeability of the endosomes was measured from confocal z-stacks. Surface label, measured from non-permeabilized control cells, was subtracted from the results of cells permeabilized on ice. Total intensity refers to the fluorescence intensity detected after permeabilization of all membranes with Triton X-100. Representative images showing permeability or total fluorescence are shown. Altogether 30 cells from three independent tests were analysed. Results are mean values ±S.E. Bars, 10 μm.

5.3 Intact cholesterol environment both on the plasma membrane and in α2-MVBs is needed for integrin ligand uptake and EV1 infection

5.3.1 Integrin internalization and EV1 infection are dependent on cholesterol

α2β1 integrin has been shown to associate with DRMs together with GPI-APs at the plasma membrane in SAOS-α2β1 cells (Marjomäki et al. 2002, Upla et al. 2004). Due to this close association with cholesterol-rich membranes, the role of cholesterol on the α2β1 integrin internalization process was studied in more detail. Two cholesterol sequestering drugs, filipin and nystatin, were used to perturb cholesterol function during integrin internalization. Pretreatment of cells with these drugs left antibody-clustered integrin on the plasma membrane (III, Fig. 1A). Calculations of the ratio between the surface-derived versus internalized integrin confirmed that both drugs halted integrin traffic at the plasma membrane. However, despite the drug treatments, integrin clustering on the plasma membrane seemed not to be inhibited.

Since EV1 is shown to cointernalize with integrin after binding (Marjomäki et al. 2002, Upla et al. 2004), it was hypothesized that filipin and nystatin would also inhibit the infection. In addition, in CV-1 (African green monkey kidney) cells EV1 infection was inhibited totally by cholesterol depletion with methyl-β-cyclodextrin (mβCD) and partially with a combined progesterone and nystatin treatment (Pietiäinen et al. 2004). This was the case also in SAOS-α2β1 cells. Both drugs halted infection completely and confocal images showed that EV1 remained on the plasma membrane (III, Fig. 1B). These results thus indicate that intact cholesterol on the plasma membrane is needed for internalization of α2β1 integrin after clustering with antibodies or EV1.

5.3.2 Disturbance of cholesterol synthesis prevents integrin internalization and EV1 infection

In addition to cholesterol sequestering drugs, another approach was used to study the role of the cholesterol. Ketoconazole is a drug that prevents cholesterol biosynthesis at the level of the earliest sterol precursor, lanosterol (Van den Bossche et al. 1980, Buttke & Chapman 1983, Iglesias & Gibbons 1989). Biochemical lipid assay verified that after 3-day ketoconazole treatment 37% of total cellular sterol content was lanosterol and the rest was cholesterol (III, data not shown). Lipoprotein-deficient serum (LPDS) treatment alone caused 13% depletion in total cholesterol content of SAOS-α2β1 cells (III, Fig. 2A). Next α2 integrin internalization in ketoconazole treated cells was determined. After 2 h internalization, in normal serum or LPDS conditions integrin was typically efficiently internalized to cytoplasmic vesicles while some integrin was left on the plasma membrane. In contrast, ketoconazole treatment led to integrin accumulation on the plasma membrane, which was seen as strong colocalization between the clustered and surface labelled integrin (III, Fig. 2B).

Quantification of the ratio between the surface-derived versus internalized integrin showed a 2-fold increase due to ketoconazole treatment compared to normal serum or LPDS conditions without the drug. When ketoconazole treated cells were studied using higher resolution, clearly less internalized integrin was detected inside the cells compared to control conditions (III, Fig. 2C). In cells treated with the LPDS medium, integrin was found in multivesicular structures already after 30 min, and after 3.5 h structures contained more ILVs as reported also previously (Karjalainen et al. 2008). Instead, the few found cytoplasmic structures in ketoconazole treated cells, were tubular-like structures that are typical at the beginning of the biogenesis of α2-MVBs (Karjalainen et al. 2008). These results suggest that in addition to inhibition of integrin internalization, the biogenesis of integrin structures is prevented by ketoconazole treatment.

In addition to inhibition of integrin internalization, EV1 infection was also inhibited by 65% when cholesterol biosynthesis was disturbed compared to control cells (III, Fig. 2D). Interestingly, also starvation due to cultivation in 5% LPDS medium caused a 35% decline in infectivity (III, Fig. 2D). However, when lipoprotein-deficient cells were overloaded with mβCD-cholesterol, the infection was blocked completely (III, Fig. 2E). This could be due to overloading of cells with cholesterol and making membranes too rigid (Ohvo-Rekilä et al. 2002). Similar overloading of the cholesterol to LEs and LYs can be achieved by treating cells with U18666A (Liscum & Faust 1989), as was also seen in this study by accumulation of filipin stain into Lamp-1 containing structures (III, Fig. 2F). This treatment did not have an effect on EV1 infection, which was not a surprise since EV1 is not found in the conventional LE/LY pathway (II, Fig. 4A-C, II, Fig. S1C-D).

Cholesterol-rich microdomains contain also sphingolipids (Lingwood & Simons 2010). Sphingolipid synthesis was perturbed with Fumonisin B_1 (Wang et al. 1991) to see if also sphingolipids are important in the clustering-induced integrin internalization pathway. Pretreatment of cells with Fumonisin for 48 h caused a small but significant decline in infection (15%), which implies that also sphingolipids are contributing to the EV1 infection pathway (III, Fig. S1).

