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268

Paula Turkki

Cellular Factors Promoting Efficient Baculovirus Internalization and Gene Delivery into Human Cells

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ABSTRACT

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Yhteenveto: Bakuloviruksen sisäänmenoon ja geeninsiirtoon vaikuttavat solun ominaisuudet

In order to efficiently infect or transfer genetic material into the cell, viruses utilize several cell surface and intracellular molecules. In this thesis, cellular factors associated with trafficking and efficient gene transfer of baculovirus (BV) were studied. BV is an insect pathogen that has a lot of potential as a human gene therapy vector. Despite intensive research and a growing number of BV-based applications, the cell surface receptor and the factors affecting virus internalization into human cells have not been identified. Here, BV was shown to use cell surface heparan sulfate proteoglycan syndecan as its binding receptor on the cell surface. Binding was mediated by the heparan sulfate chains and specifically by 6-O- and N-sulfated heparan sulfates. In the cell lines under study, syndecan-1 was found to be responsible for the virus binding while other members of the syndecan family showed no association with the virus. BV internalization was shown to lead to extensive membrane ruffling and to induce simultaneous uptake of *E. coli* into non-phagocytic cells. Even though the BV containing vesicles showed similarity to macropinosomes, common macropinosome regulators did not affect BV internalization. Neither was the clathrin-, caveolae-, flotillin-, GPI-anchored protein-enriched- or IL-2R - mediated endocytosis route involved in the BV entry. Rather, BV internalization resembled phagocytosis and was shown to be regulated by Arf6, RhoA and dynamin. BV non-permissive Ea.hy926 and MG-63 cells showed a strong vimentin network and high activity of PKC epsilon and low activity of PKC alpha. Furthermore, efficient BV transduction was shown to correlate with loose vimentin network and activation and down regulation of PKC subtypes alpha and epsilon, respectively. Remarkably these factors observed in the BV non-permissive cells, also correlated with the infection of a non-related human pathogen, a picornavirus echovirus-1.

Keywords: Baculovirus, echovirus-1, endocytosis, integrin, syndecan, virus internalization.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I Laakkonen J.P., Mäkelä A.R., Kakkonen E., Turkki P., Kukkonen S., Peränen J., Ylä-Herttuala S., Airenne K.J., Oker-Blom C., Vihinen-Ranta M. and Marjomäki V. 2009. Clathrin-independent entry of baculovirus triggers uptake of *E. coli* in non-phagocytic human cells. *PLoS ONE* 4(4): e5093.

II Makkonen KE*., Turkki P*., Laakkonen J.P., Ylä-Herttuala S., Marjomäki V*., Airenne K*. 2013. 6-O sulfated and N-sulfated syndecan-1 promotes baculovirus binding and entry into mammalian cells. *Journal of Virology*. 87:20 11148-11159.

III Turkki P*., Makkonen KE*., Huttunen M., Laakkonen J.P., Ylä-Herttuala S., Airenne K*., Marjomäki V*. 2013. Cell susceptibility to baculovirus transduction and echovirus infection is modified by PKC phosphorylation and vimentin organization. *Journal of Virology*. 87:17 9822-9835.

*Equal contribution

RESPONSIBILITIES

Article I: I participated in the transfection experiments with Arf6, BARS, Rab34, RhoA, Cdc42, Eps15, Pak-1, clathrin and Rac-1 (wt and mutant constructs) and the following BV-uptake experiments and subsequent imaging of the samples. I participated in the EIPA-dextran studies in addition to caveolae and BV colocalization studies.

Article II: In most of the BV binding experiments, Emilia Makkonen prepared the samples and I immunolabelled, imaged and analysed the data. In addition I performed the PI-PLC experiments and imaged and quantified the data. I analysed the levels of different syndecans in cells and I was responsible for the BV and syndecan (1-4) colocalization experiment and data. I wrote the article together with Emilia Makkonen, Johanna Laakkonen, Varpu Marjomäki and Kari Airenne.

Article III: I was responsible for all the experiments concerning EV1. Furthermore I was responsible for all siRNA experiments and all experiments conducted with the fluorescence microscope and subsequent data analysis. Emilia Makkonen and I performed the experiments with PMA. Emilia Makkonen, Moona Huttunen and I performed the SDS-PAGE and western blotting experiments. I wrote the article together with Emilia Makkonen, Johanna Laakkonen, Varpu Marjomäki and Kari Airenne.

ABBREVIATIONS

293	Human embryonic kidney cell line
ab	antibody
*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
Arf6	ADP-ribosylation factor (GTPase)
BuV	budded virus
BV	baculovirus
CA	constitutively active
Cdc42	cell division cycle 42 protein (GTPase)
DAG	diacylglycerol
DN	dominant negative
E. coli	*Esterichia coli*
Ea.hy926	Hybridoma of human umbilical vein endothelial and human lung carcinoma epithelial cells
ECM	extracellular matrix
EIPA	5-(N-ethyl-N-isopropyl)-amiloride
Eps15	EGFR protein tyrosine kinase substrate 15
EV1	echovirus 1
GAG	glycosaminoglycan
GEEC	GPI-anchored protein enriched early endosomal compartment
GPI	glycosylphosphatidylinositol
GTP	gunosine triphosphate
HepG2	human hepatocarcinoma cell line
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
IL2	interleukin-2
IP3	inositol 1,4,5-triphosphate
Mab	monoclonal antibody
MG-63	human osteosarcoma cells
$NaClO_3$	sodium chlorate
ODV	occlusion derived virus
PAGE	polyacrylamide gel electrophoresis
Pak1	p21-activated kinase-1
PDZ	postsynaptic density protein domain
PH	pleckstrin homology domain
PI3K	PI3 kinase
PIP2	phosphatidylinositol 4, 5-biphosphate
PI-PLC	PI-specific phospholipase C
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol ester
Rab	ras-like protein from rat brain (GTPase)

Raw264	mouse macrophage-like cell line
RhoA	ras homolog gene family member A (GTPase)
siRNA	small interfering RNA
TM	transmembrane
V	variable domain (syndecans)
WT	wild type

1 INTRODUCTION

This PhD thesis concentrated on studying virus-cell interactions using the following two viruses: an insect-specific baculovirus (BV) and a human pathogen echovirus-1 (EV1). A deeper understanding of virus-cell interactions is needed for the development of better strategies to reduce viral pathogenesis. Furthermore, knowledge of the viral life cycle provides insight into the use of viruses for medical purposes, as in the case of gene therapy. Because viruses use host cell mechanisms to invade cells, analysing the virus-cell interactions will also provide valuable information regarding the host cell functions.

In general, the virus life cycle starts on the cell membrane where the virus binds to its receptor(s). Binding of the virus usually leads to activation of signaling cascades and/or other cellular molecules and possible co-receptors. These events then lead to internalization of the virus or the virus-receptor complex. Internalization may occur via one or more cellular entry pathways that are dependent on the receptor and the signaling events initiated by the virus. These endocytic routes are common pathways that cells use in order to maintain homeostasis and several other critical physiological functions. Once the virus is internalized, it needs to get to the site of viral gene translation and replication either in the cytoplasm or in the nucleus. In order to travel there, the virus exploits cellular trafficking machinery. The cell cytoskeleton forms the main "highways" that are used for transport of organelles and molecules in the cell. Different viruses use different members of the cell cytoskeleton in their intracellular trafficking. Depending on the receptor used, the endocytic route taken and the cytoskeletal components involved, virus internalization can be inhibited or blocked with specific drugs and chemicals. In this thesis, these early virus internalization and trafficking steps were characterized for BV in human cells. In addition, the cellular factors contributing to the non-permissive phenotype of Ea.hy926 and MG-63 cells were evaluated by studying efficient infection and gene transfer of two non-related viruses, a human picornavirus EV1 and insect BV, respectively.

2 REVIEW OF THE LITERATURE

2.1 Cell surface molecules mediating virus internalization

Viruses can either directly fuse to the cell membrane or they can exploit the endocytic machinery of the cell to gain entry. In order to enter the cell by endocytosis, viruses need to interact with receptors on the cell surface. Viral receptors can act solely as attachment receptors or can mediate both the binding and internalization of the virus. Furthermore, viruses may have several receptors or co-receptors that are needed for efficient entry. It can be difficult to differentiate between receptor, co-receptor and attachment factors since they all affect the efficient entry of the virus. Various cell surface molecules can act as a viral receptor. This section will concentrate on heparan sulfate proteoglycans (HSPG) with a primary focus on syndecan-1. The role of α2β1-integrin as EV1 receptor will be discussed later in the EV1 section.

2.1.1 Heparan sulfate proteoglycans

All animal cells express HSPGs on their cell surface and in the extracellular matrix. These are glycoproteins that contain one or more covalently attached heparan sulfate (HS) chains. In addition to HS, several HSPGs also have chondroitin sulfate chains. HS and chondroitin sulfate chains are unbranched highly sulfated polysaccharides that are formed from repeating disaccharide units. HS chains have repeating units of glucuronic acid and *N*-acetylglucosamine whereas chondroitin sulfate chains are formed from glucuronic acid *N*-acetylgalactosamine. HS and chondroitin sulfate biosynthesis is mediated by enzymes found in the Golgi compartments (Kreuger and Kjellen 2012). HSPGs can be grouped according to their location: membrane, secreted extracellular matrix (ECM) and secretory vesicle HSPGs. HSPGs are known to bind various ligands, including growth factors, cytokines and several pathogens via their HS side chains (Sarrazin *et al.* 2011). Membrane HSPGs are one of the most common attachment factors for viruses. Binding to the negatively charged HS is usually considered to be electrostatic and nonspecific (Mercer *et al.* 2010).

There are several non-enveloped and enveloped viruses identified so far that use HSPGs for binding and/or entry into cells, including the following human viruses: herpes simplex virus (WuDunn and Spear 1989, Shieh *et al.* 1992), dengue virus (Chen *et al.* 1997, Germi *et al.* 2002), human immunodeficiency virus (HIV) (Patel *et al.* 1993), hepatitis C virus (Jiang *et al.* 2012), cytomegalovirus (Compton *et al.* 1993), adeno-associated virus 2 (Summerford and Samulski 1998) and human papilloma virus (Giroglou *et al.* 2001). Some of these viruses, however, have other receptors as well and the HSPG only serves as a binding receptor or co-receptor rather than the primary entry receptor. For example, HIV binding to cell surface HSPG retains virus infectivity up to a week whereas unbound virus loses its infectivity within just a day (Bobardt *et al.* 2003). The use of HSPG as a receptor can also be restricted to specific cell types, as the sulfation of HSPGs can define whether the cell line is susceptible to virus infection or not. Thus far, studies on HSPG and virus interactions have mainly focused on the role of HS chains and the relevance of the core protein itself has been less studied. The major cell surface HSPGs are syndecans and glypicans. The syndecan protein family comprises of four transmembrane receptors, syndecans 1-4 (Fig. 1).

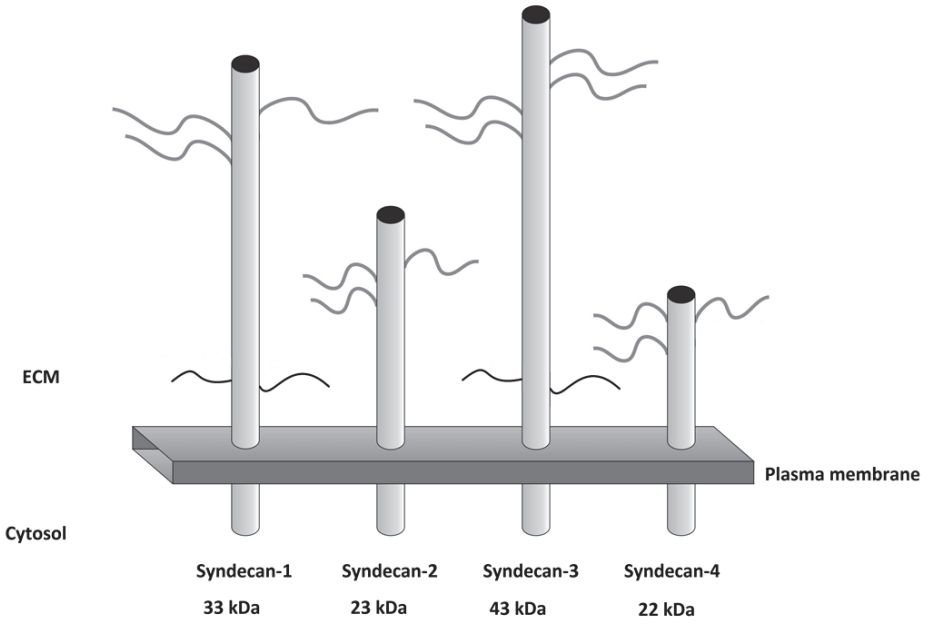

FIGURE 1 Syndecan protein family. All syndecans have HS chains (shown in gray) but syndecan-1 and syndecan-3 have also chondroitin sulphate chains (shown in black). Modified from Okina et al. (2009) and Tkachenko et al. (2005).

Syndecans are transmembrane receptors whereas glypicans are attached to the cell surface via a glycosylphosphatidylinisotol (GPI) anchor. Syndecans thus have a more diverse role as they are capable of transducing extracellular stimuli

to intracellular signals, in addition to their role in the regulation of the extracellular ligands and other cell surface receptors (Kwon *et al.* 2012).

2.1.2 Syndecans

Syndecans are involved in cell differentiation, adhesion, migration and cytoskeletal organization. Each of the four syndecan family members are encoded by their own genes. Syndecans 1-3 display tissue specific expression, whereas syndecan-4 is more ubiquitously found. The extracellular domain interacts with multiple molecules in the extracellular matrix (ECM), leading to changes in the cell function and signaling (Fig. 2).

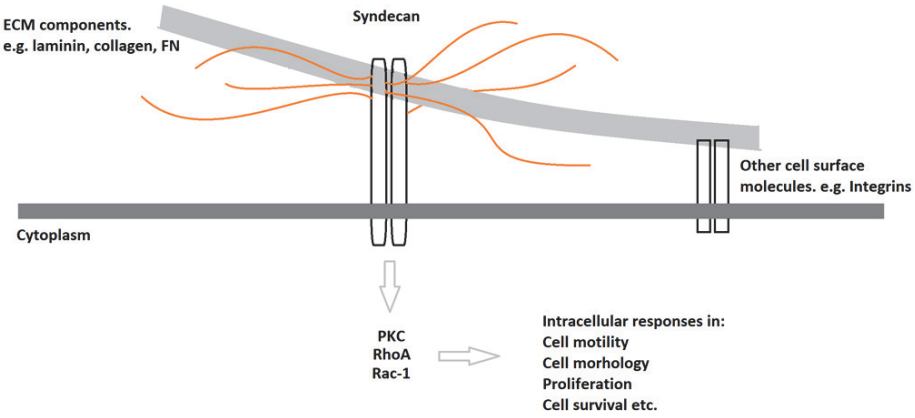

FIGURE 2 Syndecans participation in intracellular signal transduction: The syndecan ectodomain associates with extracellular components and cell surface molecules, resulting in intracellular signal transduction by the syndecans cytoplasmic domain. The grey stripe represents the ECM components. Modified from Kwon et al (2012).

In addition to syndecans role in conveying signals from the ECM to the cytoskeleton, syndecans also act as docking receptors that bind ECM ligands and regulate their availability to other cell surface receptors (Fig. 3). The intracellular function of syndecans is regulated by dimerizations, clustering and phosphorylations (Lambaerts *et al.* 2009). For example, syndecan-4 phosphorylation is the major control point in the regulation of integrin recycling and via integrins, of cell migration (Morgan *et al.* 2013).

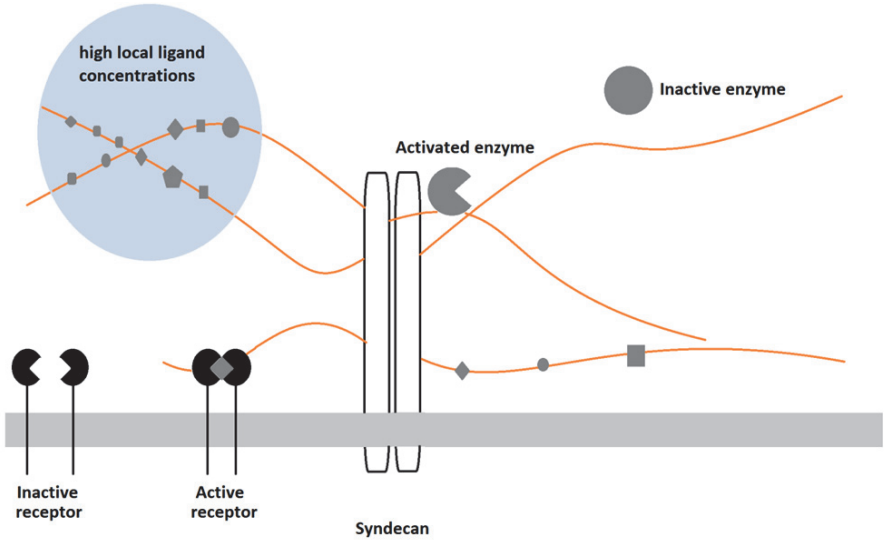

FIGURE 3 Syndecans role as a docking receptor. Syndecans have multiple roles in regulating the extracellular environment. Syndecans regulate extracellular events by activating enzymes, binding and concentrating ligands and cofactors from the ECM and thus modifying the activity of cell surface receptors. Modified from Kwon et al (2012).

The core protein is highly conserved except for the variable (V) region in the intracellular domain and the highly variable attachment sites for glycosaminoglycans (GAGs) in the extracellular domain. The short cytoplasmic domain is composed of C1 and C2 regions that are conserved among all syndecans, in addition to the variable region that defines the syndecan-specific intracellular functions (Choi *et al.* 2011). The highly conserved transmembrane domain is responsible for the dimerization and oligomerization of syndecan core proteins (Fig. 4). Non-covalent oligomerization appears to be essential for the syndecan to transmit signals from the ECM to cytosol (Choi *et al.* 2005, Dews and Mackenzie 2007). Syndecans do, however, differ in their tendency to dimerize such that syndecan-1 weakly forms homo-dimers, while syndecan-2 displays the strongest tendency to homodimerisize. Furthermore, syndecan-1 heterodimerizes with syndecans 2 and 3, but not with syndecan-4. The composition of different syndecans on the cell surface can affect the cells' characteristics, which can contribute to the various cell type specific functions of syndecans. For example, a heterodimer consisting of syndecans 3 and 4 is not as active as a syndecan-4 homodimer, therefore cells with little or no expression of syndecan-3 are more effective in syndecan-4 mediated signaling (Dews and Mackenzie 2007). Core proteins can provide the correct arrangement of the GAG chains or appropriately localize them on specific locations on the plasma membrane, for example to the lipid rafts (syndecan-1 and syndecan-4) (Fuki *et al.* 2000a, Tkachenko and Simons 2002) or focal adhesions (syndecan-4) (Dovas *et al.* 2006, Tkachenko *et al.* 2006).

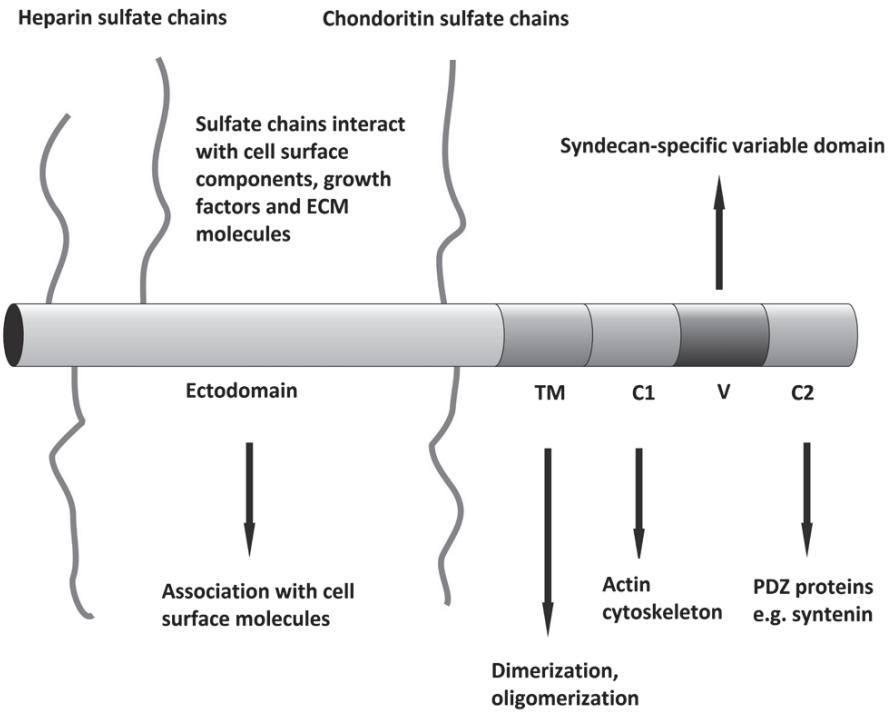

FIGURE 4 Syndecan structure showing different domains and their functions and associated proteins. TM = transmembrane domain, V = Variable domain. Modified from Couchman (2003).