5.3.3 Cholesterol-rich areas are important for soluble collagen uptake

As it was found that soluble collagen causes internalization of α2-MVBs and triggers an internalization pathway closely resembling the EV1 internalization pathway (II Fig. 10A-B, S3B), it was further studied if the uptake of soluble collagen is also dependent on cholesterol. In order to do this, cells were treated with the drug inhibiting cholesterol synthesis, ketoconazole, for 3 days. In control cells, collagen was found in intracellular structures without a clear accumulation to the cell surface after 6 h (III, Fig. 3A). Instead, in ketoconazole treated cells collagen showed a strong accumulation to the cell surface (III, Fig. 3A). The calculations of the number of the cells, showing either vesicular or cell surface phenotype, confirmed that ketoconazole treatment caused a 5-fold decrease in the vesicular phenotype. Futhermore, to reveal the extent of

internalization, collagen was labeled before and after permeabilization (III, Fig. 3B). In control cells, collagen was mostly in intracellular vesicles and showed only a minor labeling at the cell surface (III, Fig. 3B). Cells treated with 5% LPDS showed more plasma membrane labeling than cells treated with normal serum. Ketoconazole caused even more evident collagen accumulation at the cell surface, which was confirmed also by image analysis. Quantifications showed 5-fold decrease in internalization caused by the ketoconazole treatment similar to the phenotype calculations.

When α2β1 integrin localization was compared with collagen labeling, it was observed that collagen internalization induced α2β1 integrin localization to endosomal structures and these vesicles colocalized partially with collagen (III, Fig. 4). These results were in line with the results obtained in the degradation studies (II, Fig. 10A). As ketoconazole treatment led to collagen accumulation at the cell surface, also integrin accumulated in the same surface domains without significant internalization between 2 and 6 h (III, Fig. 4).

In conclusion, these results indicate that normal cholesterol environment is needed for the uptake of soluble collagen and for simultaneous collagen-induced endocytosis of α2β1 integrin.

5.3.4 EV1 infection and uncoating relies on intact cholesterol domains in α2-MVBs

Since some endosomal structures have been shown to contain domains with raft-like components (Gagescu et al. 2000, Sobo et al. 2007), it was studied, by cold Triton X-100 treatment if the formed multivesicular structures contain DRMs. Cells grown on coverslips were clustered with α2 or αV integrin antibodies and internalized for 2 h before the Triton X-100 treatment. α2 integrin structures remained unaffected by the treatment (III, Fig. 5) suggesting that α2-MVBs are rich in DRMs. Instead, αV integrin structures that are internalized via a different pathway (Upla et al. 2004) were affected (III, Fig. 5). Quantifications of confocal images showed that the Triton X-100 treatment caused a clear decrease in the intensity of αV integrin signal and in the number of structures suggesting that the drugs solubilized the vesicle content (III, Fig. 5B-C). The intensity of α2 integrin signal and the number of α2-MVBs remained stable, but a little decrease in their average size was detected (III, Fig. 5B-D).

Similar experiments were done with filipin. Cholesterol aggregating drug, filipin had no effect on αV structures but α2-MVBs were affected (III, Fig. 5A-D). Calculations showed that the number of αV structures and total αV integrin intensity remained at the same level as in non-treated control cells (III, Fig. 5B-C), only the volume of the αV structures had decreased a little (III, Fig. 5D). Instead filipin caused an increase in the amount of α2-MVBs and a decrease in the volume (III, Fig. 5B-C), however, the intensity of integrin signal remained the same. This suggests that filipin could cause some fragmentation of α2-MVBs. Altogether results of the treatments with Triton X-100 and filipin suggest that α2-MVBs contain cholesterol-rich domains. Moreover, when EV1 infected cells were labeled with filipin at 4 h p.i. when the replication had already

started, most of the viral capsid protein VP1 structures were rich in cholesterol (III, Fig. 5E).

Whether cholesterol-rich domains of internalized structures are essential for EV1 infection was further studied. First, filipin and nystatin were added 5 min p.i. Both drugs inhibited infection after EV1 internalization had started, suggesting that both internalization and maturation of structures are dependent on intact cholesterol environment (III, Fig. 6A). Therefore the time scale of cholesterol dependency of the infection was further studied by adding filipin and nystatin at different times p.i. Both drugs were able to block the infection in the early entry when added from 5 min to 3 h p.i. (III, Fig. 6B). After 4 h, when replication had already started (Upla et al. 2008), the drugs caused only a minor inhibition. In addition, replication structures, labeled with dsRNA, did not accumulate filipin staining (III, Fig. 5B). Together these results imply that EVI replication is not dependent of cholesterol.

Previous studies have shown that the uncoating of EV1 starts around 30 min p.i. (Marjomäki et al. 2002). To see if uncoating of the virus is affected by filipin and nystatin, the detailed schedule of uncoating was determined by neutral red assay. Cells were infected with neutral red-labeled EV1 and treated with light at different time points with the exception of control. If the capsid is intact at the time of light reaction, neutral red dye is crosslinked to the RNA and infection is inhibited (Madshus et al. 1984, Brandenburg et al. 2007). The assay revealed that uncoating is halted totally when RNA crosslinking is executed 5 or 15 min p.i., but it is inhibited only partially after 30 min p.i. (III, Fig. 6C) confirming the previous results that uncoating starts already at this point. When cells were treated with the bright light between 1 and 3 h, most cells were able to produce new virus and after 4 h no difference to control cells was anymore detected. On the basis of these results, uncoating and the effect of cholesterol sequestering drugs was studied also by a sucrose sedimentation assay. Filipin and nystatin were added on cells infected with ^{35}[S] methionine-labeled virus before the uncoating had started, after 15 min p.i. After 4 h cells were collected and uncoated capsids were separated from intact capsids by the sucrose gradient. In the control sample, both intact RNA containing capsids (160 S) and empty capsids (80 S) were detected (III, Fig. 6D). In filipin treated cells, instead, a clear drop in the amount of empty capsids was seen (III, Fig. 6E). Also nystatin reduced the number of empty capsids, but allowed uncoating at a low level (III, Fig. 6F) as seen also in the infection tests (III, Fig. 6A and 6B). Together these studies suggest that uncoating of the virus is dependent on intact cholesterol-rich domains.

Since filipin and nystatin are known to be able to increase membrane permeability for ions (Archer & Gale 1975, Kotler-Brajtburg et al. 1979) and high filipin concentrations are shown to cause fragmentation of endoplasmic reticulum (Axelsson & Warren 2004), it was studied whether this could explain at least partially our results. To this purpose, small fluorescent beads, Fluospheres were cointernalized together with the clustered integrin for 1 h. These particles colocalized well with α2-MVBs as was observed from confocal labelings (III, Fig. 6G). Cells were further incubated for 30 min with filipin. The

intensity of the Fluospheres inside α2-MVBs was determined and a drop of average intensity by 28% was detected (III, Fig. 6G), indicating that filipin can induce some leakeage of Fluospheres from α2-MVBs. Therefore the changes in the ion balance are possible and could contribute to the changes in viral uncoating.

6 DISCUSSION

6.1 α2β1 integrin clustering-triggered endocytic route differs from the classical acidic endosomal pathway

Classically it is thought that endocytosed transmembrane proteins are degraded in acidic LEs/LYs (Pillay et al. 2002, White et al. 2006, Luzio et al. 2009, Bruns et al. 2010). In this work, it was found that structures that are induced by integrin clustering are separate from the conventional EEs, LEs and Lys (Fig. 10). However, these structures were shown to be able to degrade the clustered integrin. Firstly, it was shown here with intraendosomal pH-measurements that internalized α2 integrin structures were not acidified during the first 3 h and only a minor drop in pH could be seen in the longer timescale from 4 to 6 h (I, II). Secondly, structures were devoid of the typical endosomal markers EEA1, Lamp-1, Rab7, LBPA, CD63 and CI-MPR (I, II) and thirdly, stimulated EGFR showed separate intracellular localization and different degradation kinetics when it was activated simultaneously with the integrin clustering (I). Interestingly, despite the differences to acidic LEs, the biogenesis of α2-MVBs is dependent on the same ESCRT machinery as conventional multivesicular structures (I).

Evidence for distinct endosomal pools in cells has been reported also by others. Lakadamyali et al. divided Rab5-positive EEs into two distict populations on the basis of their kinetics (Lakadamyali et al. 2006). The more dynamic endosomes received proteins that were targeted for degradation, whereas the proteins to be recycled were guided to the more static pool (Lakadamyali et al. 2006). In comparison, there are also reports of specific endosomes that are selective for certain receptors or ligands. Miaczynska et al. reported an endosomal subcompartment that contained a Rab5 effector, APPL-1, but was distinct from EEA1, Tf and dextran labeled endosomes (Miaczynska et al. 2004). In neuronal cells there is also a specialized early endosomal compartment containing neuron-enriched endosomal protein 21 (NEEP21) that does not colocalize with Rab5 and EEA1 (Lasiecka & Winckler 2011). In

addition, existence of distinct late endosomal pools were reported as the stimulated EGFR is targeted to LYs via a MVB subcompartment that does not colocalize with LBPA containing ensosomes (White et al. 2006). Moreover, specialized late endomal/lysosomal structures are present in certain cell types like melanosomes, lytic granules, MHC class II compartments, platelet-dense granules, basophilic granules and azurophil granules (Dell'Angelica et al. 2000). These organelles contain specific proteins needed for their function and some late endosomal/lysosomal markers too, but not necessarily all of them (Dell'Angelica et al. 2000). Interestingly, it was shown in antigen presentating dendritic cells that pH of phagosomes is alkanized in a process mediated by Rac2 and NADPH oxidase 2 (NOX2) (Savina et al. 2009). If Rac2 or NOX2 were inhibited, phagosomes were acidified, which reduced the efficient presentation of antigens possibly due to too effective degradation of antigens (Savina et al. 2009). This thus indicates that not all endosomes are necessarily acidic and that neutral endosomes can have special functions in cells.

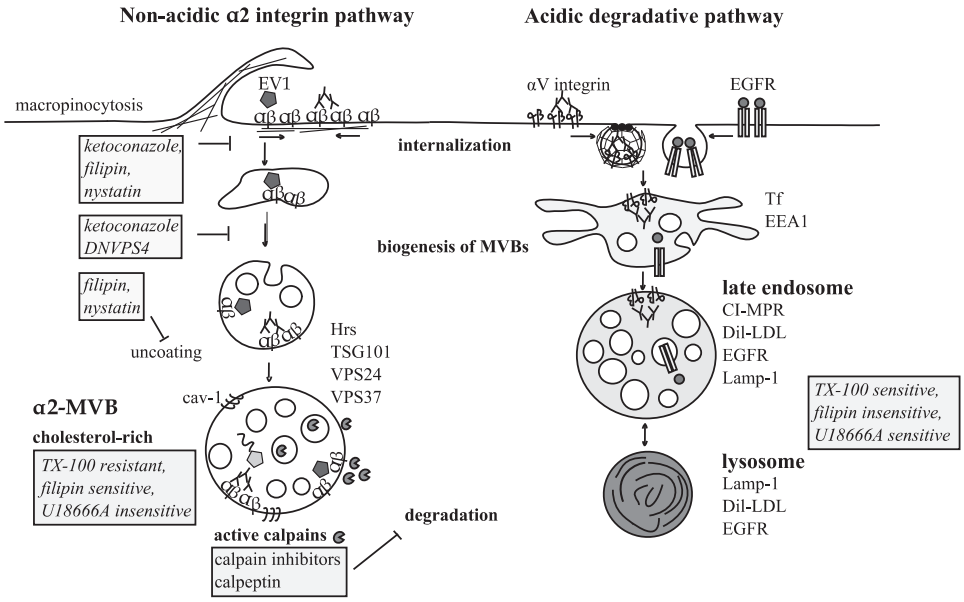

FIGURE 10. Summary of the results. Echovirus 1 and antibody-clustered α2β1 integrins are targeted to multivesicular structures that are distinct from conventional acidic endosomes and their markers. Biogenesis of the structures is dependent on ESCRT machinery and cholesterol-rich membranes. α2β1 integrins are down-regulated in the α2-MVBs independently of lysosomal hydrolases but instead is contributed by calpains. Cholesterol-rich domains at plasma membrane and in α2-MVBs are important for EV1 infection at the internalization step and for the genome uncoating. dil-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanina-labeled LDL; TX-100, Triton X-100.