Additionally, the relative position of the GAG chains is affected by the core protein, which further explains the syndecan-specific differences in ligand binding and their functional diversity (Kreuger and Kjellen 2012). The structural and functional diversity is also modified by the GAG chains themselves. GAG chains can differ in sulfation and other modifications such as charge, length and sugar composition. Both the degree and pattern of sulfation in HS side chains determines subsequent ligand interactions. The aforementioned factors all affect ligand specificity, and thereby modulate syndecan function. Cells differentially express a range of syndecan types and each syndecan type can vary in GAG quantity and size. Even though the GAG-binding sites show quite high variability, there is a conserved region composed of three GAG binding sites near the N-terminus of the core protein that is common to all four syndecans (Multhaupt *et al.* 2009, Couchman 2010, Choi *et al.* 2011, Morgan *et al.* 2013). Studies performed with syndecan-1 show that all three GAG attachments sites are crucial for syndecan function. Loss of one or two of these chains leads to decreased collagen binding, increased cell migration, and decreased cell-to-cell adhesion. Elimination of all three sites results in loss of function when the functionality is measured by the cell's ability to bind collagen, cell migration, and

cell aggregation. Furthermore, different GAG sites have distinct effects on the function (Langford *et al.* 1998). Indeed, the GAG chains might be one way for the cell to regulate its adhesiveness. One example comes from the comparison between simple and stratified epithelia. In simple epithelia, syndecan-1 has more and larger HS chains than syndecan-1 on stratified epithelia; as a result, these cells show very different adhesion properties that characterize simple and stratified epithelia (Sanderson and Bernfield 1988). Although most of the GAG chains are HS, chondroitin sulfates are also found in syndecan-1, syndecan-3 and rarely in syndecan-4 (Couchman 2003, Xian *et al.* 2010, Choi *et al.* 2011). However, also the chondroitin sulfate chains of different syndecans appear to be structurally and functionally different (Deepa *et al.* 2004). In addition to the three HS binding sites, syndecan-1 contains additional two chondroitin sulfate binding sites (Kokenyesi and Bernfield 1994). It is believed that the HS chains attract and present different proteins, whereas the chondroitin sulfate chains might play a larger role in the interactions between syndecan and other molecules (Eriksson and Spillmann 2012). Although there is little research regarding the role of the HSPG core protein in virus entry, there are some syndecan-specific reports. For example, syndecan-1 has been shown to act as a receptor for hepatitis C virus (Shi *et al.* 2013), human papilloma virus (Shafti-Keramat *et al.* 2003) and herpes simplex virus (Karasneh *et al.* 2011).

2.1.3 Syndecan-1 internalization

Previous observations on lipoprotein internalization and corresponding growth factors are used to describe ligand mediated syndecan internalization (Fuki *et al.* 1997, Fuki *et al.* 2000a, Wilsie *et al.* 2006). Syndecan-1 internalization appears to utilize a previously uncharacterized endocytic pathway (Fuki *et al.* 1997, Fuki *et al.* 2000b). Internalization is actin-dependent, clathrin- and caveolin-independent, and occurs from membrane raft areas enriched with cholesterol and sphingomyelin (Fuki *et al.* 1997, Fuki *et al.* 2000a, Wilsie *et al.* 2006, Podyma-Inoue *et al.* 2012). Distribution of syndecan-1 to the lipid raft areas is independent of the extracellular domain and can be introduced by clustering the transmembrane and cytoplasmic domains (Fuki *et al.* 2000a). In addition to syndecan-1, syndecan-4 appears to mediate internalization via raft areas (Tkachenko and Simons 2002), indicating raft-dependent internalization might be a conserved characteristic within the syndecan family. Localization to the raft areas and subsequent internalization requires ligand mediated clustering (Fuki *et al.* 2000a). Furthermore, internalization seems to depend solely on the conserved MKKK motif. During the initial internalization phase, ligand binding leads to ERK activation via the MKKK motif. ERK activation causes syndecan-1 to disassociate from α-tubulin, followed by rapid relocation to the raft areas. Next, Src kinase phosphorylates residues within the transmembrane and cytoplasmic domains and triggers association between syndecan-1 and cortactin, resulting in actin-dependent endocytosis (Chen and Williams 2013). Syndecan-1 ligand binding causes actin-mediated membrane protrusions on the cell surface. Formation of the membrane protrusions and actin interaction is dependent on the

dephosphorylation of tyrosine residues in the syndecan-1 intracellular domain (Sulka *et al.* 2009). The route after these early steps is not fully understood. Syndecans are reported to traffic to the late endosomes resulting in subsequent degradation in lysosomes. (Payne *et al.* 2007). The syndecan recycling route is controlled by ADP-ribosylation factor 6 (Arf6), phosphatidylinositol-3,5-bisphosphate (PIP2) and syntenin. The syntenin expression level functions as a rate-limiting step in syndecan recycling (Zimmermann *et al.* 2005). The switch between recycling and degradation, however, is not well understood.

2.1.4 Syntenin, PIP2 and Arf6 mediated syndecan recycling

Syntenin and syndecan interaction is mediated by the syntenin PDZ (postsynaptic density protein, disc large and zonula occludens) -domain. Syntenin is involved in organization of protein complexes on the plasma membrane, intracellular trafficking and cell surface targeting. Recent studies illustrate involvement of syntenin in cancer metastasis, and thus it has been intensively studied (Sarkar *et al.* 2008). Early internalization of syndecan appears to be independent of syntenin but somewhere between the early and recycling endosomes, syntenin becomes associated with syndecan. This association is dependent on the interaction between syntenin and PIP2, which, in turn, is dependent on Arf6 activation (Zimmermann *et al.* 2005). Syndecans associate with the syntenin PDZ domain via their C-terminus (Grootjans *et al.* 1997). Syntenin overexpression correlates with increased invasion and migration properties in several cancer cell lines (Sarkar *et al.* 2008). In several studies syntenin overexpression correlates with induction of membrane protrusions (Grootjans *et al.* 1997, Zimmermann *et al.* 2001) and with formation of polarised actin structures (Sala-Valdes *et al.* 2012). Overexpression of syntenin induces the formation of membrane ruffles, lamellipodia and long projections. However, this phenotype can be reversed by overexpression of RhoA constitutively active (CA) mutant (Zimmermann *et al.* 2001) that is deficient in GTPase activity and thus exhibits constitutive activity, suggesting that syntenin overexpression is associated with actin dynamics. It is unclear whether this is due to the regulation on the syntenin amount or function, and whether the syndecans are involved or not.

PIP2 is a membrane phospholipid and a common regulator of several important cellular processes in higher eukaryotes, including endosomal trafficking and intracellular signaling (Ho *et al.* 2012). PIP2 recruits syntenin to the cell surface by binding to its PDZ domains. The high affinity binding of PIP2 to syntenin requires both PDZ domains. The PDZ1 domain has higher affinity for PIP2 and the PDZ2 domain shows higher affinity for syndecan. Local changes in PIP2 concentration can therefore control the association of PDZ proteins with their target receptors and plasma membrane (Zimmermann *et al.* 2002). A high level of PIP2 expression can inhibit syndecan and syntenin interaction. Another inhibiting mechanism involves syndecan phosphorylation, since it appears that the phosphorylation of the syndecan cytoplasmic domain regulates syndecan-syntenin interaction (Sulka *et al.* 2009).

Arf6 is a GTP binding protein that is commonly found in all eukaryotes. It functions in regulating membrane trafficking and the actin cytoskeleton on the plasma membrane. Arf6 activation and inactivation are regulated by GTP binding and hydrolysis, respectively. Active Arf6 not only facilitates internalization from the plasma membrane, but also participates in subsequent recycling to the plasma membrane (Donaldson 2003, D'Souza-Schorey and Chavrier 2006, Schweitzer *et al.* 2011). Arf6 activates PIP5-kinase and thus induces the synthesis of PIP2 (Honda *et al.* 1999). The inability of syntenin to bind PIP2 causes the accumulation of syntenin, Arf6 and syndecan (with its ligand) in recycling endosomes. Interestingly, though syndecan and syntenin are trapped in these vesicles, transferrin is still able to enter and exit these compartments. This notion, along with other evidence, prompted the authors to propose that syntenin defective in PIP2 binding does not block general endocytic recycling, but it specifically participates in Arf6 mediated recycling. Interestingly, also β1-integrin accumulates in these compartments due to defective syntenin and PIP2 binding (Zimmermann *et al.* 2005).

2.2 Endocytosis as a viral entry pathway

Several viruses have evolved to use endocytosis in order to get inside their host cells and travel to the site of replication (Mercer *et al.* 2010). Endocytosis provides a rapid internalization mechanism that also hides the virus from the host cells immune response. Endocytosis is a universal mechanism, through which cells transport fluids, solutes, and particles from the extracellular environment into the cytoplasm. In addition to transport, it is also a crucial part of cell signaling. By internalizing membrane components and receptors, endocytosis regulates cell behavior in the presence of certain stimuli. Additionally, by targeting the receptors either for degradation or recycling back to the membrane, the endocytosis network can control the cell sensitivity to stimuli. Furthermore, endosomes can act as controllable microenvironments by spatially combining molecules and signaling cascades into specific compartments. Thus by regulating endocytosis, cells can also either activate or inhibit these endosome-specific signaling events (Platta and Stenmark 2011).

Researchers have identified and characterized several endocytosis routes (Fig. 5). The first identified and most studied route is clathrin-mediated endocytosis. Because of its status as the first and most studied route, the other routes are sometimes all classified together as clathrin-independent routes. Novel clathrin-independent routes are emerging and the classification can vary depending on the publication. The following four sections go through the most common endocytotic routes: clathrin-mediated, caveolin-mediated, macropinocytosis and phagocytosis. A few less studied raft-associated routes are described as well. These include GPI-anchored protein-enriched early endosomal pathway (GEEC), flotillin and IL2-receptor-mediated pathways. Typically, each route has its own regulators and characteristics by which the route can be

classified. However, many of these regulators are shared with other routes, thus forming a complex network (Doherty and McMahon 2009, Mercer *et al*. 2010).

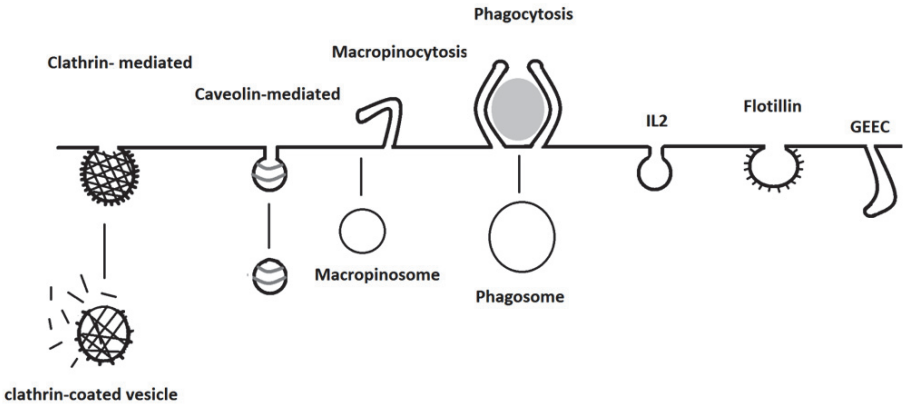

FIGURE 5 The main endocytic routes in the cell. In clathrin-, caveolin- and flotillin-mediated endocytosis, a specific coat protein is involved in the budding and vesicle formation. Macropinocytosis occurs at random locations on the cell surface and internalises mainly fluids. In phagocytosis, the membrane protrusions tightly cover the ligand. Modified from Mercer et al. (2012).

2.2.1 Clathrin -mediated endocytosis

Clathrin-mediated endocytosis is one of the most common endocytic pathways that cells use to transfer membrane components, membrane-bound receptors and soluble ligands. Usually receptors are internalized after ligand binding but some of the receptors (transferrin receptors) using the clathrin-mediated route are constantly recycled, independent of ligand binding. Clathrin is a self-assembling protein that forms a dense coat at the cell membrane. This coat serves as a scaffold for proteins but it cannot directly bind to the membrane. Clathrin adaptor proteins link the membrane components and clathrin. Furthermore, the adaptor proteins used in clathrin-mediated internalization appear to differ depending on the cargo. Transferrin receptors and several other cargos need adaptor protein 2, whereas, for example, EGFR internalization is dependent on epsins and Eps15 (Le Roy and Wrana 2005, Mercer *et al*. 2010). In clathrin-mediated endocytosis, a clathrin coated pit is first formed in the membrane. This is formed from clathrin triskelia which are organised into the plasma membrane to form a lattice. Once these pits are pinched of from the membrane, they are called clathrin-coated vesicles. Vesicle budding from the membrane is mediated by dynamin-dependent scission and actin polymerization. After internalization vesicles are rapidly uncoated and they fuse with the early endosome. From the early endosomes, the cargo is moved to the late endosomes and subsequently to the lysosomes for degradation. Alternatively, the internalized cargo can be recycled back to the plasma membrane by recycling endosomes. Clathrin-coated

pits and vesicles can be readily distinguished by electron microscopy as clathrin forms a dense region on the plasma membrane (Doherty and McMahon 2009, Brodsky 2012). The clathrin mediated pathway is the most commonly observed pathway used by viruses. Virus entry by clathrin-mediated endocytosis is generally a fast process. Viruses are found in the early endosomes within 1-2 min (Mercer *et al.* 2010).

2.2.2 Caveolin-mediated endocytosis

Caveolae are 60 to 80 nm-wide pits in the plasma membrane that contain high amounts of caveolin proteins. The main component is usually caveolin-1. In the electron microscope, caveolins are also readily distinguished and they are described as flask-shaped or open cups with a wide opening rather than a tight neck (Parton and del Pozo 2013). They can be distinguished from clathrin-coated pits by several characteristics; they show no distinct stages in their closure, they do not have an electron-dense coat and their coat structure is different. Furthermore, the density of caveolae can differ within an individual cell, e.g. the basolateral surface can show more abundant caveolin concentration than other surfaces. Interestingly, also different tissues show differential expression of caveolin suggesting that caveolin has an important role in the cell type-specific signaling (Parton and del Pozo 2013). Caveolin mediated endocytosis originates in the raft areas and similar to the clathrin-mediated route, dynamin is responsible for pinching off the vesicle. With caveolin- and several other raft-mediated pathways, ligand uptake and further trafficking is usually slow. Internalization leads to early endosomes and subsequently to the late endosomes. From the late endosomes, ligands are often transported to the endoplasmic reticulum. Several viruses use these raft-dependent pathways (Doherty and McMahon 2009, Mercer *et al.* 2010).

2.2.3 Macropinocytosis

Macropinocytosis is used as an entry route by over 20 different viruses (Mercer and Helenius 2012), including several known pathogens such as HIV (Marechal *et al.* 2001), influenza virus (Rossman *et al.* 2012), and adenovirus (de Vries *et al.* 2011). It differs from the other routes by its non-specific nature. Macropinocytosis is commonly used for the uptake of fluids, solute molecules, nutrients and antigens from the cells surrounding. It can be activated by ligands but once activated, the membrane protrusions can form anywhere in the cell membrane, thus the ligand itself might not even become internalized. Only in macrophages and dendritic cells, macropinocytosis is continuously active since it is used for antigen presentation by the major histocompatibily complex. The vesicles formed, called macropinosomes, initiate from the membrane ruffles which fold back and form large fluid-filled cavities. Macropinosomes do not have any special coat protein and thus the vesicles are irregular in shape (Doherty and McMahon 2009, Mercer and Helenius 2012). One of the most specific markers for macropinocytosis (not affecting other routes) is its sensitivity to Na+/H+

exchanger inhibitors, such as amilorides. Amilorides change the pH and thus inhibit the function of Rac1 and Cdc42 (Koivusalo *et al.* 2010). Rac1 and Cdc42 are Rho family GTPases that function in actin regulation and membrane ruffling together with RhoA (Ridley *et al.* 1992, Kozma *et al.* 1995, Nobes and Hall 1995, Allen *et al.* 1997). As a downstream target of Rac1 and Cdc42, p21 activated kinase 1 (Pak1) (Manser *et al.* 1994) activity is also required for macropinocytosis. Another macropinosome regulator, PI-3 kinase (PI3K) (Amyere *et al.* 2000), can also activate Pak1 independently of Cdc42 and Rac1 and can similarly lead to actin reorganization (Papakonstanti and Stournaras 2002). Also the downstream targets of PI3K, PLC (Amyere *et al.* 2000), Rab34 (Sun *et al.* 2003) and protein kinase C (PKC) (Keller 1990) are shown to regulate macropinocytosis.

Macropinosomes form from the membrane ruffles initiated usually by an external stimulus. In response to the stimulus, Ras GTPases are activated. Rac1, Rab5, Arf6 and PI3K are activated and function in ruffle formation, macropinosome closure and membrane trafficking. Rab5 interacts with F-actin to promote ruffle-associated actin cross-linking. Arf6 recycles Rac1 to the membrane and in addition affects the intracellular macropinosome trafficking. Pak1 regulates the cytoskeleton and is needed throughout the stages of macropinocytosis. The exact role of PKCs is not clear. Rab34 is needed in the macropinosome closure but macropinocytosis without Rab34 has also been reported, likely implying cell type specific differences in macropinocytosis. Dynamin prevents the targeting of Rac1 to the ruffles. CtBP1/Bars is needed for the closure of the macropinocytic vesicles. When macropinosome is closed, it travels into the cytoplasm and undergoes acidification. In macrophages macropinosomes finally fuse with lysosomes but in A431 cells, most macropinosomes travel back to the plasma membrane and release their contents to the ECM. Thus the macropinocytotic processes appear to be cell type-specific (Mercer *et al.* 2010, Mercer and Helenius 2012).

2.2.4 Phagocytosis

Phagocytosis is part of the innate immunity response. It is used by cells to destroy large microbial pathogens (e.g. bacteria, yeast and parasites) and apoptotic and necrotic cell debris. It also has a role in the acquired immune response, as it is used by dendritic cells to direct antigens to the major histocompatibility complex. The cells with high phagocytic activity (e.g. macrophages, monocytes, dendritic cells and neutrofils) are called professional phagocytes and cells with low phagocytic activity are often referred to as non-professional phagocytes (Rabinovitch 1995). The main difference between these two is the more limited amounts of particles the non-professional phagocytes can take up because of the lack of appropriate phagocytic receptors. However, it has been suggested that effective phagocytosis can be achieved by non-professional cells by their cell surface fibronectin or laminin receptors or HS (Rabinovitch 1995). Clustering of the phagocytic receptors by its ligand initiates the phagocytic process. The actin-directed membrane protrusions start to form from the membrane ruffle areas. The regulation of actin dynamics and the specific form of

the phagocytic cup are ligand-specific (Swanson 2008). The cup formation and subsequent internalization are dependent on Cdc42 and Rac1 (Doherty and McMahon 2009). Furthermore, PKC and myosin motors are involved in actin remodeling (Allen and Aderem 1995). For PKC activation, PLC-generated diacylglycerol (DAG) is essential. Other kinases in phagocytosis include MEK and ERK2. These seem to play a role in the pseudopod formation (Mansfield *et al.* 2000, Plows *et al.* 2004). Additionally, Arf6 is activated and it is needed for the membrane recycling and pseudopod formation (Niedergang *et al.* 2003).

Phagocytosis has many characteristics in common with macropinocytosis. These include large vacuole size, membrane protrusions, the non-constant nature, dependency on actin, and in addition, these pathways also share several common regulators (e.g. PKCs, PI3K and small GTPases). However, there are some fundamental differences. Phagocytosis is a cargo-triggered event and specialized to engulf only the ligand in question, whereas macropinocytosis is non-specific and can happen anywhere on the cell membrane and not only in the region of the initial ligand binding. To add to the specifity, phagocytosis forms a tight cup around the ligand while in macropinocytosis membrane protrusion engulfs randomly both extracellular fluid and particles (Swanson 2008).

2.2.5 Novel raft-mediated endocytic routes

In addition to the caveolin-mediated route, other raft-mediated routes have been recently discovered, including the GEEC-, IL2- and flotillin–mediated routes. GEEC is a route used by GPI-anchored proteins. Interestingly, caveolin-1 can be found in the tubular GEEC endosomes. Together with the observation that caveolin cargo is associated with the GEEC route, this finding has led to speculations whether previous results on caveolin-mediated endocytosis could be applicable to the GEEC route as well. Furthermore, the GEEC route is dynamin-independent and it is regulated by Cdc42 and Arf1. (Doherty and McMahon 2009, Mercer *et al.* 2010). The IL2 –mediated route is dependent on dynamin, RhoA, Rac1 and PAK1 and PAK2 kinases. Some of the ligands using this pathway associate with cargo traveling on the clathrin mediated pathway, suggesting a link between these routes. For example some cytokine receptors are shown to travel the IL2-route (Doherty and McMahon 2009, Mercer *et al.* 2010). Flotillin is a coat protein in the flotillin-mediated route. It is highly homologous with caveolin and believed to have a similar role in the organization of membrane lipids (Doherty and McMahon 2009). Interestingly, the ligand-mediated internalization of cell surface HSPG has been shown to be associated with and dependent on flotillin in Hela cells. However, it was not studied which members of the HSPG protein family were using this pathway (Payne *et al.* 2007).

2.3 Protein kinase C

Protein phosphorylation is one of the corner stones in cell signalling and regulation. Phosphorylation is mediated by protein kinases that can by phosphorylation of serine, threonine or tyrosine residues, alter their substrates biological activity and function. PKCs are a large family of serine and threonine kinases. This family can be further divided onto three subfamilies; classical (α, β1, β2 and γ), novel (δ, δ1, δ2, δ3, ε, η and θ) and atypical (ι, ζ, N1, N2 and N3) PKCs. This categorization is based on the subtypes demand for second messengers. Isoforms in the classical subfamily require calcium in addition to diaglycerol (DAG) and other phospholipids for their activation whereas novel subtypes require DAG and some other lipids but their activation is independent of calcium. The atypical subtypes do not need DAG or calcium for their activation but are activated by other lipids (Freeley *et al.* 2011). Phorbol esters (PMA) can mimic DAG and bind to the PKC C1 domain and thus are commonly used as activators of classical and novel PKC subtypes. Atypical PKCs cannot be activated by phorbol esters. Factors needed for activation of different PKC subfamilies are listed in table 1.

TABLE 1 Factors needed for activation of PKC subfamilies

	PMA / DAG	phospholipids	Ca+
Classical	Yes	Yes	Yes
Novel	Yes	Yes	No
Atypical	No	Yes	No

PKC activity appears to be self-inhibiting, as in the inactive state the protein interacts with itself so that the substrate is unable to access it. When activated by binding of DAG or PMA the conformation changes so that the kinase is able to phosphorylate its substrate. However, in order to become activated by these second messengers, PKCs need to be primed by phosphorylations (Fig. 6). PKC has three different phosphorylation sites and PDK-1 and mTOR are shown to act as the upstream kinases that directly mediate phosphorylation. Altogether PKCs are regulated by four mechanisms: co-activator binding, phosphorylations, protein-protein interactions and regulation by degradation. Regulation affects their structure, function and subcellular localization. In order to display full enzymatic activity, PKCs need to be first phosphorylated and then activated by second messengers. After removal of DAG, PKCs translocate from the membrane into the cytocol and their signaling is terminated. As cells are unable to degrade PMA, its binding to PKCs leads to persistent activation which then leads to efficient downregulation by degradation of the whole enzyme (Newton 2010, Freeley *et al.* 2011). Upon activation with phorbol esters, different subtypes translocate to different organelles in the cell (Duquesnes *et al.* 2011).

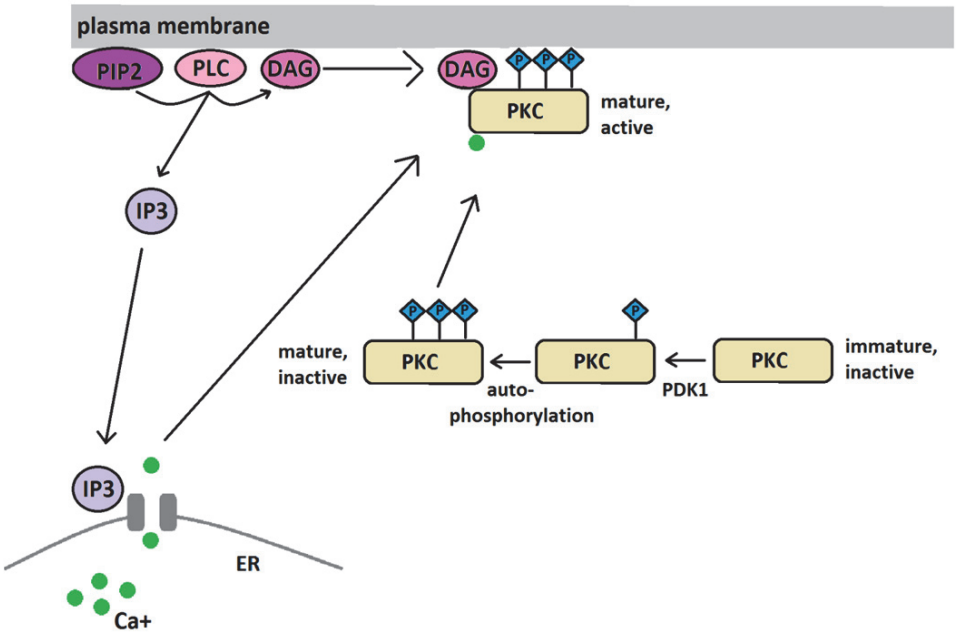

FIGURE 6 Mechanism of PKC activation. In order to be activated by the second messengers, PKC needs to be phosphorylated. DAG and inositol triphosphate (IP3) are formed from PIP2 cleavage by phospholipase C (PLC) on the plasma membrane. Modified from Griner and Kazanietz (2007).

The regulation of PKC signaling appears to form its own complex network and thus it is hard to pinpoint specific functions to specific subtypes. For example, the activity of a specific PKC can be regulated by the activity of another PKC (Lipp and Reither 2011). Since the classical α and novel ε are the isozymes that were found to be associated with BV and EV1 internalization, some of their specific interactions are described in more detail.

2.3.1 PKCα

PKC isozymes are ubiquitously expressed, PKCα being one of the most abundant subtype in various cells. Furthermore, drastic changes in PKCα expression are seen in several cancer types, PKCα activity usually leading to increased motility and invasion. The role on cancer cell growth, however, seems to be more complex and shows cell type-specific characteristics on PKCα regulation (Koivunen *et al.* 2006). PKCα specifically interacts with β1-integrin and syndecan-4. PKCα expression causes upregulation of β1-integrin on the cell surface and PKCα activation causes the β1-integrin to internalize (Ng *et al.* 1999). PKCα binds to the cytoplasmic tail of syndecan-4 and this association has not been shown with other members of the syndecan protein family. Additionally, PIP2 binding further leads to the activation of PKCα. Furthermore, PKCα activation leads to syndecan-4 localization to the site of focal adhesions. Whether this is due to

direct phosphorylation of syndecan-4 or some of its upstream regulators, are not known. As a member of the classical PKC subtypes, PKCα normally requires calcium, phospholipids and DAG for its activation. However, in the presence of PIP2 and syndecan-4, it is able to reach up to 80% of its maximal activity suggesting a new mode of activation for PKCα. Whether this activation is common to the whole PKC superfamily and/or to the syndecan family, has not been thoroughly studied yet. However, preliminary studies with PKCδ and syndecan-4 or PKCα and syndecan-2 show no signs of activation by syndecan and PKC interaction (Simons and Horowitz 2001).

2.3.2 PKCε

PKCε is an important regulator especially in cells of the cardiac, immune and nervous systems. Because of its essential role in such central tissues it is also the target of active drug discovery research. Similar to PKCα, PKCε can also be activated by other lipids such as arachidonic acid and PIP2. PKCε shows some unique structural features when compared to other PKCs such as the actin-binding motif between the C1a and C1b subdomains. In an inactive state PKCε is unable to bind actin but once activated with DAG, PMA or arachidonic acid, PKCε and actin can interact. This interaction then leads to the formation of F-actin and inhibition of actin depolymerization (Newton and Messing 2010). Furthermore, PKCε appears to be the upstream regulator of the actin regulators RhoA and RhoC (Pan *et al.* 2006). PKCε is associated with signaling cascades involving Akt, PKD1, Stat3 and ion channels. In addition to actin, it also associates with other elements of the cytoskeleton such as the microtubules and the intermediate filaments peripherin, vimentin and keratin (Newton and Messing 2010). As phosphorylation is essential for the regulation of intermediate filament network (Eriksson *et al.* 2009, Toivola *et al.* 2010), these effects are probably due to PKCε mediated phosphorylations. Furthermore, PKCε regulates β1-intergrin recycling and thus plays an important role in cell motility. Similar to other PKCs, the activity and intracellular location of PKCε are regulated by phosphorylations. Also, the basic mechanism of phosphorylation is similar. Phosphorylation of the activation loop by PDK-1 further triggers the autophosphorylations of the hydrophobic motifs (Freeley *et al.* 2011). Despite these similarities the regulation of the classical and novel subtypes is different since with PKCε the phosphorylation is regulated whereas in classical subtypes, the phosphorylation is constitutive (Cenni *et al.* 2002). Furthermore, the functional effects of all of the PKCε phosphorylation sites are not yet known (Newton and Messing 2010).

2.4 The cytoskeleton and viral trafficking

Cells are spatially organised and in order to maintain the correct distribution of specific molecules and organelles, the cell cytoskeleton is used as a mode of

transport. The cell cytoskeleton is formed by three types of filaments, actin, microtubules and intermediate filaments. Furthermore, through the interactions of associated proteins, all three of these components are associated with each other. In addition to maintaining the correct cell shape and providing the mechanism for intracellular trafficking, the cytoskeleton has vital roles in cell migration, cell division and membrane trafficking. The cytoskeleton often functions as a transport track for viruses. It has been calculated that if viruses used simple diffusion as an intracellular trafficking method, for herpes virus it would take 231 years to travel 1 cm in the cell cytoplasm and for vaccinia virus it would take more than 550 years (Sodeik 2000). Although 1 cm sounds absurdly long in cellular scale, in neuronal cells, for example, the virus has to travel several centimetres or even meters in order to get from the site of internalization to the site of replication. This kept in mind, it is no wonder that viruses have evolved to use the host cell cytoskeleton in order to efficiently traffic in the cells.

2.4.1 Actin

Actin is expressed in all known organisms. Actin has functions in the cytoplasm as well as in the nucleus, and there is a huge amount of actin-associated proteins and an even larger amount of molecules that are affected by actin dynamics. In cells, actin can be found in two forms. Under physiological conditions, actin prefers the double-helical filament form, called F-actin, whereas the monomeric form is called G-actin, although it seems that there can be other conformations as well (Schoenenberger *et al.* 2011). Actin is an essential part of the endocytic machinery. First, actin can induce membrane protrusions that are essential in the entrapment of extracellular material. Second, actin is involved in membrane invaginations, elongations and in the pinching off of the vesicles from the membrane. Third, the majority of vesicle trafficking is actin-dependent (Mooren *et al.* 2012). Actin plays a role in the clathrin-, caveolin- and several raft-mediated endocytic pathways as well as in phagocytosis and macropinocytosis (Mercer *et al.* 2010). The actin cytoskeleton is highly dynamic and mainly regulated by the Rho-family of GTPases, the major regulators being RhoA, Rac1 and Cdc42 (Schmitz *et al.* 2000). Because of the universal role of actin in the cell, several viruses exploit actin in several stages of their life cycle. For example, actin is essential for the internalization, intranuclear capsid transport and virion egress of herpesvirus (Roberts and Baines 2011).

2.4.2 Microtubulus

Microtubules are found in all eukaryotic cells. On the contrary to network-forming actins, microtubules act alone or in small groups. Similarly to actin, microtubules are also highly dynamic structures. Microtubules form from α and β tubulin subunits that are assembled into hollow tubes. In line with their highly structural role in cells, microtubules are the most rigid components of the cytoskeleton and able to support large cellular extensions, such as axons and cilia (Hawkins *et al.* 2010). Similar to actin, microtubule dynamics and organization

are regulated by the Rho-family GTPases RhoA, Rac1 and Cdc42 (Watanabe *et al.* 2005). Several viruses use microtubules for their intracellular trafficking. In addition, several viruses directly interact with the microtubule motor proteins dynein and kinesin. Usually, microtubules are used for initial internalization or for the intracellular trafficking of the virus. The trafficking through microtubules happens either by vesicles or by naked viruses (Dohner *et al.* 2005). Microtubules are associated with macropinocytosis, phagocytosis and clathrin- and caveolin-mediated endocytosis (Mercer *et al.* 2010).

2.4.3 Intermediate filaments

The intermediate filament network is composed of several different members of the intermediate filament protein family (over 70 genes) and the overall composition depends on the cell type. Intermediate filaments have important roles in signal transduction, cytoskeletal cross-talk and correct organization of the cell organelles (Goldman *et al.* 2008, Eriksson *et al.* 2009). Vimentin is the major intermediate filament in several cell types. Its expression is altered during development and in certain diseases. Vimentin overexpression in cancers correlates with increased tumor growth, invasion and poor prognosis. Vimentin is regulated by a complex phosphorylation network, it is phosphorylated by several kinases such as PKA, PKC, p21-activated kinase and RhoA-binding kinase (Sin *et al.* 1998, Goto *et al.* 2002, Eriksson *et al.* 2004). Vimentin has a high degree of homology among species suggesting that it has a vital role in cells. Vimentin acts in organising signalling and adhesion molecules. Several viruses, such as African swine fever virus (Stefanovic *et al.* 2005), vaccinia virus (Ferreira *et al.* 1994), retrovirus (Snasel *et al.* 2000), adenovirus (Belin and Boulanger 1987), parvovirus (Nuesch *et al.* 2005) bluetongue virus (Bhattacharya *et al.* 2007), dengue virus (Kanlaya *et al.* 2010), Japanese encephalitis virus (Das *et al.* 2011, Liang *et al.* 2011), herpesvirus (Miller and Hertel 2009), hepatitis C virus (Ghosh *et al.* 2011), cowpea mosaic virus (Plummer *et al.* 2012), foot-and-mouth disease virus and bovine enterovirus (Armer *et al.* 2008) depend on or are responsible for vimentin rearrangements during virus infection, suggesting that vimentin could be one of the common regulators associated with effective virus infection. Interestingly, vimentin seems to have a broader role in the virus life cycle than just in viral transport. For example two viruses from the picornavirus family, foot and mouth disease virus and cowpea mosaic virus are shown to rearrange cytosolic vimentin filaments into specific structures that enable efficient replication of the viruses (Armer *et al.* 2008, Gladue *et al.* 2013). Furthermore, surface-expressed vimentin has been recently shown to be associated with the cell surface binding of viruses (Kim *et al.* 2006, Koudelka *et al.* 2009).

2.5 Baculovirus

BVs belong to a family of insect pathogens, mainly infecting insects from the orders Lepidoptera, Diptera and Hymenoptera. They are a major factor responsible for controlling insect populations in nature and due to their natural abundance, they are also often found in the human diet (Rohrman 2011). BVs are large (30-60 x 250-300 nm), rod-shaped enveloped viruses with a double stranded DNA genome. The genome is large, from 80 to 180 kb in size and it codes for 90 to 180 genes. The prototype virus, *Autographa californica* multiple nucleopolyhedrosis (*Ac*MNPV), is the most studied and applied BV, both in the areas of virology and biotechnology. *Ac*MNPV is also the BV studied and referred to in this thesis. BVs were first studied and characterized as a potential biological pesticide as they efficiently infect insects and are commonly found in nature, making them safer alternative to chemical pesticides. Thus, BVs were closely studied by molecular biologists, virologists and geneticists, explaining our vast knowledge on BV today. Later, when the highly efficient polyhedrin- and p10 promoters were identified, BVs became famous as a widely used expression vector system in biotechnology (Summers 2006). Nowadays, chemical pesticides have mostly overtaken the use of BVs in agriculture but BVs are increasingly used as protein expression vectors in insect cells. In addition, BV has gained an increasing amount of interest in biomedicine.

2.5.1 BV life cycle in insect cells

In the BV life cycle, there are two types of virions that work in consort in order to efficiently infect the host and spread the infection to new hosts (Fig. 7). These virion types are called occlusion-derived virus (ODV) and budded virus (BuV). The ODVs are found in two forms; in large structures called occlusion bodies which accompany several ODVs or as singular virions (only once the occlusion body is disrupted). As insect populations are usually cyclic/seasonal, insect viruses must survive outside their hosts for long periods of time. Because of this, insect pathogens have developed different strategies in order to survive. BVs have developed occlusion bodies in order to preserve the virions and keep them safe from environmental threats. Occlusion bodies or polyhedra are highly stable structures that can withstand all normal environmental condition thus making the virions remain infectious basically indefinitely. Occlusion bodies are responsible for the spread of the virus in nature. It seems that they can even survive the passage though the gastrointestinal tract of birds and thus spread by way of birds (Rohrmann 2011). The singular ODVs are responsible for the primary infection as they infect the insect's midgut cells and BuVs are responsible for systemic infection (cell to cell) within the host (Volkman and Summers 1977). The main structural difference between these two virions is their envelope: in the case of BuV it is gained from the host cell surface, and in the case of ODV it is derived from the nuclear envelope. Because of their different origins, the protein composition of the membranes differ significantly (Rohrmann 2011).

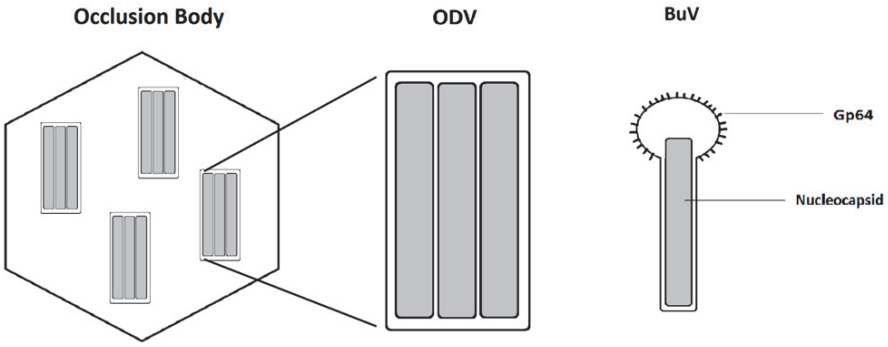

FIGURE 7 Two types of BV virions. ODVs contain several nucleocapsids and are packaged into environmentally resistant occlusion bodies. BuVs contain only one nucleocapsid per virus. Modified from Rohrman (2011).

The BV life cycle starts as the insect ingests, usually within its food, BV occlusion bodies. When the occlusion bodies get to the midgut, the high pH (up to pH 12) and the proteinases dissolve the occlusion bodies and release the ODVs. ODVs then infect the midgut epithelium cells and thus BuVs are produced. BuVs then are responsible for the cell to cell infection of the host. In the late stage of the infection, production of BuVs ceases and the production of ODVs starts in the nucleus (Fig. 8). Polyhedrin, which is the main component of occlusion bodies, accumulates in the nucleus and crystallises into a lattice that protects the ODVs and occlusion bodies are formed. Briefly after that, the host dies leaving the occlusion bodies on leaves etc. and the occlusion bodies are then eaten by another host. Interestingly, some BVs are able to affect the behaviour of the host so that in the later stage of infection, the host travels higher in the plant or the tree. After this movement, the infection leads to death of the host. Host death by BV infection has been described as melting or disintegration of the insect. The host flattens out, the tissues melt and liquefy so that the body fluids drip from the upper leaves to the lower ones thus leaving the occlusions bodies on the leaves for other insects to digest. As the host travels higher before its death, it thus facilitates the spread of occlusion bodies when the host dies (Rohrmann 2011).

2.5.2 BV in human cells

Despite the insect-specific nature of BVs, they are able to enter and transduce a variety of mammalian cell lines including human, rodent, rabbit, porcine, bovine, fish and avian cells (Hu 2006, Airenne *et al.* 2009). However, they are unable to replicate in these cells and that is why BV is considered as a safer alternative in medical applications when compared to human pathogenic viruses. In addition to safety, BVs show several features that can be exploited in biomedical applications. The large cloning capacity of BVs allows the transfer of multiple

genes and regulatory elements. Furthermore, BVs can be easily manipulated using molecular biology methods and they can be produced in high titers using simple and robust culturing methods. So far BV has been under extensive studies in the fields of protein production, virus and virus-like particles production, eukaryotic protein display, vaccine development, cell-based assay development, tissue engineering and in gene therapy (Chen *et al.* 2011). In BV-based mammalian cell applications, only the budded form of the virus is usually used. The ODV form has also been studied for use in human cell applications but the results were not encouraging (Makela *et al.* 2008). In the studies and discussions here, all data is gathered with BuVs.