A central question considering the non-acidic endosomes is what keeps them neutral. Since bafilomycin A1, an inhibitor of V-ATPase, was able to increase the pH of α2-MVBs a little, it is probable that the structures contain these

pumps. Unfortunately, we were not able to evidence the presence of V-ATPase in our studies due to faint and diffuse labelling of used antibody. The function of V-ATPases can be regulated e.g. by the density of the pumps, distribution of V-ATPase isoforms on membranes or by function of other ion pumps or channels (Jefferies et al. 2008, Marshansky & Futai 2008). Therefore it can be speculated that the amount of the V-ATPase might be low in α2-MVBs and/or the structures might have some other ionic pumps or channels that prevent the acidification. Our previous studies showed that drugs inhibiting Na^+/H^+ exchanger inhibited the biogenesis of α2-MVBs and EV1 infection. Also our unpublished observations suggested that these MVBs contained a NHE6-antibody reactive exchanger. It remains to be shown in future studies what is the repertoire of ion pumps or channels in the α2-MVBs, and how they are maintaining the ionic conditions of the structures. E.g. it would be interesting to look at the distribution of Ca^{2+} channels and pumps, since Ca^{2+} could be one factor explaining the results obtained in this study. In addition to regulation effect of pH by cation channels, Ca^{2+} has been shown to be important in mediating the fusion to lysosomes. Furthermore, Ca^{2+} is also needed for the calpain activation.

Targeting to the degradative LYs could be unfavourable for pathogens in general and therefore they have exploited many strategies to avoid these structures (Gruenberg & van der Goot 2006). The acidic pH can function as a trigger to escape the endosomes before the LYs as was shown with e.g. Semliki forest virus and FMDV (Helenius et al. 1982, Berryman et al. 2005). Some bacteria like *Mycobacterium tuberculosis* or *Salmonella* species that enter the cells by phagocytosis or macropinocytosis, are able to prevent the deletorious fusion with lysosomes by modifying cell function with their own effector molecules (Harrison et al. 2004, Li & Xie 2011). Here, we showed that also EV1 avoids the lysosomes since the α2-MVBs are not acidified and they do not contain Rab7 that is essential for the fusion process (Luzio et al. 2007, Huotari & Helenius 2011).

Macropinosomes are reported to mature differently in distinct cells. In macrophages they are able to gain markers of the canonical endosomal pathway and finally fuse with lysosomes (Racoosin & Swanson 1993, Kerr et al. 2006). In some cells, EGF-induced macropinosomes are able to fuse with each other and have only minor colocalization with convential endosomes (Hewlett et al. 1994). They were also suggested to be recycled back to the plasma membrane (Hewlett et al. 1994, Bryant et al. 2007). In our studies here, a high concentration of EGF that is reported to stimulate macropinocytosis, led to the downregulation of EGFR (I). However, although EV1 uses the macropinocytosis pathway for its entry, the created structures lacked the conventional endosomal markers and were distinct from EGF-induced structures (I,II). In addition to EV1, many viruses are known to utilize macropinocytosis or macropinocytosis-like entry as was recently reported with human cytomegalovirus (HCMV) and human polyomavirus type-16 (Haspot et al. 2012, Schelhaas et al. 2012). The entry of HCMV was cholesterol-dependent whereas HPV-16 was cholesterol-independent. HPV-16 travelled through Rab5 but not EEA1-positive endosomes

to Lamp-1 structures, whereas HCMV structures contained EEA1 but remained Lamp-2-negative. Both of the viruses showed very slow entry kinetics and stayed in endosomes for several hours. Altogether these examples, in addition to the studies shown in this thesis, demontrate that the viral entry via macropinocytosis can show a large variety between the viruses.

6.2 Calpains contribute to degradation in the non-recycling integrin pathway

Currently, there are only a few articles concerning the downregulation of integrins. The ECM fibronectin was shown to be internalized together with α5β1 integrin, which leads to fibronectin turnover in the lysosomes (Shi & Sottile 2011). Whether the co-internalized integrin is also degraded is not known (Shi & Sottile 2011). Another study proposed that integrin itself could be ubiquitinated and targeted to the lysosomal degradation, when cells are plated on soluble fibronectin (Lobert et al. 2010). In comparison, there is much more evidence about recycling of integrins than degradation (Pellinen & Ivaska 2006, Caswell et al. 2009). In our studies here, integrin clustering by virus or antibodies led to the integrin degradation without any detectable recycling (II). The degradation pathway was not connected to the classical late endosomal/lysosomal pathway and avoided proteolysis by the lysosomal hydrolases (II). Instead, calpains were shown to promote the degradation, which was proved by several experiments (II).

Calpains are central proteases that regulate cell migration and they have several substrates in focal adhesion sites (Franco et al. 2004, Franco & Huttenlocher 2005, Chan et al. 2010). Calpains are known to cleave the cytoskeletal linkage proteins, like talin, FAK and paxillin and thus promote the disassembly of FAs (Franco et al. 2004, Chan et al. 2010, Cortesio et al. 2011). Also β integrin tails, that mediate the adhesion to the actin cytoskeleton, can be cleaved by the calpains (Du et al. 1995, Pfaff et al. 1999). In addition to the cleavage of β integrins, our study showed that calpain-1 and -2 are able to proteolyse also the α2 subunit *in vitro* and that proteolysis can be prevented by calpain inhibition (II). Protease inhibitor studies have previously suggested that the β1 integrin downregulation is mediated by calpains, in addition to lysosomal degradation, in normal prostate cells (Moro et al. 2004). Here, calpain inhibition, both by using calpeptin or isoform specific inhibitors, inhibited the degradation of α2β1 integrins, whereas the lysosomal protease inhibitor, leupeptin had only a minor effect (II, Fig. 10). The inhibitory effect of calpains was not due to prevention of biogenesis of α2-MVBs, since calpeptin was able to inhibit the degradation also when added after α2-MVBs were formed (II).

Acidicity of the LEs has been shown to be crucial for protein degradation (Bayer et al. 1998, Baravalle et al. 2005, Mindell 2012). The acidic hydrolases can be abundant already at the early endosomes but in general, they are not stable

or active in neutral or slightly acidic environment. Instead, many acidic hydrolases become activated only at acidic pH and have a low pH optimum (Pillay et al. 2002). Lysosomal protein degration is restricted to the lumen of membrane bound acidic organelles where conditions are optimal for degradation: lysosomal hydrolases are stable and active, whereas their targets are unstable and denatured (Repnik et al. 2012). In contrast to the acidic hydrolases, calpains have a neutral pH optimum and they are present in the cytosol (Goll et al. 2003). Therefore, the non-acidic or slightly acidic pH of α2-MVBs enables the calpain-mediated degradation rather than degradation by lysosomal hydrolases (II).

However, as the degradation of the luminal domain of integrin was also detected and not only the tail, it raised a question how calpains reach the endosomal lumen. Our unpublished results (Soonsawad et al., unpublished) and the permeability assay in this thesis suggest that EV1 infection is associated with breakages in the limiting membrane of α2-MVBs, which could provide a portal for calpains to reach the luminal side of integrins. The breakages are also needed for EV1 genome release to the cytoplasm since the uncoating of virus is known to occur inside the α2-MVBs (Pietiäinen et al. 2004). Similarly, some other picornaviruses are suggested to release their genome first inside the endosome from where it is then released to the cytosol (Tuthill et al. 2010). The breakages can be either small, pore-like openings as with human rhinovirus serotype-2 (Prchla et al. 1994, Schober et al. 1998, Brabec et al. 2005) or alternatively the whole endosome can dissociate as shown with human rhinovirus serotype-14 or adenovirus (Schober et al. 1998, Meier & Greber 2004, Brabec et al. 2005).

Since calpains are constantly in contact with their substrates in the cytosol, their activation must be highly regulated (Goll et al. 2003). The first requirement for calpain activation is the increased Ca^{2+} concentration in the cytosol (Goll et al. 2003, Friedrich 2004, Hood et al. 2006). Overall Ca^{2+} concentration in the cytosol is clearly under 1 μM, whereas calpain activation *in vitro* requires about 10-1000 times higher Ca^{2+} concentrations (Friedrich 2004, Hood et al. 2006). Since such Ca^{2+} levels are unphysiological in cells, the need for Ca^{2+} is lowered by the calpain association with membranes (Friedrich 2004, Hood et al. 2006). It was shown here that the calpains were associated with α2-MVBs and they were activated after the integrin clustering (II). The increase of the activation status of calpains was similar as shown earlier with EV1 infection (Upla et al. 2008), myocardial cells after injury (Matsumura et al. 2001) or when calcium is released from ER with thapsigargin (Rosser et al. 1993). Sometimes calpain activation is coupled to calpain autolysis (Suzuki & Sorimachi 1998, Goll et al. 2003, Franco & Huttenlocher 2005, Hanna et al. 2008). Autolysis can be detected in SDS-PAGE as a decrease of the molecular weight of calpain from 80 kDa protein to 78 or 76 kDa form (Hathaway et al. 1982, Zimmerman & Schlaepfer 1991, Schoenwaelder et al. 1997). When β1 integrins were immunoprecipitated from uninfected or EV1 infected cells, a difference in the molecular weight was detected upon integrin clustering. The 80 kDa form of calpain-1 was detected from untreated cells whereas it was absent from EV1 treated samples (II). These

results together suggest that calpains are autolysed and activated during EV1 infection.

6.3 Specific cholesterol environment is important for EV1 infection

6.3.1 Cholesterol-dependency of EV1 internalization

Cholesterol has been shown to be important for infection of various viruses and during different phases of infection including internalization, uncoating, replication and assembly (Chazal & Gerlier 2003, Mercer et al. 2010, Vieira et al. 2010). The lipid rafts and caveolae that are abundant in cholesterol are entry sites for many viruses such as for simian virus 40 (Anderson et al. 1996, Stang et al. 1997, Damm et al. 2005), human papillomavirus type-31 (Smith et al. 2007) and FMDV (O'Donnell et al. 2008). EV1 was previously thought to internalize via caveolae since it was isolated together with caveolin-1 and it was seen in caveolae resembling invaginations (Marjomäki et al. 2002). However, more detailed research revealed that caveolin-1 is not needed in the very early internalization step, but it is accumulated gradually to the internalized structures (Pietiäinen et al. 2004, Karjalainen et al. 2008). Cholesterol depletion with mβCD or lovastatin is reported to disturb the infection with many viruses including both enveloped viruses like herpes simplex virus (Gianni et al. 2010, Rahn et al. 2011) or human immunodeficiency virus-1 (Carter et al. 2009) and non-enveloped viruses such as echovirus 11 (Sobo et al. 2011) and EV1 (Marjomäki et al. 2002). It was shown here that plasma membrane cholesterol is needed for EV1 internalization, since cholesterol sequestering drugs and disturbance of cholesterol synthesis halted the virus and its receptor to the cell surface (III, Fig. 10). Interestingly, the LPDS treatment alone was able to prevent partially the EV1 infection (III), which could be due to the fact that LPDS treatment alone can cause a reduction of cholesterol as was seen in our results (III) and by others (Martin et al. 1993, Calleros et al. 2006, Dorobantu et al. 2011). Additional treatment with cholesterol did not restore infection, but instead prevented the infection. Actually, it has been shown that the addition of cholesterol may not only cause replenishment of cholesterol levels but it may cause the enrichment of cholesterol on the membranes (Martin et al. 1993, Zidovetzki & Levitan 2007). This could make membranes too rigid and thereby prevent the infection (Ohvo-Rekilä et al. 2002). Furthermore, addition of cholesterol may also suppress cholesterol synthesis of cell and thereby influence the infection (Brown et al. 2002, Goldstein et al. 2006, Mackenzie et al. 2007). Altogether, these results imply that the normal cholesterol balance on the plasma membrane is crucial for successful EV1 infection.