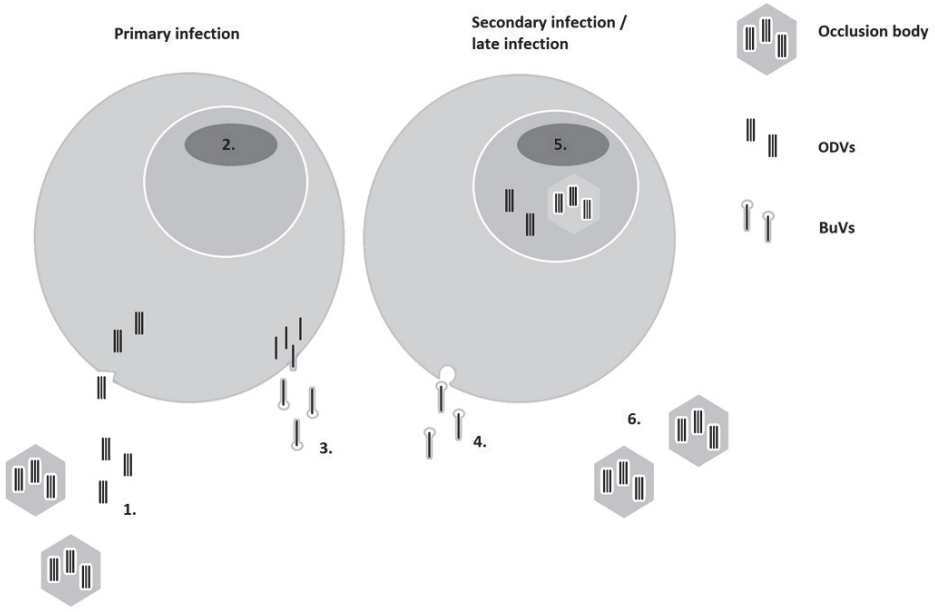

FIGURE 8 BV life cycle in insect cells. Primary infection is mediated by ODVs and systemic infection occurs via BuVs. 1. Occlusion bodies dissolve in the insect midgut releasing the ODVs. 2. After internalization, ODVs start the primary infection by replicating in the midgut cells. 3. During the early stages of infection, BuVs are produced. 4. BVs are responsible for spreading the infection to all cells. 5. In the late stage of infection, ODVs are produced and occlusion bodies are formed in the nucleus. 6. As the cells and host die, ODVs are released. Modified from Rohrman (2011).

2.5.3 BV entry into human cells

The exact receptor or mechanism of entry for BV into mammalian cells is not known. Because of its broad tropism, the receptor is thought to be some common plasma membrane molecule such as a phospholipid or HSPG (Duisit *et al.* 1999, Tani *et al.* 2001). Electrostatic charges, including HSs, are suggested to be

involved in BV binding to human cells (Duisit *et al.* 1999). BV transduction into mammalian cell is dependent on the major glycoprotein 64 (gp64) (Tani *et al.* 2001, O'Flynn *et al.* 2012). A heparan-binding sequence identified in gp64 is essential for mammalian cell transduction (Wu and Wang 2012). Studies with vesicular stomatitis viruses pseudotyped with BV gp64 suggest that gp64 binds to a receptor that can be devoid of raft areas but due to clustering or co-receptors, the final entry is from raft areas (Kataoka *et al.* 2012). Other studies suggest receptor-independent entry and direct fusion of the virus with the cell membrane. However, the membrane fusion is dependent on low pH (Dong *et al.* 2010). In the most recent paper (O'Flynn *et al.* 2012) one of the arguments for receptor-independent entry was simply the lack of identified receptor so far. These contradicting speculations can be due to cell type-specific differences in BV internalization.

The BV entry route into mammalian cells is not thoroughly understod. BV is suggested to use a clathrin-dependent (Matilainen *et al.* 2005, Long *et al.* 2006) entry pathway and/or macropinocytosis (Matilainen *et al.* 2005). However, these results are merely suggestive. Interestingly, genistein, which is an inhibitor of caveolin-mediated endocytosis, is shown to increase BV transduction suggesting that caveolae-mediated endocytosis could have an inhibitory role in BV trafficking (Long *et al.* 2006). After internalization, BV releases its nucleocapsid from the endosome into the cytoplasm by acid-induced g64-mediated fusion. Subsequent nuclear transport of the capsid is mediated by actin filaments (Kukkonen *et al.* 2003, Matilainen *et al.* 2005, Salminen *et al.* 2005). The whole viral capsid enters the nucleus and the nuclear transport is independent of disintegration of the nuclear membrane (Laakkonen *et al.* 2008). BV binding to mammalian cells is shown to concentrate on specific areas on the plasma membrane. Overall, BV internalization is a slow process, virus clusters are evident in the membrane at 5 to 15 min p.t. (post transduction). After 30 min p.t., viruses are seen in perinuclear compartments and some of the viruses are colocalizing with early endosomal markers. Capsids are seen in the nucleus around 3 to 4 h p.t. and the transgene expression is detected after 6h p.t. (Matilainen *et al.* 2005). BV transduction is actin-dependent (Kukkonen *et al.* 2003, Matilainen *et al.* 2005, Salminen *et al.* 2005) and microtubules appear to act as a mechanical barrier to virus entry, since the dissociation of the microtubule network supports efficient viral trafficking (van Loo *et al.* 2001). The intermediate filament protein vimentin appears to have some role in effective BV transduction but the nature of this role is not clear (Mahonen *et al.* 2010).

2.6 Echovirus-1

Echovirus-1 is a non-enveloped virus with a single-stranded RNA genome of 7-8.5 kb in size (Bedard and Semler 2004) and an icosahedral capsid (approximately 30 nm in diameter) (Filman *et al.* 1998). It is a human pathogen belonging to the enterovirus genus of the *Picornaviridae* family. Picornaviruses

are significant pathogens to humans and among picornaviruses, enteroviruses cause the most severe diseases. EV1 causes a variety of diseases in humans, for example, meningitis, encephalitis, carditis and mild respiratory and enteric infections (Grist *et al.* 1978). On the cell surface, EV1 uses α2β1 integrin as its binding and entry receptor into the cell (Bergelson *et al.* 1992, Bergelson *et al.* 1993). Once internalized, virus is found in endosomes and later on the genome is released to the cytoplasm and the plus-stranded RNA can readily act as an mRNA (Fig. 9). The genome encodes for one large polyprotein which is further processed into capsid proteins, non-structural proteins and enzymes needed for the virus replication. The infection leads to cell death by lysis, subsequently releasing the newly formed virions into the surroundings.

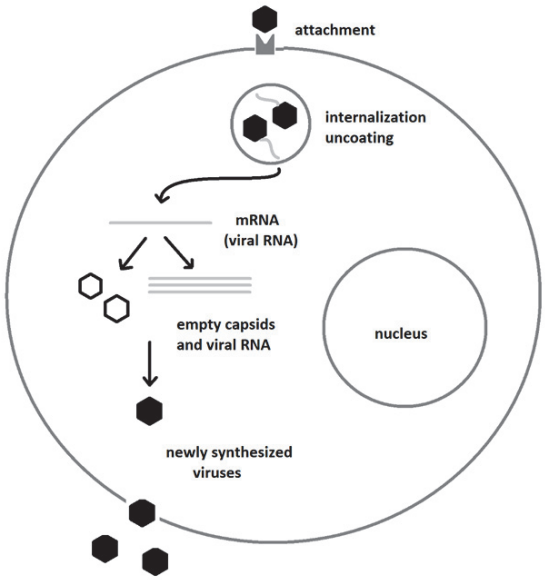

FIGURE 9 EV1 life cycle in human cells. Virus binds to its receptor on the cell surface leading to virus internalization. Viral RNA can readily act as mRNA. New virions are produced and released by cell lysis.

2.6.1 α2β1-integrin as EV1 receptor

Integrins are a large family of transmembrane receptors that link the ECM to the intracellular environment. Integrins are constituted from 18 α- and eight β-subunits, forming altogether 24 different integrin heterodimers in mammalian cells. Integrins have a large extracellular domain and short cytoplasmic part. Integrin conformation is essential for its activity state. In a bent conformation, integrin is inactive, whereas in the extended form it is in the high affinity state and thus active. This conformational variety enables inside-out signaling (intracellular changes affect the activity state of the intergin and thus the ECM binding). In addition, integrins can mediate outside in signaling (extracellular

ligand binding causing intracellular changes) (Campbell and Humphries 2011). Furthermore, integrins are regulated by a cycle of internalization, degradation and recycling back to the cell membrane (Bridgewater *et al.* 2012). Integrins are common receptors for several pathogens including various viruses, such as adenovirus, herpesvirus, hantavirus and foot-and-mouth disease virus (Stewart and Nemerow 2007).

As stated earlier, α2β1-integrin is the cell surface receptor used by EV1 for its binding and internalization into human cells. The α2β1-integrin is abundantly expressed in several cell types (Mizejewski 1999). Naturally, α2β1 integrin binds to the ECM components collagen and lamin (Elices and Hemler 1989). EV1 and collagen both bind to the I domain of the α2-subunit (Elices and Hemler 1989, Bergelson *et al.* 1992, Bergelson *et al.* 1993) but there are differences in their binding. First, the exact binding sequences differ (King *et al.* 1995); second, the binding affinity of EV1 is ten times higher compared to collagen (Xing *et al.* 2004); and third, EV1 binds to the inactive bendt state of the integrin (Jokinen *et al.* 2010), whereas collagen binds to the active state. When the α2β1 integrin is clustered with EV1, it is not able to induce the same signaling routes as when the integrin is clustered with collagen (Jokinen *et al.* 2010), probably due to binding of the virus to the inactive conformation. Also, the internalization route of EV1 bound to α2β1 is different from the route of integrins natural ligand, collagen. EV1 bound integrin does not use the normal recycling pathway back to the plasma membrane but instead is degraded by calpains and thus the overall α2β1 integrin level is downregulated in response to EV1 infection (Rintanen *et al.* 2012).

2.6.2 Echovirus entry route

Usually virus entry follows the pathway used by the physiological ligand of its receptor, but in the case of EV1 entry, this is not true. As mentioned above, EV1 binding to α2β1-integrin receptor causes virus-integrin internalization but the intracellular route of this complex is different from the route of integrins natural ligand, collagen. Binding of EV1 causes lateral clustering of the integrin on the cell surface leading to internalization of the virus-receptor complex (Upla *et al.* 2004). Internalization is dependent on PKCα activation (Upla *et al.* 2004, Jokinen *et al.* 2010) and independent of flotillin, GPIs, caveolae, dynamin or clathrin (Pietiainen *et al.* 2004, Upla *et al.* 2004, Karjalainen *et al.* 2008). The dynamin-dependency might however be cell line specific as there are also contradictory reports (Pietiainen *et al.* 2004, Karjalainen *et al.* 2008). Several macropinocytosis regulators are involved in the entry pathway, strongly suggesting macropinocytosis as the entry route (Karjalainen *et al.* 2008). Even though the early entry is independent of caveolae, EV1 associates with caveolin-1 later after internalization (Pietiainen *et al.* 2004, Karjalainen *et al.* 2008, Karjalainen *et al.* 2011). The internalization as well as genome release are dependent on cholesterol-rich membrane areas (Siljamaki *et al.* 2013). After internalization, EV1 is seen in tubulovesicular endosomes that mature into multivesicular bodies resembling late endosomes. However, these structures are distinct from the

multivesicular late endosomes. Even though, the biogenesis of these virus-induced multivesicular bodies, is dependent on the ESCRT complex that is known to function in late endosomal multivesicular bodies. These virus-induced structures show neutral pH and they were shown to be essential for virus infection (Karjalainen *et al.* 2011).

3 AIM OF THE STUDY

Studying virus-cell interactions provides valuable information concerning viral-mediated gene transfer and virus-host interactions. In both of these areas, the lack of basic knowledge can hamper the development of future treatments and applications. BVs are insect pathogens that are commonly found in nature. They can not replicate but are able to enter and deliver genetic material to human cells. Due to their non-pathogenic nature and efficient gene delivery, they have gained the interest of researchers in the area of biomedicine. However, even though several BV-based biomedical applications are being developed and studied, its trafficking in human cells is still poorly understood. Furthermore, the efficiency of BV-mediated gene transfer is cell type-dependent and the cellular factors affecting it have not yet been identified. Here we have studied BV internalization and gene transfer in human cells. The specific aims of this doctoral thesis were:

1. To define the entry pathway and regulators that are associated with BV internalization into human cells
2. To characterize the role of HSPG in BV binding and internalization to human cells
3. To characterize cellular factors contributing to the permissiveness of the cells leading to efficient gene delivery by BV

4 SUMMARY OF MATERIALS AND METHODS

Detailed descriptions of all materials and methods can be found in the original publications (I-III). An overall view of the materials and methods are summarised in the tables below (Tables 1-6).

TABLE 1 Methods

Method	Publication
BV binding and internalization assays	I , II, III
BV transduction	I , II, III
Confocal microscopy	I, II, III
Drug treatments	I, II, III
Electron microscopy	I
EV1 binding and internalization assays	III
EV1 infection	III
Flow cytometry	I, II, III
Immunofluorescence labeling	I, II, III
Plasmid transfections	I, III
Quantification of microscopic data	I, III
Receptor clustering assay	II, III
SDS-PAGE and immunoblotting	I, III
siRNA-mediated mRNA inhibition	I, II, III
Sulfation assays	III

TABLE 2 Cell lines

Cell line	Cell type	Publication
293(T)	Human embryonic kidney cells	I, II, III
Ea.hy926	Hybridoma of human umbilical vein endothelial and human lung carcinoma epithelial cells	II, III
HepG2	Human hepatocarcinoma cells	I, II, III
MG-63	human osteosarcoma cells	III
Raw264.7	Mouse macrophage-like cells	III

TABLE 3 Primary antibodies

Antibody	Supplier	Publication
A211E10 (α2-integrin)	From F. Berditchevski	III
AcMNPV ab	From L. Volkman	I, II, III
Actin ab	Sigma Aldrich	I
Afr6 Mab	Thermo fisher scientific	I, III
cd59	SantaCruz biotechnology	II, III
cd81 Mab	SantaCruz biotechnology	III
Clathrin ab	Abcam	I
Dynamin-2 ab	From M. McNiven	I
EEA-1 Mab	Transduction laboratories	I, III
EV1	Marjomäki et al. 2002	III
FLAG M2 Mab	Sigma Aldrich	I
Flotillin Mab	BD biosciences	I
gp64 Mab	from L. Volkman	II, III
Lamin Mab	Novocastra laboratories	I
Luciferase Mab	Serotec	I
NTb ab (IL-2)	Gift from A. Dautry-Varsat	I
PIP2	Molecular probes	III
PKCα	Transduction leboratories	III
PKCε	SantaCruz biotechnology	III
pPKCα	Upstate Biotechnology	III
pPKCε	Upstate Biotechnology	III
Rac-1 Mab	Millipore	I
RhoA Mab	SantaCruz biotechnology	I
Syndecan -2 ab	SantaCruz biotechnology	II, III
Syndecan -3 ab	SantaCruz biotechnology	II, III
Syndecan-1 ab	SantaCruz biotechnology	II, III
Syndecan-4 ab	SantaCruz biotechnology	II, III
Syntenin Mab	SantaCruz biotechnology	III
Tubulin Mab	Sigma Aldrich	I
Vimentin Mab	Leica microsystems	III
vp39 Mab	Gift from L. Volkman	I, II, III

TABLE 4 Constructs and siRNAs

Construct	Description	Publication
Arf6	siRNA	I
Arf6 Q67L	Constitutively active	I
Arf6 T27N	Dominant negative	I
Arf6 WT	Wild type	I, III
Cdc42 17N	Dominant negative	I
Cdc42 WT	Wild type	I
Clathrin	Wild type	I
Dynamin-2	siRNA	I
Eps15 DN	Dominant negative	I
Eps15 WT	Wild type	I
GPI-EGFP	Wild type	I
IL-2R beta-chain	Wild type	I
Pak1AID	Constitutively active	I
Pak1T423E	Dominant negative	I
PH-GFP	PIP2 PH-domaind binding	III
PKCα DN	Dominant negative	III
PKCα WT	Wild type	III
PKCε k/m	Kinase dead	III
PKCε WT	Wild type	III
Rab34 CA	Constitutively active	I
Rab34 DN	Dominant negative	I
Rab34 WT	Wild type	I
Rac1	siRNA	I
Rac1 12V	Constitutively active	I
RhoA	siRNA	I
RhoA 14V	Dominant negative	I
RhoA 19N	Constitutively active	I
RhoA WT	Wild type	I, III
Sdc-1 WT	Wild type	
Syntenin	siRNA	III
Vimentin	siRNA	III

TABLE 5 Reagents and drugs

Reagent / Drug	Description		Publication
Amiloride (EIPA)	Macropinocytosis regulator/inhibitor	Sigma Aldrich	I
Chlorpromazine	Inhibition of clathrin coated pit formation	Sigma Aldrich	I
Conjugated transferrin	Ligand for clathrin mediated endocytosis	Molecular Probes	I
Cycloheximide	Inhibition of protein synthesis	Sigma Aldrich	I
Desulfated heparans	2-O-, 6-O- and N-desulfated heparans	Iduron	II
Dynasore	Dynamin inhibitor	Kirchhausen lab	I
Filipin	Cholesterol binding	Sigma Aldrich	I
HRP	Fluid-phase marker	Sigma Aldrich	I
PI-PLC	Digestion of GPI-anchored proteins	Sigma Aldrich	II
PMA	Activation of PKCs	Sigma Aldrich	III
Recombinant Sdc-1	Competition assays	Sigma-Aldrich	II
Sodium chlorate	Prevention of GAG sulfation	Sigma Aldrich	II
TRICT-dextran (10 kDa)	Macropinocytosis probe	Molecular Probes	I
TRICT-phalloidin	Filamentous actin probe	Sigma Aldrich	I, III

5 REVIEW OF THE RESULTS

5.1 BV receptor in human cells

As previously stated, BV can transduce a wide variety of cell types. Because of this, the cell surface receptor for BV has been thought to be a general cell surface molecule. However, the exact receptor that BV interacts with in mammalian cells has not been identified yet. HSPGs are one of the most common molecules found on the cell surface and they associate with several different viruses. In order to identify the specific binding receptor for BV in mammalian cells, we studied the role of HSPGs in BV binding to HepG2, 293 and Ea.hy926 cells by flow cytometry and confocal microscopy.

5.1.1 BV binds to 6-O and N-sulfated heparans

In order to define the role of HSPGs on BV binding, we started by studying the effect of sulfation on BV binding. $NaClO_3$ is a substance that prevents sulfate donation to new polysaccharide chains leading to undersulfated GAGs. With $NaClO_3$ treatment, we were able to decrease the binding of the virus on the cell surface in Ea.hy926 and HepG2 cells (II, Fig. 2A). In addition, we showed that the BV transduction efficiency decreased in a dose-dependent fashion in HepG2 cells (II, Fig. 2B). Thus, it can be concluded that sulfation was essential for binding and efficient internalization of the virus.

As we showed that the overall sulfation is essential to efficient BV transduction, we wanted to further assess the optimal sulfation. We then performed a competition assay using differentially desulfated heparans. Virus was first incubated with heparans and the virus was then allowed to internalize cells and the transduction efficiency was determined. As the heparans showed differential desulfation, we could determine the specific sulfations that were needed and those that did not have an effect. Preincubation with wild type heparan and 2-O-desulfated heparan efficiently inhibited virus transduction and preincubation with 6-O-desulfated and N-desulfated heparans did not affect virus transduction in 293T and HepG2 cells (II, Fig. 2C). These results suggested

that BV binds to 6-O and N-sulfated heparans, and the binding was not mediated by 2-O-sulfations.

5.1.2 Syndecan-1 acts as a binding and entry receptor for BV

As it was clear that BV associated with specifically sulfated HSPG, we wanted to further define which members of the HSPG family mediated the binding. HSPG contain two main subfamilies, glypicans and syndecans. Glypicans are GPI-anchored proteins that can be cleaved from the plasma membrane with PI-PLC enzyme. We thus studied the effect of PI-PLC treatment on BV internalization. PI-PLC treatment did not significantly inhibit BV binding in HepG2 or Ea.hy926 cells (II, Fig.3A) or transduction (data not shown) demonstrating that glypicans were not essential in BV binding or efficient internalization. PI-PLC treatment did, however, efficiently remove GPI-anchored proteins from the cell surface as demonstrated with the aid of Cd59 antibody (II, Fig. 3A and 3B).

As the other major HSPG family is composed of syndecans, we then proceeded to study their role in more detail. We started by assessing the amount of different syndecans in 293, HepG2, MG-63 and Ea.hy926 cells. All of the studied cells expressed all syndecans and there seemed to be a clear trend between the cell lines, as syndecan-1 was the most expressed or one of the most expressed syndecan and syndecan-4 showed the lowest expression (II, Fig. 4A). In addition, we assessed virus binding in the cells and the results showed that binding correlated with the expression of syndecan-1 in the HepG2, Ea.hy926 and MG-63 cells (II, Fig. 4B).

As HepG2 and Ea.hy926 cells showed the highest amounts of bound virus on the cell surface we continued our studies with these two cell lines. We then studied the colocalization of BV with different syndecans. BV was allowed to internalize cells for 4h and the cells were then fixed, immunolabelled and imaged with a confocal microscope. A clear colocalization was observed with BV and syndecan-1 whereas only background colocalization was seen with other members of the syndecan family (II, Fig. 5A). Clear colocalization between syndecan-1 and BV was also evident in earlier timepoints (30 min and 2h p.t.) (II, Fig. 5B). To gain more information on the role of different syndecans in BV internalization, we performed antibody inhibition experiment in the HepG2 cells. Prior to virus addition, cells were treated with primary antibodies against all syndecans. Only the antibody against syndecan-1 was able to inhibit BV transduction and internalization (data not shown). Antibody against syndecan-1 showed dose-dependent inhibition of BV transduction in HepG2 cells (II, Fig. 6A). Furthermore, a competition assay with recombinant syndecan 1 protein also showed dose-dependent inhibition of BV transduction (II, Fig. 6C). Additionally, overexpression of syndecan-1 in HepG2 cells induced BV transduction significantly (II, Fig. 6D). The main experiments leading to identification of syndecan-1 as a BV-binding receptor are summarized in figure 10.

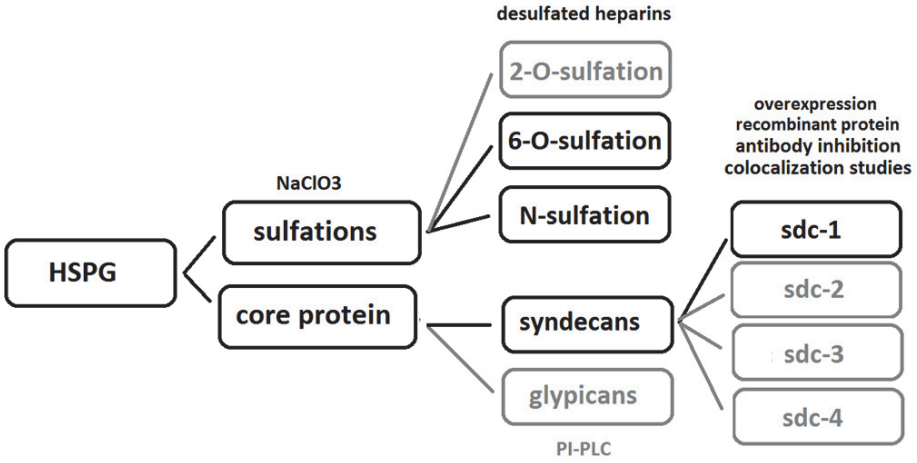

FIGURE 10 Main experiments and conclusions leading to identification of syndecan-1 as a BV binding receptor. Factors shown to play a role in BV binding are shown in black. Factors uninvolved in BV binding are shown in grey.

5.2 BV internalization into permissive cells

In insect cells, BV uptake is mediated by endocytosis. In mammalian cells BV uptake has been suggested to be either clathrin-mediated or macropinocytotic. These previous results, however, were only suggestive and were monitored from the aspect of transduction and not the actual internalization (Matilainen *et al.* 2005, Long *et al.* 2006, Kataoka *et al.* 2012). Here we studied the regulators of several endocytic pathways (macropinocytosis, phagocytosis, clathrin, caveolin, GEEC, flotillin and IL-2R), and their effect on BV internalization and nuclear entry. BV entry pathways were studied by confocal microscopy, electron microscopy and flow cytometry in permissive HepG2 and 293 cells.

5.2.1 BV is not internalized through clathrin-, caveolin-, flotillin-, IL-2-, or GEEC-mediated pathways, or macropinocytosis

Using confocal microscopy, we first studied the possibility of clathrin mediated endocytosis in BV entry. Several lines of evidence suggested that clathrin was not involved in BV internalization. We were unable to see any colocalization between BV and clathrin heavy chain during the early entry timescale (I, Fig. 1A, B and C), neither was the distribution of clathrin altered during virus entry (I, Fig. S1A, S1B). Furthermore, BV internalization was not affected by expression of the clathrin-regulator Eps15 dominant negative (DN) mutant (I, Fig. S1C and D). Eps15 DN lacks the EH-domains and efficiently inhibits the formation of clathrin

coated vesicles (Benmerah *et al.* 1999). In addition, there was no colocalization observed between BV and transferrin (I, Fig. S1E), which is a typical cargo trafficking in the clathrin-mediated route. Next, the role of caveolae was studied with confocal microscopy. As the results show, there was no colocalization between BV and caveolae during the early entry timescale (I, Fig. S1G). These results indicated that BV does not enter through the clathrin or caveolae-dependent pathway.

GPI-anchored proteins are commonly found in the raft areas. We tested the possibility of BV traveling the GEEC route. The GEEC route is a raft-derived, GPI-anchored protein enriched early endosomal pathway (Sabharanjak *et al.* 2002, Kalia *et al.* 2006). However, there was no colocalization between GPI-EGFP and BV (I, Fig. S3B). In addition, Cdc42 mutants had no inhibitory influence on BV internalization (I, Fig. S3C, S3D and S3E). Cdc42 is needed to target the GPI-anchored proteins to early endosomes (Sabharanjak *et al.* 2002) and thus Cdc42 mutants should have an effect on cargo trafficking this route. GPI-anchored proteins have also been shown to travel through the flotillin-positive route. Flotillin-1 defines a new raft-mediated entry route independent of the caveolin- and clathrin-mediated routes (Glebov *et al.* 2006). Nevertheless, flotillin and BV showed no colocalization (I, Fig. 3B and S3A). These results altogether indicated that neither the GEEC nor flotillin route is used by BV.

Next we tested the role of the IL-2 receptor pathway on BV entry. The IL2-receptor pathway is a raft-associated, clathrin-independent, dynamin-dependent route that is regulated by the Rho GTPases RhoA (Lamaze *et al.* 2001) and Rac1, and the serine/threonine kinases Pak1 and Pak2 (Grassart *et al.* 2008). The Ntb-domain of IL-2 was transfected into the cells in order to visualize the internalized IL-2. No colocalization was observed between IL-2 and BV (I, Fig. S3H), suggesting that BV does not enter through the IL-2 receptor pathway. Neither did siRNA against Rac1 or Rac1 CA and DN mutant constructs have an effect on BV internalization (I, Fig. 5C, Fig. S3F, Fig. S3G). To conclude, BV internalization was not mediated by the IL-2 receptor pathway.

One of the characteristics of macropinocytosis is an increase in the uptake of horseradish peroxidase (HRP). However, there was no difference on the HRP uptake between the control cells and BV transduced cells (I, Fig. 2E), indicating that BV does not induce HRP uptake. To further study the possible role of macropinocytosis, different inhibitors and proteins involved in macropinocytosis were tested. EIPA is an inhibitor of Na+/H+ exchanger and also one of the inhibitors for macropinocytosis. EIPA treatment had no inhibitory effect on BV transduction. In addition, Rab34 and Pak1 are common regulators of macropinocytosis (Dharmawardhane *et al.* 2000, Sun *et al.* 2003). Neither Rab34 nor Pak1 wt, DN or CA mutants had an effect on BV transduction (I, Fig. 3D, S3K, S3L, S3M, S3N and S3O). Also the Pak-1 downstream target CtBP1/BARS, another macropinocytosis regulator (Liberali *et al.* 2008), wt or DN constructs were tested and had no effect on BV nuclear entry. These results further suggested that macropinocytosis was not involved in BV entry.

5.2.2 BV internalization is regulated by Arf6, RhoA and Dynamin

Arf6 is a common regulator of membrane trafficking and we next studied its role in BV entry. Cells were transfected with Arf6 WT, CA and DN constructs. Arf6 CA (Q67L) causes the accumulation of attenuated PIP2 containing endosomes (Zimmermann *et al.* 2005). Arf6 DN (T27N) blocks the return of Arf6 recycling endosomes back to the plasma membrane (Radhakrishna and Donaldson 1997) and induces the accumulation of syntenin and retention of syndecans in the endosomes (Zimmermann *et al.* 2005). The Arf6 DN construct showed inhibition of both the virus internalization and transduction efficiency. Interestingly, the decrease in the transduction efficiency was higher than the decrease in virus entry (I, Fig. 4A, 4B and 4C) thus suggesting that Arf6 DN has some inhibitory role after virus internalization as well. Inhibition of nuclear entry was also evident in the case of the Arf6 CA contruct (I, Fig. 4C) and with Arf6 siRNA (I, Fig. 4D).

RhoA is a small GTPase that regulates the actin cytoskeleton. The experiments with RhoA CA and DN constructs markedly decreased BV internalization (I, Fig. 5A and 5B). Also siRNA against RhoA showed lower nuclear internalization of the virus (I, Fig. 5C).

Dynamin is a small molecule that is associated with several endocytosis routes. Dynamins are involved in the scission of the newly formed vesicles from the plasma membrane (Mercer *et al.* 2010). The dynamin-dependence was tested using dynasore, which is a dynamin inhibitor. Dynasore treatment was shown to cause a 67%-reduction in virus internalization (I, Fig. 1D). Altogether, these results showed that BV internalization was regulated by Arf6, RhoA and dynamin.

5.2.3 BV internalizes from raft areas and induces membrane ruffling and co-internalization of *E. coli* in to non-phagocytic cells

After BV administration, extensive ruffle formation was observed on the target cell surface (I, Fig. 2A and S2). The viruses were shown to use the membrane protrusions for attachment and also for entry into the cells (I, Fig. 2A). The percentage of ruffle-positive cells rose from 27 % in control cells to 77% in BV-treated cells (I, Fig. 2B). After co-internalization with the fluid-phase marker HRP, EM analysis showed BV together with HRP in large (603 nm ± 25 nm) vacuoles (I, Fig. 2D). When BV entry was studied in live cells, we observed that virus administration caused extensive ruffling on the cell surface in HepG2 and 293 cells. This ruffling then lead to efficient BV uptake from the ruffle areas. Cell surface areas that were active during BV entry were shown to be strongly positive for phalloidin-labelled F-actin. Using differential labeling, we monitored the internalization of BV in response to the different treatments. Filipin binds to unesterified cholesterol in solution and in membranes and thus affects specifically the raft and caveolar pathways (Schnitzer *et al.* 1994). Filipin was observed to inhibit the BV internalization by 82% (I, Fig. 3A).

Several factors, including the large size of BV, dependency of the entry on Arf6 and RhoA, association with membrane rafts and extensive ruffling, lead us to study the role of a phagocytosis-like mechanism in BV entry. The role of phagocytosis was studied with *E. coli* particles, which are commonly used markers for phagocytosis. *E. coli* was internalized into cells together with BV and these two showed clear colocalization (I, Fig. 6A and 6B) in HepG2 cells. Furthermore, using fluorescent *E. coli* particles we studied the simultaneous internalization of *E. coli* and BV. From these studies it was clear that internalization of *E. coli* into these non-professional phagocytic cells could not happen without the simultaneous inoculation with BV. The amount of internalized *E. coli* was significantly higher whe cells were simultaneously fed with BVs (I, Fig. 6C). This induced entry of *E. coli* was observed to be transient, since the bacteria could only efficiently enter when fed simultaneously with the virus and not after the virus was removed. The effect of dynamin and filipin on induced *E. coli* entry was tested and the assay showed that both filipin and dynasore decreased the co-internalization of *E. coli* and BV (I, Fig. 6D).

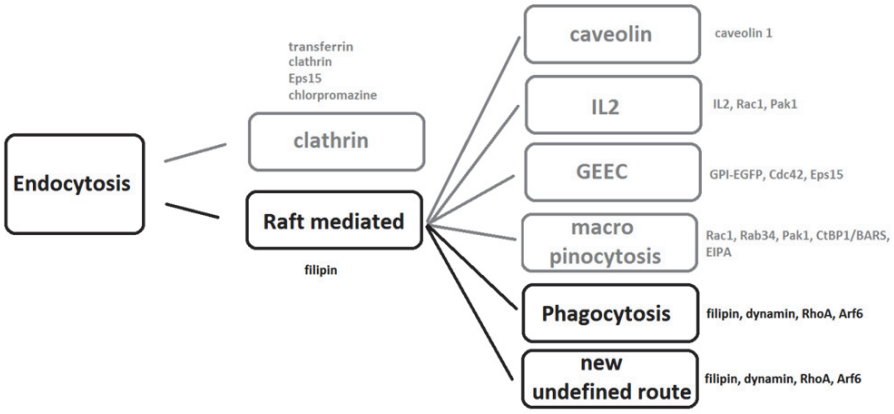

FIGURE 11 Routes and regulators mediating BV internalization in permissive cells. Drugs and experiments affecting BV internalization are shown in black. Regulators that were shown not to have a role in BV internalization are shown in grey color.

5.3 BV transduction and EV1 infection in non-permissive cells

BV is able to transduce a wide variety of mammalian cell types but the transduction efficiency can vary drastically between cell types. In order to define what are the factors mediating efficient and/or defective transduction, we studied cell lines that have been previously characterised as highly permissive and non-permissive (Kukkonen *et al.* 2003, Kost *et al.* 2005). In addition to BV, we

also studied the small human pathogen EV1. The main characteristics of both viruses are listed in Table 7.

TABLE 7 Characteristics of BV and EV1. Characteristics marked with * are in human cells.

Characteristic	Baculovirus	Echovirus-1
Family	*Baculoviridae*	*Picornaviridae*
Host	Insect	Human
Virion size	up to 250 nm	30 nm
Nucleocapsid	Helical	Icosahedral
Genome	DNA	RNA
Genome size	up to 180 kb	up to 8.5 kb
Envelope	Enveloped	Non-enveloped
Receptor*	Syndecan (II)	α2β1-integrin
Entry route*	Unclassified (I)	Macropinocytosis
Gene translation*	Nucleus	Cytoplasm

5.3.1 Permissive and non-permissive cell phenotype

Five cell lines from different origins were characterized for their ability to be transduced or infected by BV or EV1, respectively. Raw264.7 cells were practically unaffected by virus treatment while HepG2 cells showed, by far, the highest transduction and infection efficiency. 293 cells showed moderate transduction and infection, whereas Ea.hy926 and MG-63 cells were almost non-permissive to the viruses, showing only minimal transduction and infection (III, Fig. 1A and B). From these cell lines, Ea.hy926 and MG-63 were selected for further characterization as model cell lines for non-permissive cells. As the model cell line for permissive cells, HepG2 cells were chosen. We then assessed the efficiency of virus binding and the viral receptor amount on the cell surface. The results showed that all three cell lines expressed viral receptors abundantly and virus binding was equally efficient (III, Fig. 1C-F). Eventough both viruses were shown to bind to cells equally well, only EV1 was shown to internalize the non-permissive Ea.hy926 and MG-63 cells. BV accumulated on the cell surface with syndecan-1 (III, Fig. 2A, B, E andf F). As the permissive and non-permissive cell lines were further characterized, we observed that the permissive HepG2 cell line showed significantly higher amounts of F-actin, notably lower levels of syntenin and a distinguishably different vimentin expression pattern in comparison to the non-permissive cells. Additionally, the non-permissive cells had large cytoplasmic aggregates of pPKCα which were not observed in the permissive cell line (III, Fig. 3A and B).

Since RPMI medium has been shown to induce BV transduction significantly (Mahonen *et al.* 2010), we wanted to assess if this was true for these cell lines also. Additionally, we wanted to study if EV1 infection could be affected by the culture medium as well. In contrast to DMEM, BV transduction and EV1 infection efficiencies were significantly improved in Ea.hy926, MG-63 and HepG2 cells, when cells were cultured in RPMI medium (III, Fig. 4A). As the

change to RPMI culture medium was able to at least partly rescue efficient BV transduction and EV1 infection, we next monitored how F-actin, syntenin, pPKCα and vimentin were affected by the medium change (III, Fig. 4B). We could not observe any changes in F-actin and therefore its role was not further characterized. However, high syntenin expression was found to coincide with low BV transduction and EV1 infection. The effect of culture media on syntenin expression was especially drastic in Ea.hy926 cells. To directly study the effect of syntenin expression in the cells, we used siRNA knockdown experiment. Even though syntenin expression was efficiently downregulated (95% knock-down) it could not rescue BV transduction or EV1 infection (III, Fig. 4D). Also, the PKCα distribution changed in response to the medium change. In RPMI medium, the overall level of pPKCα was higher and it was more located to the cell surface (III, Fig. 4B and E). The medium change affected the vimentin contents of the cell as well. The non-permissive phenotype correlated with a tight and compact network of filamentous vimentin. As the cells became more permissive to the viruses, the level of filamentous vimentin decreased, the vimentin network became looser and more spread around the cell (III, Fig. 4B). Because of the drastic changes in the vimentin network, we also studied if PKCε was affected by the medium change. We could not see any change with the total PKCε pool (data not shown) but the phosphorylated form showed significant downregulation in response to RPMI medium (III, Fig. 5A). These phenotypic characteristics are listed in Table 8.

TABLE 8 Cellular factors affecting permissive and non-permissive cell phenotypes. PM=plasma membrane.

	Non-permissive (DMEM)	**Non-permissive (RPMI)**	**Permissive**
F-actin	low	low	high
Syntenin	high	low	low
pPKCα	aggregated in cytoplasma	localized more to the PM	in the PM
pPKCε	aggregated with BV on the PM	downregulated	diffuse
Vimentin	highly expressed, tight filamentous network	downregulated, more loose filamentous network	low expression, no filamentous network detectable

5.3.2 BV aggregates with syndecan-1 on the cell surface in non-permissive cells

As it was evident that the non-permissive nature of the cells was not due to inefficient virus binding or a lack of viral receptor, we then wanted to asses if BV

was able to internalize into the cells. Simultaneously we wanted to observe how the BV receptor, syndecan-1, was affected by BV internalization in these cells. After 5h internalization of BV, cells were fixed and immunolabelled to distinguish between the cell surface and intracellular BV or syndecan-1, respectively. The cells were subjected to thorough analysis by confocal microscopy, which revealed that BV was not able to internalize into the non-permissive cells but stayed on the cell surface in large aggregates. In addition, these aggregates were shown to colocalize with syndecan-1 (III, Fig. 2A and B) and not with other members of the syndecan family (data not shown). When these aggregates were further studied, it was noticed that in the non-permissive Ea.hy926 cells, they became larger over time and it was evident that BV was able to gather more of syndecan-1 as the time elapsed (III, Fig. 2C and D). Furthermore, pPKCε was found in these aggregates with BV and syndecan-1 (III, Fig. 5C).

5.3.3 Intracellular trafficking of EV1 is deficient in non-permissive cells

Even though the cell lines permissiveness to BV and EV1 went hand-in-hand with both viruses, the mechanism of restriction showed notable differences. EV1 was found to efficiently internalize into non-permissive cells. Furthermore, the internalization and ratio of extracellular and intracellular α2β1-integrin after clustering was similar in both non-permissive and permissive cells (III, Fig. 2E and F), suggesting that integrin internalization in the cells is normal and, in the case of EV1, the block is in the intracellular trafficking. The regulation of PKC subtypes alpha and epsilon were observed to differ between the viruses as well. PKCα was unaffected by BV internalization in permissive cells, while after EV1 internalization pPKCα was downregulated (III, Fig. 3B) as reported earlier by Upla and colleagues (2004). However, in non-permissive cells EV1 internalization did not affect pPKCα suggesting that altered PKCα function in non-permissive cells might affect the intracellular trafficking of EV1. These results also further indicated that pPKCα function is different in permissive and non-permissive cells. In addition, PKCε was downregulated in response to EV1 internalization in non-permissive cells, while BV transduction showed no alterations in pPKCε levels (III, Fig. 5C). Suggesting that the internalization of EV1 into non-permissive was dependent on PKCε activity.