Integrins, especially the activated integrins, are linked with the raft localization and are suggested to affect the cholesterol content of cells and the formation of raft domains (Pankov et al. 2005). FAs contain highly ordered

membranes that are even more ordered than GM1 ganglioside domains and caveolae (Gaus et al. 2006). The deattachment of cells leads to the internalization of many raft components and this process is regulated specifically via integrins (del Pozo et al. 2004, Gaus et al. 2006, Norambuena & Schwartz 2011). In addition, activated integrins can recruit raft components on membranes and raft components can influence integrin localization and function. For example the increase of lactosylceramide is able to increase β1 integrin clustering on the plasma memrane and promote its internalization via dynamin and caveolin-1 pathway (Sharma et al. 2005). Previously, it has been shown that α2β1 integrin is found in the DRM fractions together with caveolin-1 and GPI-AP (Upla et al. 2004). α2β1 integrin is located at same areas as GPI-APs in plasma membrane but not with caveolin-1 initially (Pietiäinen et al. 2004, Upla et al. 2004, Karjalainen et al. 2008). However, the clustering induces the separation of α2β1 integrins from GPI-APs and they are not internalized together (Upla et al. 2004, Karjalainen et al. 2008). Futhermore, caveolin-1 is not associated with integrins during early internalization, but starts to accumulate in internalized structures gradually (Karjalainen et al. 2008). Cholesterol-rich domains were shown to be important for infection in this study. Similarly, it has been reported by others that some proteins are internalized via raft-dependent macropinocytosis (Fan et al. 2007, Wadia et al. 2008). Although large macropinocytic ruffles cannot contain only raft membranes, macropinocytosis has been shown to be dependent on cholesterol (Grimmer et al. 2002). Cholesterol depletion inhibits Rac1 localization to the plasma membrane and thus prevents the actin remodelling and the membrane ruffling (Grimmer et al. 2002). Thereby, since EV1 has been shown to enter cells via macropinocytosis, it is not a surprise that the depletion or sequestering of the cholesterol inhibits EV1 infection and integrin internalization.

6.3.2 Role of cholesterol in EV1 uncoating and replication

The amount of cholesterol is thought to decrease from the plasma membrane to the LEs and LYs (Möbius et al. 2003) and cholesterol labeling with filipin does not reveal major cholesterol labeling of endosomes in control fibroblast cells (Kobayashi et al. 1999, Linder et al. 2007). In addition to plasma membrane labeling, Golgi area as well few early endosomal/late endosomal vesicles can show some accumulation of the filipin label (Höltta-Vuori et al. 2002). A similar pattern was also seen in SAOS-α2β1 cells, where most of the filipin stain was found on the plasma membrane and only some vesicles and Golgi-like area were labeled (III). Intrestingly, when EV1 infected cells were labeled with filipin at 4 h p.i., most of the viral capsid protein VP1 was associated with filipin (III). This indicates that VP1 structures contain high amount of the cholesterol as it can be detected with filipin and thereby supports the Triton X-100 results, which also suggested that α2-MVBs are rich in raft-lipids.

Here, the early stages of infection and uncoating were shown to be strongly sensitive to cholesterol disturbance whereas the later stages after 3 h were not markedly influenced (III, Fig. 10). The ketoconazole treatment

prevented the maturation of endosomes into MVBs, which has been reported to be essential for infection. Previously the inhibition of Na^{2+}/H^{+} exchanger with EIPA was shown to prevent the biogenesis of MVBs and the infection (Karjalainen et al. 2008). In addition, studies in this thesis showed that the inhibition of ESCRT components was able to inhibit the ILV formation and EV1 infection (II). Thereby, it can be suggested that specific cholesterol content on membranes is be needed for maturation of α2-MVBs and therefore provides favourable conditions for virus uncoating and RNA release from the endosomes.

Filipin and nystatin among the other polyene antibiotics have been shown to cause permebilization of the membranes and thereby affect the ion balance of the structures (Archer & Gale 1975, Kotler-Brajtburg et al. 1979). Since EV1 uncoating was sensitive to filipin and nystatin treatments, it is likely that changes in the ionic conditions could partially mediate the inhibitory effect on the uncoating. The uncoating of some viruses is reported to be dependent on specific ionic conditions (Wetz & Kucinski 1991, Chemello et al. 2002) in addition to receptor binding, acidity and temperature (Tuthill et al. 2010). The pH of the endosomes is connected to the ionic conditions of endosomes and therefore the changes in pH are reflected to the ion concentrations too (Scott & Gruenberg 2011). Although the EV1-induced structures were measured to be nearly neutral, bafilomycin A1 had some effect on infection at early stages of infection (II), similarly to filipin and nystatin. Therefore it is likely that subtle alterations in the pH maybe reflected to the ionic balance of EV1-induced structures and therefore could affect to EV1 uncoating. Perhaps subtle changes in pH could regulate e.g. the binding affinity between the virus and the receptor. Whether the separation of the receptor and the virus is an important trigger to start uncoating and what is the exact ambient ionic conditions in the endosomes needed for uncoating remains to be studied.