5.3.4 PMA modulates the vimentin network, affecting the permissiveness of the cell

As PKCα and PKCε were both shown to have a role in the permissiveness of the cells, we wanted to study how the PKC activator phorbol-12-myristate-13-acetate (PMA) would affect BV transduction and EV1 infection efficiency. Interestingly, PMA induced efficient virus trafficking in HepG2 cells, whereas in non-permissive cells, it either had no effect in DMEM or it could undo the stimulating effect of RPMI on BV transduction and EV1 infection (III, Fig. 6A and B). As the role of PMA in permissive and non-permissive cells was thus different, it was

clear that only the non-permissive cells contained some negative regulator that was affected by PKC activation. As PKCε is one of the kinases that regulate the vimentin network and the permissive cell lines did not appear to have a proper vimentin network (III, Fig. 4B and 7B), we thus further studied the association between PMA treatment and the vimentin network. These experiments altogether showed that in Ea.hy926 cells cultured in RPMI medium, PMA caused similar alterations in the vimentin network as does the change to DMEM medium. Furthermore, in cells cultured in DMEM, PMA was able to even further tighten the vimentin network (III, Fig. 7A) indicating that the vimentin dynamics were associated with the efficiency of BV transduction and EV1 infection.

These results thus suggested that in non-permissive cells, efficient virus transduction and infection could be introduced by loosening and downregulating the filamentous vimentin network. In the permissive HepG2 cell line, vimentin showed no changes in response to PMA treatment (III, Fig. 7B). In addition to modulating the vimentin network, both the change to DMEM and PMA treatment appeared to also increase the total amount of filamentous vimentin. Furthermore, the overall level of vimentin in the cells was highly different between the permissive and non-permissive cells (III, Fig. 4B) and thus we wanted to see if vimentin knockdown could modulate the cells permissiveness. Inhibition of vimentin expression was efficient (knockdown of 77%) but it could not rescue BV transduction or EV1 infection (III, Fig. 7C), suggesting that the overall amount of vimentin in the cells is not the main restricting factor. Altogether these results are in agreement with the hypothesis that regulation of PKC activation and subsequent vimentin dynamics are the main factors associated with the permissiveness of the cells (figure 12).

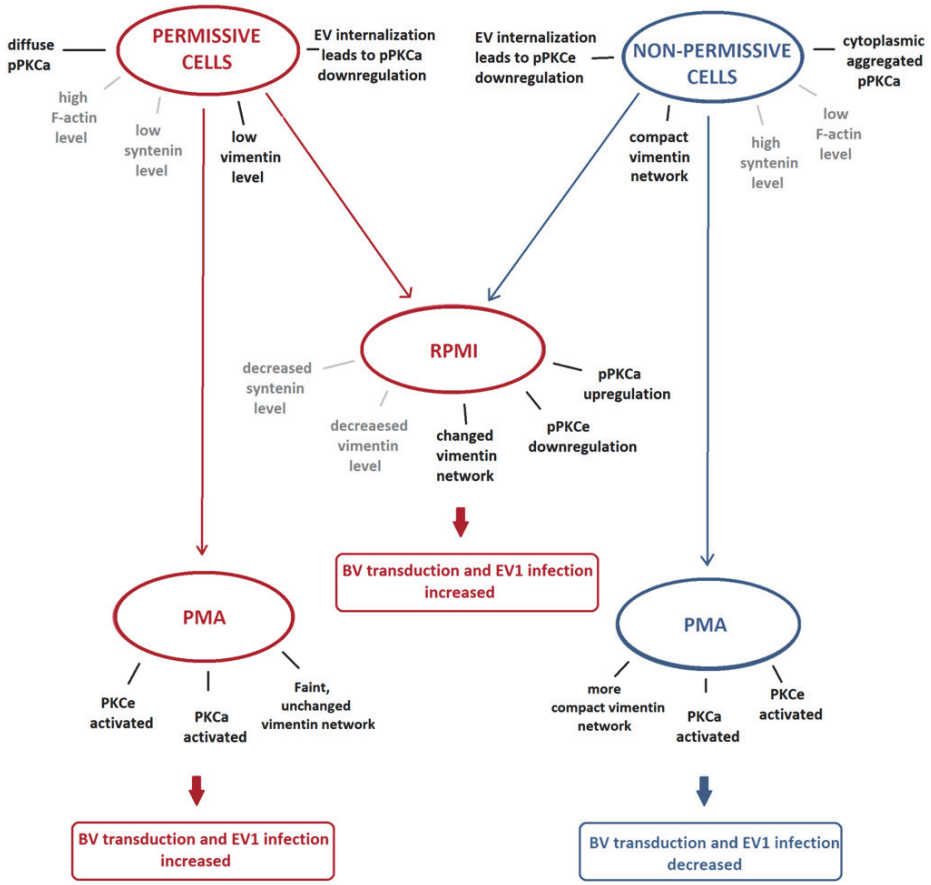

FIGURE 12 Cellular characteristics associated with permissive and non-permissive cell phenotype. Factors not affecting the efficiency of BV transduction and EV1 infection are shown in grey colour.

6 DISCUSSION

BV holds great promise in the field of gene therapy, vaccine research and other biomedical applications. Therefore, the cellular factors affecting its life cycle in human cells are important in further defining its applicability. This thesis aimed to study the aspects of BV binding, internalization and efficient transduction in human cell lines. Interestingly, as we studied the factors limiting successful BV transduction in non-permissive cells, we noticed similarities with another, highly different virus, EV1. In order to define the non-permissive cell phenotype in more detail and additionally gain knowledge on EV1 trafficking, in the third publication, these two viruses were studied side-by-side.

6.1 BV binding on the cell surface

BV showing potential as a human gene transfer vector, its life cycle in human cells has been studied extensively. Surprisingly, despite several studies, the BV receptor in human cells is still unidentified. Although some common cell surface molecules have been suggested and studied, no specific molecules affecting BV binding in mammalian cells have been identified so far. Due to their ubiquitous expression on the cell surface, HSPG has been suggested to play a role in BV binding to mammalian cells (Duisit *et al.* 1999). We studied HSPGs in more detail and identified syndecan-1 as a specific cell surface receptor for BV.

6.1.1 6-O- and N-sulfated syndecan-1 mediates binding and entry of BV to human cells

The sulfation status of HSPG is crucial for the interactions of HS moieties with its ligands. With $NaClO_3$ treatment, we showed that sulfation is also crucial in the binding of BV to the HS moieties. Furthermore, with undersulfated heparans, we showed that BV specifically binds to *N*- and 6-*O*- but not to 2-*O*-sulfated HSPG, indicating that BV binding to the mammalian cell surface is not solely due to random electrostatic interactions (II). There are two main families of HSPG;

syndecans and glypicans. On the contrary to transmembrane syndecans, glypicans are GPI-anchored HSPG molecules. In order to determine if both of these molecules affected BV binding, we enzymatically removed glypicans from the cell surface. Glypican removal did not affect BV binding or transduction suggesting that syndecans were the major family responsible for BV binding and entry. Furthermore, BV colocalization with different syndecans, the antibody inhibition, recombinant protein competition and overexpression experiments all suggested that syndecan-1 was specifically associated with BV binding and internalization into the cells (II). Furthermore, several other cell lines permissive for BV transduction, such as PC-3, MCF-7, A-431 and A-549 express high levels of syndecan-1 (Human protein atlas). Since syndecan-1 is abundantly expressed in various cell types and syndecan-1 upregulation can be detected in several cancer types and several severe diseases, these results further justify the use of BV in biomedical applications.

As the sulfation status of different syndecans differ between cell types, it is possible that the preference for a specific syndecan might change in response to different sulfation. However, despite the fact that the cell lines used in our studies were derived from highly different cell types (liver carcinoma cells, embryonic kidney cells, hybridoma of endothelial and epithelial cells and osteosarcoma cells), they all showed specific preference of syndecan-1 as the BV binding receptor over the other members of the syndecan family. With 293 cells, this was not as evident as with the other cell lines (II), suggesting that there might be some other factor, in addition to syndecan-1, that mediates BV binding but do not lead to efficient transduction. Actually, there was a small indication that in addition to syndecan-1, syndecan-2 might also be involved in the binding of BV to 293 cells (data not shown). This was specific to 293 cells only and was not studied in more detail.

6.1.2 BV and syndecan-1 internalization is halted on the cell surface in non-permissive human cells

As reported previously by Mähönen et al (2010), Ea.hy926 and MG-63 cell are deficient for BV transduction and virus accumulates into the cells. Since syndecan sulfation is dependent on the cell type and thus can differ between cell lines, we first assumed that the aggregation and block in the internalization could be because some other member of the syndecan family showed the optimal sulfation and this "decoy" receptor then lead the virus to a deficient pathway. However, as we studied these virus aggregates in more detail, we noticed that BV only colocalizes with syndecan-1 and not with other members of the syndecan family (III). Furthermore, we noticed that these virus aggregates are located on the cell membrane rather than in cytoplasm, as previously suggested (Kukkonen *et al.* 2003, Mahonen *et al.* 2010). Since the aggregation was not due to BV binding to a decoy receptor but rather due to deficient BV and syndecan-1 trafficking, we studied regulators that affect syndecan trafficking in the cells, including Arf6, PIP2 and syntenin. Even though Arf6 regulated BV entry in permissive cells (I), it had no rescuing effect on the block seen in non-permissive

cells (ata not shown). Additionally, we were not able to see differences in PIP2 distribution in response to DMEM and RPMI media (data not shown). Furthermore, even though syntenin expression was found to be upregulated in the non-permissive cells, syntenin knockdown had no effect on BV transduction (III).

In permissive cells, antibody-mediated syndecan-1 clustering leads to efficient BV internalization. In non-permissive cells, however, BV-induced aggregates were not able to enter the cells or clear up from the membrane since they were visible 24 h after virus incubation (data not shown). Syndecan clustering occurs naturally also without ligand but ligand-induced clustering appears to be essential for the internalization of the ligand (Fuki *et al.* 1997, Fuki *et al.* 2000a). In order to determine if these atypically large aggregates were occurring also naturally in non-permissive Ea.hy926 cells, we monitored the trafficking of syndecan-1 without BV or antibody-mediated clustering. We noticed that even though syndecan-1 seemed to aggregate into small clusters on the cell surface, most of these clusters entered the cells within 2h, and after 24h; none of the clusters were detected on the cell surface (data not shown). This suggested that syndecan-1 trafficking was not naturally attenuated on the cell membrane in Ea.hy926 cells.

Syndecan heterodimerization/oligomerization can also be one factor explaining the cell type specific differences in syndecan function. However, we characterized the syndecan composition in Ea.hy926 and HepG2 cells and there were no significant differences in the syndecan levels between these cell lines (II). Furthermore we were not able to see any colocalization of BV with any other syndecan, suggesting that differential syndecan function due to different syndecan expression patterns was not the explanation for deficient virus internalization.

In addition to BV and syndecan-1, also the phosphorylated form of PKCε was found from the cell surface BV aggregates (III). In non-permissive cells, EV1 infection was also deficient. However, on the contrary to BV, EV1 was able to internalize the cells and EV1 internalization was shown to lead to subsequent pPKCε downregulation. PKCε is one of the kinases that phosphorylate vimentin, thus PKCε might provide a link between EV1 and vimentin. Vimentin is emerging as one of the regulators controlling vesicular membrane trafficking (Ivaska *et al.* 2007). Additinally, several viruses use cell surface vimentin for their entry (Das *et al.* 2011, Liang *et al.* 2011, Plummer *et al.* 2012).

6.2 BV internalization

6.2.1 BV internalization is not mediated by clathrin-, caveolin-, flotillin-, GEEC- and IL-2R -mediated pathways or macropinocytosis

The BV entry route to mammalian cells has not been thoroughly studied. BV is suggested to use a clathrin-dependent entry pathway and macropinocytosis

(Matilainen *et al.* 2005, Long *et al.* 2006, Kataoka *et al.* 2012). Matilainen et al based their suggestion on the observation that BVs can be seen associated with clathrin-coated pits. However, the authors were not able to detect any viruses in clathrin-coated vesicles and they concluded that also the attachment of the virus to clathrin-coated pits was rare. BVs are, however, found frequently in macropinosome-resembling vesicles (Matilainen *et al.* 2005). Long et al. and Kataoka et al. base their conclusions on result showing inhibition of BV transduction by inhibitors of the clathrin-mediated pathway (chlorpromazine and Eps15) (Long *et al.* 2006, Kataoka *et al.* 2012). In both of these studies, BV transduction efficiency is measured instead of directly monitoring virus internalization. Hence, these inhibitors can have an affect also some later aspect of the virus life cycle, not the internalization step. As these previous studies concerning BV internalization are inconclusive, we wanted to assess the role of different entry pathways in more detail. In our studies with HepG2 and 293 cells, we were not able to see any indications of clathrin-dependent endocytosis (I). BV did not colocalize with any common markers or cargo from this route. We also studied the effect of chlorpromazine and an Eps15 mutant and, contrary to Long et al. (2006), we monitored the amount of internalized viruses. We were not able to detect any inhibition of BV internalization, suggesting that either there are cell type specific differences or chlorpromazine and the Eps15 mutant construct affected BV trafficking only in the later steps leading to a decrease in the transduction efficiency.

As Matilainen et al also suggested macropinocytosis (Matilainen *et al.* 2005), we next studied the involvement of macropinocytosis in BV entry. Similar to Matilainen et al. (2005), we also observed BV in large macropinosome-resembling vesicles (I). However, common macropinocytotic inhibitors and regulators (EIPA, Rab 34, Pak1 and CtBP/BARS) did not affect BV internalization. Furthermore, even though BV was shown to cointernalize with fluid-phase markers, we were not able to observe any effect of BV on the HRP uptake arguing against macropinocytic entry. Additionally, we were not able to detect any colocalization between BV and flotillin-1, GPI-EGFP, caveolin-1 (5, 15 and 30 min. p.t.) or the IL-2 receptor (5 to 60 min. p.t.), suggesting that BV did not use these pathways to gain entry into human cells.

Altogether, these studies indicated that BV internalized permissive human cells by a clathrin-, caveolin-, flotillin-, GEEC- and IL-2R–independent pathway. BV was seen in large vesicles resembling macropinocomes but entry was unaffected by the classical macropinocytosis regulators.

6.2.2 Efficient BV internalization is regulated by Arf6, RhoA and Dynamin

Arf6 is a well known regulator of membrane trafficking and the actin cytoskeleton. Syndecan recycling occurs by an Arf6 mediated route. Recycling is regulated by syntenin and PIP2 interaction. In our studies, both DN and CA mutants of Arf6 inhibited BV internalization (I). Since the function of Arf6 is GTP-GDP cycle -dependent, both mutants can stall the function of Arf6. CA inhibits the budding from the plasma membrane and DN inhibits the recycling

step back to the plasma membrane. Interestingly, the Arf6 DN mutant was shown to inhibit BV transduction to an even higher extent than the initial BV internalization (30 min p.t.) (I), suggesting that Arf6 has a role in the initial early entry of BV and in the subsequent trafficking. The later control point could be explained by the regulation of syndecan-1 recycling back to the plasma membrane. The Arf6 DN mutant has been shown to block the return of the cargo from the recycling endosomes back to the plasma membrane, and induce the accumulation of syntenin and retention of syndecan in the recycling endosomes (Zimmermann *et al.* 2005). Loss of syndecan on the plasma membrane could then affect the internalization of new virus particles. Expression of Arf6 CA results in formation of PIP2 positive actin-coated vesicles that are unable to recycle back to the plasma membrane (Brown *et al.* 2001). As RhoA also regulated efficient BV internalization (I), the inhibitory effect of the Arf6 CA mutant could be due to RhoA inactivation since the Arf6 CA mutant has been shown to inhibit RhoA activation (Boshans *et al.* 2000). Interestingly, in addition to the role of RhoA in actin reorganization, it also affects the vimentin network (Chang and Goldman 2004) which might also have play role in BV internalization (III).

In addition to Arf6 and RhoA, dynasore and filipin inhibited BV internalization suggesting that entry is dynamin-dependent and originates from the raft areas, or depends on cholesterol otherwise (I). Raft areas have been associated previously with the GEEC, flotillin and IL2R pathways but we were not able to see any association of BV with any of these aforementioned routes. Furthermore, syndecans 1 and 4 specifically associate with membrane rafts (Podyma-Inoue *et al.* 2012).

Our results showed that the BV internalization pathway resembled phagocytosis in several ways, including BV induced extensive ruffling on the cell surface and virus internalization in these ruffle-areas (I). Additionally, BV internalization occurred together with fluid-phase markers but BV itself was not able to induce the fluid-phase marker uptake. Furthermore, BV induced simultaneous uptake of *E. coli* particles into non-phagocytic cells. Herpes simplex virus -1, which is also shown to bind and internalize through HSPG (Shieh *et al.* 1992, Tiwari *et al.* 2006), is suggested to exploit phagocytosis-like entry pathway in both non-professional and professional phagocytes (Clement *et al.* 2006, Tiwari and Shukla 2012). Similar to our findings here, herpes simplex virus-1 internalization into non-phagocytic human cells is regulated by RhoA but independent of regulation by Rac1 or Cdc42 (Clement *et al.* 2006). Furthermire, it is suggested that non-phagocytic cells are capable of phagocytosis and this could be mediated by cell surface molecules expressing HS chains (Rabinovitch 1995).

In addition to the extensive membrane ruffles and RhoA-depedent regulation of entry, the cell membrane areas that BV used to internalize were shown to be high in F-actin content (I). Furthermore, when cell lines were compared for their F-actin contents in general, we noticed that the highly permissive HepG2 cells contained significantly more F-actin than the non-permissive cell lines (III), which supported the previously suggested role for F-actin in BV internalization. However, F-actin levels were not altered in response

to RPMI medium change, suggesting that the low F-actin levels seen in non-permissive cells were enough to support BV internalization.

6.2.3 Syndecan-1 mediated BV internalization

Results from BV entry studies are in line with our receptor studies further suggesting syndecan-1 mediated entry of BV. Similar to BV internalization, also syndecan-1 internalization is clathrin- and caveolin-independent, raft-associated, Arf6 regulated and actin-dependent (Fuki *et al.* 1997, Fuki *et al.* 2000a, Chakravarti *et al.* 2005, Wilsie *et al.* 2006). Both BV and syndecan internalization are also dependent on cholesterol. Interestingly, the disruption of cholesterol inhibits the internalization but not the overall binding of BV (Kataoka *et al.* 2012). Similarly, only the internalization of syndecan-1 is dependent on cholesterol-enriched raft areas where it translocates upon internalization (Fuki *et al.* 2000a). Furthermore, BV internalization is tubulin-independent since disruption of the tubulin network is not able to inhibit BV transduction. However, disruption of the tubulin network by nocodazole treatment leads to enhancement of BV transduction. This has been suggested to be due to tubulin acting as a barrier for BV trafficking (van Loo *et al.* 2001). In light of our studies concerning BV and syndecan-1 interaction, it is plausible that α-tubulin might be restricting syndecan-1 internalization on the cell surface. It was recently reported by Chen et al (2013) that, in order to efficiently internalize, syndecan-1 needs to be dissociated from α-tubulin. Dissociation was mediated by the ERK kinase.

Gp64 is essential for BV internalization into mammalian cells (Liang *et al.* 2005, Kataoka *et al.* 2012, Wu and Wang 2012). Internalization mediated by gp64 occurs in the cell surface raft areas and removal of cholesterol from the membrane inhibits virus internalization (Zhang *et al.* 2003, Kataoka *et al.* 2012). Recently, a heparan-binding sequence was identified in gp64 and this was found to be essential for BV internalization into mammalian cells (Wu and Wang 2012), suggesting that phospholipids and HSPGs might act in co-operation mediating BV internalization. Our hypothesis is therefore that phospholipids and HSPGs co-operatively mediate BV entry. First, virus attachment is mediated by 6-O- and N-sulfated heparans. Subsequent signaling and transport of the virus to specific membrane areas is dependent on syndecan-1. Finally, the membrane fusion is mediated by gp64 and phospholipids interactions. This is a highly simplified scenario and other receptors and cell surface molecules are likely to be involved.

6.3 Human cell lines in BV research

Since BV was studied here as a gene transfer vector for biomedical applications rather than an insect-infecting pathogen, the cell lines used in the studies are from human origin. The cell lines studied here (HepG2, 293, Ea.hy926 and MG-63) showed significant differences with regard to BV binding, internalization and efficiency of BV-mediated gene delivery. HepG2 cells showed high virus

binding, active internalization and efficient transduction (II and III). This cell line is thus model cell line that can be used in all BV studies. 293 cells showed a moderate transduction rate but the binding of the virus was not in line with the transduction efficiency. Additionally, 293 cells showed relatively higher binding of BV in relation to the transduction efficiency. However, the transduction levels coincided with the amount of syndecan-1 on the cell surface, suggesting that in 293 cells BV might bind to some additional receptors, in addition to syndecan-1, but this binding does not lead to efficient transduction (II). Thus, 293 cells are not ideal for the receptor binding studies but can be exploited for the entry and transduction studies. Ea.hy926 and MG-63 cells were deficient for BV transduction even though BV binds to syndecan-1, as it did in the permissive cells, and the binding of virus on the cell surface was efficient (III). These cell lines thus can be used to quantitatively study BV binding but not the subsequent internalization steps. Highest BV binding efficiency and syndecan-1 expression levels were in fact found in the non-permissive Ea.hy926 and the permissive HepG2 cells (II). Interestingly, also another HSPG exploiting virus, adeno-associated virus 2, is shown to accumulate into aggregates in Ea.hy926 cells leading to deficient adeno-associated virus-mediated gene delivery (Pajusola *et al.* 2002). This therefore suggests that in Ea.hy926 cells the internalization of other HSPG-exploiting viruses might be defective as well.

6.3.1 Cellular factors affecting the permissiveness of the cell to BV transduction

In order to determine which cellular factors affected the permissiveness of the cell to BV transduction, we studied the highly permissive HepG2 cells and the non-permissive Ea.hy926 and MG-63 cells (III). The non-permissive cell phenotype showed low F-actin content, high syntenin expression, cytoplasmic aggregates of phosphorylated PKCα, and highly expressed and tightly organized vimentin network in the cells. In addition to aggregated pPKCα in these cells, also the regulation of pPKCα in response to α2β1-integrin internalization (studied by EV1 internalization) appeared to differ between the permissive and non-permissive cells. Normally, α2β1-integrin and EV1 internalization leads to efficient PKCα activation which then leads to subsequent PKCα downregulation in the cells (Upla *et al.* 2004). However, we were not able to detect any changes in pPKCα in non-permissive Ea.hy926 cells after EV1 internalization whereas the permissive cells showed typical downregulation of pPKCα (III, Fig. 3B).

Furthermore, the non-permissive nature of the cells could be modulated by changing the culture medium (III). A change to RPMI medium significantly induced BV transduction in both permissive and non-permissive cells. The RPMI medium phenomenon is previously shown for BV, lentivirus, adeno-associated virus and adenovirus (Mahonen *et al.* 2010). We showed that EV1 infection could also be induced with a culture medium change from DMEM to RPMI (III). The diverse group of viruses affected by the culture medium phenomenon suggests that some highly common regulator(s) are affected by the change of culture medium.

A change to RPMI medium was shown to reduce syntenin levels, upregulate pPKCα, and downregulate pPKCε and modulate the vimentin network (III). F-actin levels were not affected, suggesting that even the fairly low level of F-actin in non-permissive cells was sufficient to promote efficient BV transduction. Additional to RPMI culture medium, the PKC activator PMA was able to modulate BV transduction efficiency in the cells (III). However, PMA's effect on BV transduction between the cell lines was not that straightforward as observed with different media. Our hypothesis is that PMA has a dual effect on BV transduction. First, PKCα activation induces BV transduction. Similarly this effect could also be seen when cells were cultured in RPMI medium. The overall amount of pPKCα was increased and relocation of pPKCα to the membranes could be observed (III, Fig. 4B and E). Second, pPKCε activation reduced BV transduction in cells where vimentin is in a filamentous form. Similarly, this effect on pPKCε (III, Fig. 5A) and the vimentin network (III, Fig. 7A and B) was also seen when cells are changed from RPMI to DMEM culture medium. Thus, in permissive HepG2 cells where vimentin was unaffected by PMA treatment, PMA induced BV transduction. In contrast, in non-permissive cells where PMA altered the organization of vimentin network, PMA either had no effect or was even able to prevent the effect of the RPMI medium on BV transduction (III). The highly permissive HepG2 cells showed no proper vimentin network but vimentin appeared to be in non-filamentous form. In addition to the non-filamentous appearance of vimentin, it seemed that the overall vimentin level was extremely low in permissive cells. However, vimentin targeted siRNA was not able to rescue the virus transduction suggesting that the overall vimentin amount might not be the problem. Therefore, it could be that successful BV transduction is dependent either on the availability of free vimentin subunits or alternatively, the highly expressed and compact vimentin network may work as a physical barrier to prevent virus trafficking.

6.4 Cellular factors affecting efficient EV1 infection

HepG2, Ea.hy926 and MG-63 cells were also studied in the context of EV1 infection efficiency. These cell lines showed similar EV1 infection rates to those observed with BV transduction. Like with BV, the differences between non-permissive and permissive cells were not due to EV1 virus binding or amount of receptor on the cell surface. Additionally, EV1 infection efficiency could also be induced by a change to RPMI culture medium. However, the nature of restriction in the non-permissive cells differed markedly between BV and EV1. Whereas BV was shown to aggregate on the cell membrane, EV1 was shown to efficiently internalize the non-permissive cells, thus suggesting that the block was in the intracellular trafficking (III).

PKCα activation is essential for EV1 internalization and efficient EV1 internalization leads to PKCα downregulation (Upla *et al.* 2004). In permissive HepG2 cells we observed this same phenomenon but in the non-permissive cells

pPKCα was unaffected (III), suggesting that the function of PKCα was different in the non-permissive cells. This hypothesis was further supported by the fact that when the cells became more permissive as they were cultured in the optimal RPMI medium, pPKCα was activated and relocated in the cell. Previously, PKCα is shown to have a role in the internalization step (Upla *et al.* 2004) and as non-permissive cells were able to internalize the EV1, these results suggest that PKCα might regulate the intracellular trafficking of the virus as well. How and where in the virus life cycle PKCα functions remains to be studied further.

In addition to PKCα, PKCε and vimentin were also associated with the cell permissiveness. Furthermore, pPKCε was downregulated in response to EV1 internalization in non-permissive cells (III) whereas permissive cells showed no changes in pPKCε levels (data not shown).Therefore, these result indicated that PKCε could also have an important role in cell permissiveness to EV1 infection. Alltogether, these results suggest that PKCα, PKCε and vimentin were associated with the internalization of EV1. Interestingly, some viruses use vimentin as their receptor on the cell surface. Additionally, other picornaviruses are able to modify vimentin in order to cause efficient infection (Koudelka *et al.* 2009, Plummer *et al.* 2012, Gladue *et al.* 2013). However, in our preliminary studies, we were not able to observe any visible changes in the vimentin network in response to EV1 internalization (15 min and 2h p.i., data not shown).

7 CONCLUSIONS

The main conclusions of this thesis are:

1. Baculovirus internalization into permissive human cells depends on cholesterol and is regulated by Arf6, RhoA and dynamin. Virus binding causes extensive ruffling of the cell surface and leads to subsequent cointernalization of *E. coli* particles into non-phagocytic cells. Of the common classified endocytic routes, baculovirus uptake resembles phagocytosis.
2. Baculovirus binds to cell surface HSPGs, preferring 6-O and N-sulfated heparans. Glypicans are not involved in efficient baculovirus binding or transduction. From the syndecan family, syndecan-1 specifically mediates the binding and internalization of baculovirus to permissive human cells.
3. In non-permissive cells baculovirus internalization is halted on the cell surface where baculovirus aggregates with syndecan-1 and pPKCε. Additionally, intracellular trafficking of echovirus-1 is defective in these BV non-permissive cells. Trafficking of both viruses can be improved by a change from DMEM to RPMI medium and by PMA treatment in permissive cells. The medium change and PMA affect vimentin network and PKC α and ε activation in the studied cell lines.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Bakuloviruksen sisäänmenoon ja geeninsiirtoon vaikuttavat solun ominaisuudet

Virukset ovat pieniä biologisia partikkeleita jotka tarvitsevat isäntäsolua lisääntyäkseen. Virukset koostuvat proteiineista sekä geneettisestä materiaalista joka voi olla joko RNA:ta tai DNA:ta. Joillain viruksilla proteiinikuorta voi myös peittää lipidivaippa. Viruksia on useita eri luokkia ja perheitä mutta yhteistä niille kaikille on että ne ovat erikoistuneet kuljettamaan oman geneettisen materiaalinsa isäntäsolun sisään ja valjastaa isäntäsolun molekyylikoneiston valmistamaan uusia viruspartikkeleita. Voisi siis sanoa viruksien olevan eksperttejä viemään geneettistä materiaalia soluihin. Tästä syystä virukset ovat lupaava vaihtoehto geeniterapiahoitoihin.

Bakulovirus on yksi potentiaalinen kandidaatti geeniterapiahoitoihin. Bakulovirus on luontaisesti hyönteisiä infektoiva virus joka ei ole infektiivinen ihmisille. Virus kykenee menemään ihmisen soluihin sisään ja tehokkaasti ilmentämän niissä haluttuja geenejä mutta ei kykene muodostamaan uusia viruspartikkeleita. Vaikka bakuloviruksella on paljon potentiaalia sekä kehitteillä on useita bakulovirusvälitteisiä biolääketieteen sovelluksia, sen vaikutuksia ja elinkiertoa ihmisen soluissa ei ole vielä tarkasti tutkittu. Tämä jarruttaa bakuloviruspohjaisten hoitojen kehitystä sekä käyttöönottoa.

Tämä väitöskirja koostuu kolmesta osatyöstä, joissa on tutkittu bakuloviruksen elinkiertoa ihmisen syöpäsoluissa. Ensimmäisessä osatyössä on määritetty bakuloviruksen käyttämää sisäänmenoreittiä sekä siihen vaikuttavia solun säätelijöitä. Toisessa osatyössä on identifioitu bakuloviruksen reseptori eli solun pinnan molekyyli jota virus käyttää sitoutuakseen ja internalisoituakseen ihmisen soluihin. Kolmannessa osatyössä on määritetty kohdesolujen ominaisuuksia jotka voivat heikentää bakulovirusvälitteisen geeninsiirron tehokkuutta. Kolmannessa osatyössä bakuloviruksen lisäksi on tutkittu myös ihmiselle patogeenista virusta, echovirus-1:stä. Echoviruksen osalta on määritetty tekijöitä jotka vaikuttavat sen infektion tehokkuuteen.

Bakuloviruksen sisäänmenoreitin määrittämiseksi tutkimme viruksen assosiaatiota solun yleisimpien kuljetusreittin kanssa. Useimmat virukset pääsevät soluun sisään käyttäen klatriini- tai caveoliinivälitteistä reittiä tai makropinosytoosia. Bakuloviruksen ei kuitenkaan havaittu käyttävän yhtäkään näistä tunnetuista reiteistä. Osa reittien säätelijöistä tai tunnusmerkeistä sopivat yhteen viruksen internalisaation kanssa mutta reittien inhibiittorit eivät kyenneet estämään virusta eivätkä reittien säätelijät vaikuttaneet viruksen internalisaatioon. Bakuloviruksen käyttämä reitti muistutti eniten fagosytoosia vaikka käytössä olevat syöpäsolulinjat olivat ei-fagosytoottisia. Viruksen sitoutumisen havaittiin aiheuttavan solukalvon poimuuntumista sekä internalisaaatio tapahtui kolesterolirikkailta solukalvon alueilta. Internalisaatiota säätelivät dynamiini, Arf6 sekä RhoA. Viruksen internalisaatio myös lisäsi E. Coli bakteerin yhtäaikasita sisäänmenoa.

Viruksen käyttämä solun pinnan reseptori on usein se tekijä mikä määrittää tietyn solutyypin olevan permissiivinen tietylle virukselle ja sen infektiolle tai virusvälitteiselle geeninsiirrolle. Useista tutkimuksista huolimatta bakuloviruksen käyttämää reseptoria ihmisen soluissa ei ole vielä identifioitu. Tässä väitöskirjatyössä osoitettiin että bakulovirus käyttää hepraanisulfaatti proteoglykaaneihin kuuluvia syndekaaneja sitoutuakseen ihmisen soluihin. Sitoutuminen on riippuvaista heparaanisulfaattien sulfaatioasteesta ja bakuloviruksen osoitettiin sitoutuvan spesifisesti N- ja 6-O-sulfaation omaaviin hepariineihin. Hepariinisulfaattien lisäksi myös hepariinisulfaatit omaavalla proteiinirungolla oli merkitystä viruksen sitoutumisessa. Syndekaaniperheeseen kuuluu neljä (syndekaanit 1-4) proteiinia joiden ilmentyminen soluissa vaihtelee solutyypin mukaan. Näistä neljästä proteiinista ainoastaan syndekaani-1:n osoitettiin lokalisoituvan viruksen kanssa samoille alueille soluissa sekä syndekaani-1:n internalisaation näytettiin johtavan lisääntyneeseen bakuloviruksen internalisaation. Nämä tulokset osoittivat syndekaani-1:n toimivan bakuloviruksen sitomis- ja internalisaatioreseptorina ihmisen syöpäsoluihin. Syndekaani-1 on usein yliekspressoitu syöpäsoluissa ja sen on useissa eri syöpätyypeissä todettu olevan markkerina pahalaatuiselle syöpätyypille. Tästä syystä juuri syndekaani-1:n toiminta bakuloviruksen reseptorina on hyvä uutinen bakulovirusperäisten syöpähoitojen kehitykselle. Nämä tulokset tukevat ja selittävät hyvin myös aiempia saamiamme tuloksia liittyen bakuloviruksen sisäänmenoreittiin. Aiemmat raportit syndekaani-1:n internalisaatioreitistä ovat hyvin samankaltaisia kuten ensimmäisessä osatyössä määrittelemämme reitti bakuloviruksen internalisaatiolle.

Kolmannessa osatyössä tarkastelimme solutyyppien ominaisuuksia jotka voivat johtaa heikentyneeseen bakuloviruksen geeninsiirtotehokkuuteen. Geeniterapiasovelluksissa tärkeää on riittävän tehokas geeninsiirto soluihin jotta vaste hoidolle olisi mahdollisimman hyvä sekä että käytettävä virusmäärä saataisiin pidettyä mahdollisimman pienenä. Yksi bakuloviruksen hyvistä ominaisuuksista geeninsiirtovektorina on sen kyky päästä sisään useisiin ihmisen kudosten soluihin ja useisiin eri solutyyppeihin. Kuitenkin on tiedossa että mm. ihmisen osteosarkooma soluissa sekä endoteeli-epiteeli fuusiosolulinjassa bakuloviruksen geeninsiirtotehokkuus on todella huono. Ihmisen maksasyöpäsolulinjoissa geeninsiirto on yleisesti hyvin tehokasta. Tutkimalla näitä solulinjoja sekä bakuloviruksen käyttäytymistä niissä, halusimme selvittää mitkä ovat ne solun ominaisuudet jotka vaikuttavat geeninsiirron tehokkuuteen. Jotta saisimme tietää oliko tämä defektiivisyys ainoastaan bakulovirusta koskeva, tutkimme lisäksi echovirus-1:n infektiivisyyttä näissä soluissa. Echovirus-1 ja bakulovirus ovat kaikinpuolin hyvin erilaisia viruksia mm. koon, isäntäeliön, geneettisen materiaalin, solun sisäänmenoreitin sekä solunpinnan reseptorin suhteen. Tästä huolimatta bakulovirusvälitteisen geeninsiirron lisäksi myös echovirus infektio oli heikko kyseisissä solulinjoissa. Viruksien defektiivisen liikennöinnin soluissa havaittiin korreloivan PKC-perheen kinaasien PKCα ja PKCε aktivaation sekä solun vimentiiniverkoston muutoksien kanssa. Vimentiiniverkoston sekä sen muutoksien on aiemmin todettu olevan tärkeä tekijä usean eri

viruksen infektioissa ja soluun sisäänpääsyssä. Nämä tulokset vihjaavat sen olevan tärkeä säätelytekijä myös baculoviruksen ja echoviruksen internalisaatiossa ihmisen soluihin.

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ORIGINAL ARTICLES

I

CLATHRIN-INDEPENDENT ENTRY OF BACULOVIRUS TRIGGERS UPTAKE OF E. COLI IN NON-PHAGOCYTIC HUMAN CELLS

by

Laakkonen J.P., Mäkelä A.R., Kakkonen E., Turkki P., Kukkonen S., Peränen J., Ylä-Herttuala S., Airenne K.J., Oker-Blom C., Vihinen-Ranta M. and Marjomäki V. 2009.

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Clathrin-Independent Entry of Baculovirus Triggers Uptake of *E. coli* in Non-Phagocytic Human Cells

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Abstract

The prototype baculovirus, *Autographa californica* multiple nucleopolyhedrovirus, an insect pathogen, holds great potential as a gene therapy vector. To develop transductional targeting and gene delivery by baculovirus, we focused on characterizing the nature and regulation of its uptake in human cancer cells. Baculovirus entered the cells along fluid-phase markers from the raft areas into smooth-surfaced vesicles devoid of clathrin. Notably, regulators associated with macropinocytosis, namely EIPA, Pak1, Rab34, and Rac1, had no significant effect on viral transduction, and the virus did not induce fluid-phase uptake. The internalization and nuclear uptake was, however, affected by mutants of RhoA, and of Arf6, a regulator of clathrin-independent entry. Furthermore, the entry of baculovirus induced ruffle formation and triggered the uptake of fluorescent *E. coli* bioparticles. To conclude, baculovirus enters human cells via a clathrin-independent pathway, which is able to trigger bacterial uptake. This study increases our understanding of virus entry strategies and gives new insight into baculovirus-mediated gene delivery in human cells.

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Introduction

The baculovirus under study, *Autographa californica* multiple nucleopolyhedrovirus, is a large, enveloped, dsDNA virus that belongs to the family of *Baculoviridae*. Baculoviruses are arthropod-specific viruses ubiquitously found in the environment, of which members have been isolated from more than 600 host insect species. They play an important ecological role in regulating the size of insect populations, and their complexity in form and function suggest a long evolutionary lineage. Most baculoviruses have been isolated from the order Lepidoptera, and Lepidopteran baculoviruses are also the best characterized [1]. Baculoviruses are unique compared to other virus families by having two distinct viral phenotypes, occlusion-derived virion and budded virion, but with a shared genotype [1,2,3]. The occlusion-derived virion is specialized and only infects the highly differentiated columnar epithelial cells within the alkaline conditions of the larval midgut. The budded virion, on the other hand, is generalized and highly infectious to the tissues of the host as well as cultured insect cells. Traditionally, baculoviruses have been applied as targeted biocontrol agents and for heterologous gene expression in insect cells and larvae [4]. Most of the established data on baculovirus and mammalian cell interactions and baculovirus display technology relates to the budded virion [5,6].

Apart from efficiently infecting arthropods and cultured insect cells, baculoviruses are also capable of successfully transducing various mammalian cell types [7,8]. The receptor(s) promoting cellular binding and subsequent uptake of the virus into insect or mammalian cells are currently unidentified. As the virus is able to enter a vast variety of cell types, abundant plasmalemmal molecules, such as heparan sulfate [9] and phospholipids [10] have been suggested to participate in the binding process. Low early endosomal pH was shown to be crucial for the release of the viral capsid, and for efficient transduction in both insect and mammalian cells [11,12,13,14]. Despite early endosomal targeting and occasional viral attachment to plasma membrane-bound coated pits, no baculoviruses have been observed within budded clathrin-coated vesicles [12]. Recently, Long *et al.* [15] demonstrated inhibition of baculovirus-mediated transduction in baby hamster kidney, BHK21 cells treated with chlorpromazine, suggestive for clathrin-mediated endocytosis (CME). In contrast, we detected enveloped baculovirus in numerous large plasma membrane invaginations and non-coated vesicles associated with plasma membrane ruffling, indicating that a more efficient endocytic pathway could be involved [12].

Since the discovery that baculoviruses are able to transduce cells of mammalian origin this viral vector system has been used in versatile applications in biomedicine, including vaccination as well as in cancer and immunotherapy [16,17,18]. So far, baculovirus vectors have been used for *in vivo* applications in animal models including rabbits, mice and rats [6]. In recent years, the transduction efficiency and the method of delivery have been optimized in serum-free environments, leading to successful transduction of neural cells [19], carotid arteries [20] and ocular

tissue [21], for example. In this study, we focused on elucidating the nature of baculovirus entry in human cells. We show that the functional entry of baculovirus occurs via clathrin-independent large smooth-surfaced vesicles and that it induces the uptake of *E. coli* in non-phagocytic human cells.