The replication step has been shown to be dependent on cholesterol with several viruses such as with hepatitis C virus (Shi et al. 2003, Aizaki et al. 2004). In addition, many flaviviruses like Japanese encephalitis virus (JEV), dengue virus (DENV) and West Nile virus (WNV) are shown to be dependent on cholesterol in the later stages of their entry (Mackenzie et al. 2007, Lee et al. 2008, Poh et al. 2012). JEV and DENV replication is inhibited by mβCD and filipin, whereas earlier events are not that sensitive to cholesterol manipulations (Lee et al. 2008). Furthermore, replication of DENV and WNV needs efficient cholesterol synthesis (Mackenzie et al. 2007, Poh et al. 2012). It was shown for WNV that the replication sites labeled with dsRNA accumulated cholesterol and 3-hydroxy-methyglutaryl-CoA reductase that is needed in cholesterol synthesis (Mackenzie et al. 2007). In comparison, our findings here showed that during the replication start-up of EV1, the cholesterol-sequestering drugs were not able to block the infection and filipin labeling was absent from dsRNA containing replication sites (III). Taken together, these results thus imply that EV1 replication is not sensitive to cholesterol modifiying compounds, however the early entry and uncoating are dependent on cholesterol.

6.4 Collagen uptake pathway, the possible physiological pathway hijacked by EV1

α2β1 integrin is able to bind both fibrillar and soluble collagen (Knight et al. 2000, Jokinen et al. 2004). Here, the uptake of soluble collagen promoted also the integrin internalization (II). As they were partially colocalizing in intracellular structures and their co-accumulation was enhanced during calpeptin treatment, they presumably use the same uptake pathway. Furthermore, both the uptake of collagen and integrin were similarly sensitive to cholesterol depletion (III). It would be interesting to test if 3D-collagen or proteolysed fibrillar fragments would cause a similar effect on the integrin, since it has been reported that different receptors can mediate the endocytosis of soluble and fibrillar ligands. For example the endocytosis of soluble fibronectin and collagen can occur independently of the β1 integrins (Shi & Sottile 2008, Shi et al. 2010, Madsen et al. 2011), whereas endocytosis and turnover of fibrillar fibronectin and collagen is regulated by the β1 integrins (Shi & Sottile 2008, Shi et al. 2010). Soluble collagen endocytosis is regulated by uPARAP that can bind several collagen types, including collagen type-I (Engelholm et al. 2003, Madsen et al. 2007).

The degradative pathway of endocytosed collagen is poorly known. The turnover of internalized collagen type-IV is suggested to occur in lysosomes (Kjoller et al. 2004). Colocalization with Lamp-1 label was shown to be only partial after 3 h but more apparent after 16 h (Kjoller et al. 2004). Cysteine protease inhibitor, E64d, that inhibits both lysosomal proteases and calpains, caused accumulation of collagen to Lamp-1 containing structures at the later time point (Kjoller et al. 2004). Although no significant evidence of collagen accumulation in lysosomes was found in our study, it is probable that at least some of the internalized collagen is targeted to lysosomal degradation. It is also possible that some of the collagen could be recycled back to the ECM for reuse in newly forming fibers. Altogether our results suggest that collagen uptake induces the integrin internalization and degradation in a similar manner as EV1 does and therefore could be a physiological route that EV1 has learned to utilize. However, detailed studies of the pathway of soluble collagen and integrin and their interactions will remain as topics for future study.

7 CONCLUSIONS

The main conclusions of this thesis are:

1. The clustering-triggered α2-MVBs are distinct from the conventional late endosomes and lysosomes and their formation is dependent on the ESCRT machinery.
2. Clustered integrins are targeted to a non-recycling pathway that leads to the degradation of integrins. Integrin turnover occurs inside the non-acidic α2-MVBs where calpains contribute to the degradation.
3. The normal cholesterol content of the plasma membrane is needed for internalization of EV1, integrin and collagen. The cholesterol-rich domains of intracellular integrin structures are essential for maturation of α2-MVBs and for uncoating of the virus.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Kasautuneen α2β1 integriinin endosytoosireitti

Integriinit ovat solukalvon läpäiseviä proteiineja, joiden tehtävänä on välittää solun kiinnittymistä. Solun ulkopuolisella osalla ne pystyvät tarttumaan soluväliaineeseen ja samanaikaisesti solun sisäpuolisella häntäosallaan ne voivat ankkuroitua solun tukirankaan. Kiinnittyminen soluväliaineeseen aiheuttaa lukuisten erilaisten viestinvälitysketjujen aktivoitumisen, jotka vaikuttavat esimerkiksi solun elinkykyyn, jakautumiseen tai liikkumiseen. Solujen liikkumisen kannalta on oleellista, että integriinien kiinnittymiskohdat eivät ole pysyviä vaan, että niitä voidaan purkaa solun takaosasta ja uusia muodostaa solun etuosaan niiden liikkumissuunnan mukaisesti. Tämä tapahtumasarja on kytketty integriinien kierrättämiseen, joka edellyttää integriinien sisäänottoa solun sisäisiin rakkuloihin. Sieltä ne ohjataan uudelleen takaisin solun pinnalle paikkoihin, joissa uusia kiinnittymiskohtia on tarve muodostaa.

Virukset ovat loisia, jotka koostuvat perintöaineksesta ja sitä ympäröivästä suojaavasta proteiinikuoresta. Vaipallisilla viruksilla proteiinikuorta ympäröi vielä lipidikaksoiskalvo, joka on peräisin virusta tuottaneesta solusta. Lisääntyäkseen virusten on pystyttävä vapauttamaan perintöaineksensa solun sisään, jossa sen sisältämän tiedon mukaisesti monistetaan uusia viruspartikkeleita solun tuotantokoneistoja hyväksikäyttäen. Monet virukset hyödyntävät solun sisäänpääsyssä mekanismeja, joilla solut normaalisti ottavat solun ulkopuolisia aineita sisäänsä. Tässä tutkimuksessa käytetty virus, ja jonka sisäänmenoreittiä työssä tarkasteltiin, on Echovirus 1 (EV1). Se on pieni vaipaton virus, joka kuuluu pikornavirusten perheeseen. Perheen muita tunnettuja jäseniä ovat esimerkiksi poliovirus ja rinovirukset. EV1-tartunta aiheuttaa ihmisissä yleensä lieviä hengitystie- tai suolistotulehduksia, mutta voi vakavimmillaan johtaa aivokalvontulehdukseen. Päästäkseen solun sisään, EV1 tarttuu solukalvon α2β1 integriineihin aiheuttaen niiden kasautumisen solukalvolla. Reseptorien kasautuminen aktivoi virus-integriinikompleksin sisäänoton solun sisäisiin rakkuloihin makropinosytoosin kaltaisella mekanismilla. Muodostuvat rakenteet ovat ensin putkimaisia, mutta vähitellen niistä muodostuu pyöreitä rakkuloita, jotka pitävät sisällään pienempiä vesikkeleitä.

Tässä vaitökirjatyössä osoitettiin, että EV1:n ja integriinin sisäänotto on kolesterolista riippuvaista. Kolesterolin, yhdessä sfingolipidien ja GPI-ankkuroitujen proteiinien kanssa, on havaittu muodostavan dynaamisia, vahvasti järjestäytyneitä kalvoalueita. Osa solun sisäänottoreiteistä lähtee tällaisilta kalvoalueilta ja useiden virusten on havaittu käyttävän näitä alueita hyväkseen elinkierrossaan. Runsaskolesterolisten alueiden häiritseminen esti sekä integriinin että EV1:n sisäänottoa solukalvolta. Myös solun sisällä integriini sijaitsi kolesterolirikkailla alueilla ja nämä alueet olivat tärkeitä rakenteiden kypsymisen ja myös EV1-infektion etenemisen kannalta. Erityisesti RNA:n vapautuminen oli riippuvaista kolesterolirikkaista alueista, mutta infektion myöhemmät vaiheet,

kuten RNA:n ja kuoriproteiinien tuotto eivät enää olleet herkkiä kolesterolitasapainon muutoksille.

Solun sisäänottaman materiaalin on perinteisesti ajateltu yhdistyvän varhaisiin endosomeihin. Ne toimivat eräänlaisina lajittelurakenteina, joissa kierrätettävät aineet erotellaan hajotukseen ohjattavista. Integriinien on osoitettu olevan proteiineja, jotka pääsääntöisesti kierrätetään takaisin solukalvolle joko suoraan varhaisten endosomien putkimaisista osista tai erillisen kierrättävän endosomin kautta. Sen sijaan hajotettavaksi tarkoitetut proteiinit, kuten kasvutekijäaktivoitu epidermaalinen kasvutekijäresptori (EGFR), ohjataan endosomin sisälle sisävesikkeleihin. Sisävesikkeleihin päätymisen uskotaan olevan signaali, joka ohjaa proteiinit edelleen myöhäisiin endosomeihin ja lysosomaaliseen hajotukseen. Sisävesikkelien muodostumisesta vastaavat ESCRT-proteiinikompleksit ja niihin liittyvät proteiinit. Nykykäsityksen mukaan varhainen endosomi kypsyy vähitellen myöhäiseksi endosomiksi, jolloin sen ulkomuoto ja koostumus muuttuvat: rakenne happamoituu, sisävesikkeleiden lukumäärä kasvaa, putkimaiset ulokkeet häviävät ja sekä lipidi- että proteiinikoostumus muuttuvat. EV1:n tai vasta-aineiden aiheuttaman integriinien kasautumisen seurauksena syntyneet monirakkulaiset rakenteet (α2-MVB:t) muistuttavat ulkonäöltään suuresti myöhäisiä endosomeja. Kuitenkin tarkemmat analyysit paljastivat, että rakenteet pysyivät lähes neutraaleina eikä niihin kertynyt myöhäisille endosomeille tyypillisiä merkkiproteiineja. Rakenteet pysyivät myös erillisinä EGFR-rakenteista. Vaikka α2-MVB:t olivat erillisiä perinteisistä myöhäisistä endosomeista, niiden muodostumiseen tarvittiin samoja ESCRT-proteiinikompleksien proteiineja kuin klassisten myöhäisten endosomien muodostumiseen. Monirakkulaisten rakenteiden muodostuminen oli myös edellytys onnistuneeseen EV1-infektioon.

Toisessa osatyössä monirakkulaisiin endosomeihin päätyvän integriinin kohtalo haluttiin selvittää tarkemmin. Työssä selvisi, että integriinin kasautumisesta johtuva sisäänotto soluun johti integriinin hajotukseen, eikä integriineille tyypillistä kierrättämistä takaisin solukalvolle havaittu. Integriinin hajotus oli huomattavasti nopeampaa kuin kasautumattoman integriinin. Kuitenkin verrattuna EGFR:n hajoamisnopeuteen kasautunut integriini hajosi hitaammin, mikä edelleen vahvisti näkemystä siitä, että reitit ovat toisistaan erilliset. Lisäksi integriinien hajotus ei ollut estettävissä lysosomien eikä proteasomien toimintaa estämällä eikä hajotukseen liittynyt rakenteen happamoitumista. Sen sijaan kalpaiinit, jotka ovat neutraalissa solulimassa esiintyviä ja toimivia proteaaseja, edistivät integriinien hajoamista. Erilaiset kalpaiiniestäjät estivät hajotuksen täydellisesti, myös silloin kun rakenteiden annettiin ensin kypsyä monirakkulaisiksi. Kalpaiinien todettiin myös aktivoituvan integriinien kasautumisen ja sisäänoton seurauksena ja olevan läsnä muodostuneissa rakenteissa. Väitöskirjatyössä saatiin myös vihjettä siitä, että liukoisen kollageenin sisäänottoreitti sisältää yhteneväisyyksiä integriinien kasautumisesta aiheutuvalle sisäänottoreitille ja voisi mahdollisesti olla reitti, jota EV1 on oppinut hyödyntämään päästäkseen solun sisään.

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