Results

Clathrin-mediated endocytosis is not required for baculovirus transduction

To obtain evidence as to whether or not CME is employed by the baculovirus in mammalian cells, we used various approaches; e.g. marker proteins, small interfering RNA (siRNA), chemical inhibitors, and transfection of plasmids encoding dominant negative (DN) and constitutively active (CA) protein factors. First, the baculovirus was cointernalized or consecutively fed with Alexa546-labeled transferrin (A546-TF), the endocytic marker for the clathrin-mediated pathway, in 293 and HepG2 cells (5–60 min) and observed by confocal microscopy. In both cell types, A546-TF was efficiently internalized to cells, whereas the viral uptake appeared to progress at a slower rate. No colocalization of A546-TF with baculovirus was detected, suggesting that the baculovirus did not enter along with transferrin and was not further directed to the recycling pathway (Figure S1E). Further, the clathrin light chain tomato fusion protein was expressed in 293 cells to examine if baculovirus entry affected the cellular distribution of clathrin. Live confocal microscopy revealed that the distribution of the expressed clathrin light chain was not altered in the presence of baculovirus (Figure S1A, S1B). After 1 h post transduction (p.t.), baculovirus was internalized into large, distinct vesicles in contrast to clathrin, which was localized in small vesicles present throughout the cytoplasm. Additionally, hardly any colocalization between the virus and clathrin heavy chain antibody was detected at 5–15 min p.t. in 293 cells by confocal microscopy (Figure 1A, 1B, and 1C) or in clathrin coated vesicles by EM (MOIs 500–1000; data not shown). We also tested the effect of chlorpromazine for baculovirus uptake during 30 min of internalization. The results showed no statistically significant effect on baculovirus entry in chlorpromazine treated cells (control uptake set to 100%±14% SE compared to 83%±9% SE; $p = 0.17$ by one-tail t-test).

To test the involvement of dynamin in baculovirus uptake, a small molecule called dynasore was used. Dynasore is an inhibitor of

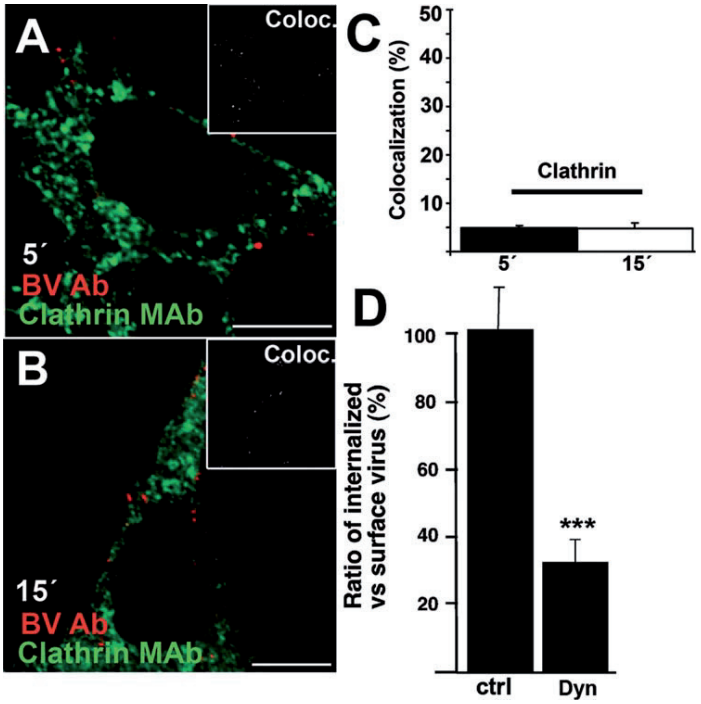

Figure 1. CME is not involved in baculovirus entry. 293 cells that were allowed to internalize baculovirus (wt) for 5 (A) and 15 min (B) were immunolabeled for baculovirus (red) and clathrin heavy chain (green). Colocalized voxels are shown in the inset. Scale bar, 10 μm. (C) Colocalization between baculovirus and clathrin heavy chain in 293 cells were calculated from confocal sections using the colocalization algorithm in the BioimageXD software (See Materials and Methods) from 3 separate samples, from 30 cells. Mean values and standard deviations are shown. (D) Differential labeling of intracellular (baculovirus Ab, Alexa488) and surface-bound baculovirus (baculovirus Ab, Alexa555) at 30 min was measured in dynasore-treated (Dyn, 80 μM) HepG2 cells from three separate samples (30 cells). Confocal sections were further analyzed by BioimageXD using the internalization algorithm. Mean values and standard deviations are shown.
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dynamins, which blocks the formation of clathrin coated vesicles [22]. Dynamin is also involved in various other entry routes (e.g. caveolae-pathway, clathrin-independent IL2-receptor pathway and phagocytosis [23]). In these experiments, internalization of A546-TF was used to control of the functionality of the drug. The entry of transferrin into live 293 cells treated with dynasore was efficiently inhibited (internalization level dropped to 4.3%±1.5% SD; Figure S1F) as compared to untreated control cells (100%). Immunolabeling of the baculoviruses before and after cellular permeabilization in the presence and absence of dynasore was performed to differentiate the virus outside and inside the cell (Figure 1D). The extent of viral internalization was quantified using the colocalization and internalization algorithms embedded in the BioimageXD software [24]. The results showed that dynasore caused a 67% ($P = 0.0005$) reduction in baculovirus internalization. In line with these results, only weak baculovirus-mediated expression of the EGFP reporter protein was observed in dynasore-treated 293 cells (data not shown). The use of DN dynamin (K44A) and siRNA against dynamin-2 in 293 cells did not result in sufficient inhibition of transferrin entry, which is used as a proof of their effect, and therefore reliable baculovirus uptake studies could not be performed using these approaches (data not shown). Overall, we can conlude that the results obtained with dynasore suggest that the virions enter human cells via dynamin-dependent means, however, the results are merely indicative and not conclusive.

Baculovirus internalizes along with fluid-phase markers

The binding and entry of the baculovirus (0–15 min p.t.) was next studied in live 293 and HepG2 cells by confocal microscopy. Interestingly, extensive ruffle formation on the cell surface of both cell types was detected early after administration of the virus (Figure 2A, Figure S2). The virions seemed to utilize the extended cellular protrusions for their attachment and further movement to the plasma membrane (Video S1). Additionally, engulfment of viruses from cellular ruffles was detected (Figure 2B). The cellular protrusions as well as cell surface areas, which were active in virus entry, were strongly positive for actin labeled with phalloidin rhodamine (Figure S2). Quantification of cells positive for ruffles showed that under control conditions 27% of the cells (±0.4% SD) showed ruffles, whereas at 30 min p.t. the number of cells showing ruffles was increased to 77% (±9% SD; n = 100).

Since many efficient uptake pathways have been connected recently with fluid-phase endocytosis, baculovirus was cointernalized with fluid-phase markers to human cells. Analyzed by EM, cellular ruffles and large endosomes were detected in baculovirus transduced 293 (Figure 2C) and HepG2 cells. The fluid-phase marker horseradish peroxidase (HRP) was detected in baculovirus-filled endosomes at 15 min p.t. in both cell types (Figure 2D) whereas at 5 min p.t., a majority of the endosomes were free of HRP and thus possibly connected with the plasma membrane. The ratios of HRP-positive vesicles containing baculovirus after 5, 15 and 30 min p.t. were 23.3%±11.7% (SD), 58.8%±9.1%, and 59.3%±10.1%, respectively. Measurement of the size of HRP- and baculovirus-filled endosomes by EM revealed that the structures were relatively large in diameter (603 nm±25 nm). Due to the high amount of ruffling and the large size of the endosomes, we next studied whether the fluid-phase uptake was inducible by baculovirus transduction, which is typical for macropinocytic uptake. HRP was allowed to internalize for 30 min alone or together with varied amounts of wild-type (wt) baculovirus (200, 500 and 1000 MOI; Figure 2E). After 30 min, cells were extensively washed with BSA containing buffer in order to remove the plasma membrane-bound HRP. The measurement of HRP activity after homogenization showed clearly that there was no difference in the uptake of HRP in cells with or without baculovirus transduction (Figure 2E). These results thus suggest that baculovirus induces ruffling and is internalized along with fluid-phase markers in large endosomes but does not itself induce fluid-phase uptake.

Plasma membrane rafts are involved in baculovirus uptake

Since several fluid-phase pathways are known to originate from the plasma membrane raft areas, we investigated whether internalization of the baculovirus is affected by drugs that interfere with the cholesterol content or affect its function on the plasma membrane. Since methyl-beta cyclodextrin has effects also on the clathrin pathway we used filipin, which has been shown to specifically affect raft and caveolae pathways [25]. The internalization assay showed that filipin inhibited the uptake of baculovirus by 82% (Figure 3A).

To elaborate on these results, we further tested the role of dynamin-independent, raft-derived GPI-anchored protein-enriched early endosomal compartment (GEEC; [26]) and flotillin pathways in baculovirus transduction. Flotillin-1 has been recently shown to define a dynamin and clathrin-independent entry pathway from the raft areas in mammalian cells [27]. In 293 or HepG2 cells, flotillin-1 and baculovirus showed no colocalization between 5 and 30 min p.t (Figure 3B, Figure S3A). Additionally, the colocalization of baculovirus with glycosyl-phosphatidylinositol (GPI)-anchored protein GPI-EGFP was investigated. GPI-EGFP was chased to the plasma membrane of 293 cells by a 4 h treatment with cycloheximide before viral administration. No apparent colocalization between baculovirus and GPI-EGFP was observed at 5–15 min p.t. (Figure S3B). Moreover, wt, CA or DN mutant forms of Cdc42, which is required for targeting GPI-anchored proteins further to early endosomes, showed no inhibiting effect on the cytoplasmic internalization of baculovirus in 293 cells (Figure S3C, S3D, S3E). Furthermore, immunolabeling of caveolin-1 and baculovirus in 293 cells after 5, 15 and 30 min internalization showed no detectable colocalization (Figure S1G). The results indicate that the baculovirus is internalized from the raft areas but does not use caveolae, GEEC or flotillin pathways for its uptake.

Macropinocytosis is not involved in functional baculovirus entry

Since the baculovirus was observed in cell surface ruffles and in large cytoplasmic endosomes, the involvement of alternative uptake mechanisms including macropinocytosis was studied [12]. Here, different inhibitors and proteins involved in macropinocytosis were tested (Figure 3C and 3D). First, the effect of EIPA, an inhibitor of the Na^+/H^+ exchanger, which is frequently associated with macropinocytic uptake, was analyzed. No decrease in baculovirus-mediated marker gene expression was observed even at rather high concentrations of EIPA (0.05–0.1 mM; Figure 3C). The functionality of EIPA in these concentrations was verified by its inhibitory effect on entry of the fluid-phase marker TRITC-De (Figure S4).

Next, more specific regulators of macropinocytosis, namely Rab34 [28] and the p21-regulated kinase-1 (Pak1) [29] were studied. The DN and CA forms of Rab34 did not affect viral transduction efficiency at 6 h p.t. in 293 cells (Figure 3D, Figure S3K, S3L, S3M, S3N, S3O). As a control, the internalization of TRITC-De into Rab34 DN mutant transfected 293 cells was partially inhibited, whereas wt and CA constructs allowed more efficient internalization of De (wt 64.3%±6.0% SD, CA

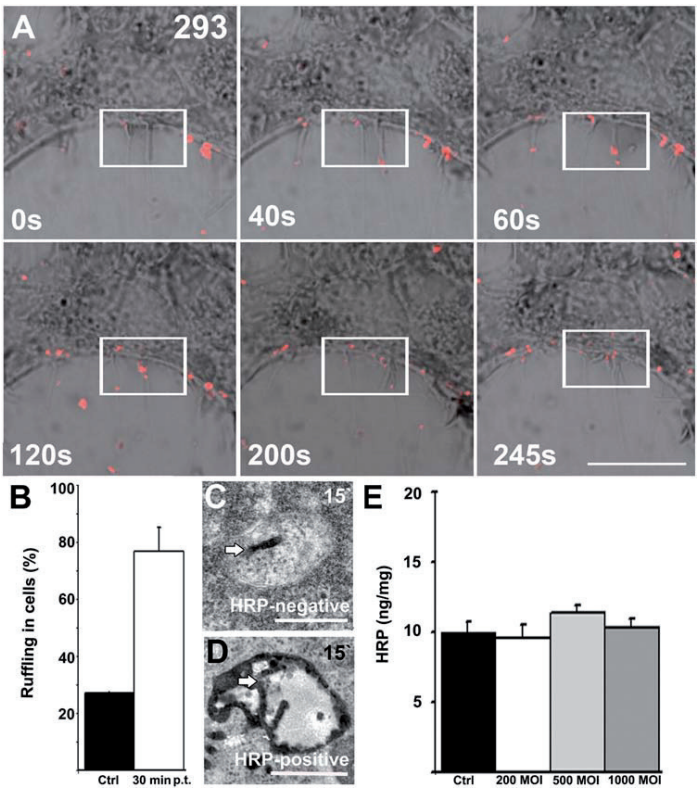

Figure 2. Baculovirus induces ruffling and internalizes along with fluid-phase markers. (A) Still images of baculovirus (p24mCherry, MOI 400) internalization in living 293 cells by confocal microscopy. Differential contrast image, baculovirus (red) and fixed time frames (0–245 s) are shown. Imaging was started at 5 min p.t. (0 s = 5 min). Some of the cellular protrusions guiding the baculovirus to the peripheral cytoplasm are within the rectangular box. Scale bar, 10 μm. (B) Cells positive for ruffles were calculated from control and baculovirus -treated cells 30 min p.t. (100 cells calculated). Mean values and standard deviations are shown. (C–D) Co-internalization of baculovirus (wt, MOI 500) with the fluid-phase marker HRP (10 mg/ml) was studied in 293 cells between 5 and 30 min p.t. HRP-negative (C) and positive (D) vesicles containing baculovirus (arrows) at 15 min p.t. are presented. Scale bar, 500 nm (C, D). (E) HRP (2 mg/ml) uptake after 30 min was measured with or without various amounts of baculovirus (200, 500 and 1000 MOI) co-internalized in cells. The amount of HRP is normalized to cellular protein content. Mean values and standard deviations are shown.
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60.2%±0.3% SD). Moreover, Pak1 had no effect on baculovirus transduction in 293 cells transfected with CA or DN mutants (Figure 3D). Control studies assured that the entry of TRITC-De into DN transfected 293 cells was partially inhibited (19.7%±0.20% SD), while CA transfected cells allowed more efficient internalization (39.8%±3.39% SD). In addition, we tested the effect of CtBP1/BARS (c-terminal binding protein 1/brefeldin A ADP-ribosylated substrate) for baculovirus entry. CtBP1/BARS was shown recently to regulate macropinocytosis donwstream of Pak1 kinase, which activates CtBP1/BARS by phosphorylation [30]. Thus we first verified that the DN CtBP1/BARS mutant construct was functional by preventing TRITC-De entry. The cells transfected with the WT CtBP1/BARS construct showed that 93% of the cells allowed dextran entry whereas the uptake was inhibited in 73% of the DN cells (inhibited cells showed less than 3 vesicles of dextran per cell). The results with baculovirus showed that the DN mutant construct (NBD-YFP) had no inhibitory effect on nuclear entry in 293 cells (Figure S3I, S3J). Similar results were gained in A431 cells (77.1% and 75.5% for NBD-YFP and BARS WT-YFP, respectively; over 100 cells calculated), which were used for CtBP1/BARS studies by Liberali et al (30). The data altogether suggest that the functional entry of baculovirus is not directly associated with macropinocytosis.

Arf6-GTPase regulates baculovirus uptake and transduction

The ADP-ribosylation factor 6 (Arf6) plays an important role in regulating clathrin-independent entry and recycling [31,32]. In

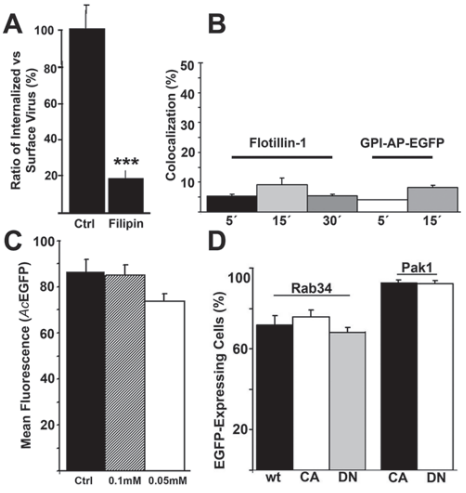

Figure 3. Plasma membrane rafts and macropinocytosis in baculovirus uptake. (A) The ratio of surface-bound (baculovirus Ab, A555) vs. intracellular baculovirus (baculovirus Ab, A488) was measured after 30 min p.t. during treatment with filipin in HepG2 cells from three separate samples (30 cells). Confocal sections were analyzed by BioimageXD. Mean values and standard deviations are shown. (B) Quantification of baculovirus colocalization with Flotillin-1 or GPI-AP-EGFP were analyzed by confocal microscopy in 293 cells after 5, 15 or 30 min p.t. from 30 cells from three separate experiments. Mean values and standard deviations are shown. (C) Macropinocytosis inhibitor EIPA (0.05 mM and 0.1 mM) was tested for its effects on baculovirus-mediated (*Ac*-EGFP, MOI 200) EGFP expression. Mean values of fluorescence intensity and standard deviations from FACS analysis are shown. (D) Baculovirus mediated EGFP expression was quantified at 6 h p.t. in the presence of transfected (48 h) macropinocytosis regulators Rab34 and Pak1 in 293 cells. The proportion of nuclei positive for EGFP expression of transfected cells was calculated from two separate experiments by confocal microscopy. In all studies, p-values were determined by unpaired Student's *t* test with a two-tailed *P* value. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.
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this study, 293 cells were transfected with the wt, CA or DN mutant forms of Arf6. In the presence of wt Arf6 protein, baculovirus (30 min p.t.) was efficiently internalized (Figure 4A). However, in cells transfected with the CA or DN forms of Arf6, the amount of internalized, cytoplasmic viruses was reduced. In order to reliably measure the internalization of baculovirus in the cells transfected with the DN construct, we performed an internalization assay in which the baculovirus was labeled with virus antibodies before and after permeabilization [33] (Figure 4A). This quantification showed that the internalization of baculovirus was reduced in DN transfected cells in comparison with wt Arf6 transfected cells (Figure 4B). Interestingly, in DN mutant cells, the percentage of baculovirus-mediated luciferase expression in the nucleus was decreased by 5-fold (Figure 4C) in comparison to cells transfected with the wt form of Arf6. Therefore, the results suggest that the DN Arf6 may have some additional inhibitory effect after internalization, on the translocation step to the nucleus. Furthermore, a 2-fold decrease was detected in nuclear entry in 293 cells transfected with the CA mutant (Figure 4C). In control studies, internalization of TRITC-De into DN and CA transfected cells was inhibited, while wt Arf6 allowed normal internalization verifying the importance of Arf6 regulating the fluid-phase entry (Figure 4C). We also used a siRNA approach to lower the expression of endogenous Arf6. Simultaneous transfection with a SiGlo transfection marker was used to pinpoint cells successfully transfected with siRNAs. In SiGlo positive cells, baculovirus internalization into 293 cell nuclei revealed a subtle decrease (Figure 4D) compared to the entry in scramble siRNA transfected and transduced cells at 6 h. Western blotting confirmed a relatively efficient knock-down (89%) of Arf6 expression in siRNA transfected cells. These results suggest that baculovirus uptake is regulated by Arf6, a regulator of clathrin-independent entry.

RhoA regulates baculovirus uptake and transduction

RhoGTPases modify actin filaments and are key regulators of cell growth, cell cycle progression and cell survival. Since actin is supposedly involved in the early events of baculovirus uptake causing e.g. ruffling, we tested the involvement of RhoGTPases in baculovirus entry. A preliminary inspection of 293 cells transfected with RhoA EGFP mutants showed that the cells transfected with the CA and DN mutants contained lower cytoplasmic amounts of baculovirus at 2 h p.t. (Figure 5A). Additional live imaging of RhoA CA mutant and fluorescent virus verified that the virus entry was decreased (1 h p.t., 2.5-fold reduction). The internalization of A546-TF and TRITC-De into cells transfected with the CA mutant was clearly inhibited (50.4%±12.6% SD and 79.8%±8.00% SD, respectively) in contrast to wt transfected cells, in which A546-TF and TRITC-De were efficiently internalized (91.8%±6.3% SD and 93.7%±2.8% SD, respectively). In order to verify further that the internalization of baculovirus was truly reduced in RhoA CA mutant cells, an internalization assay using differentially labeled baculoviruses before and after cell permeabilization was performed (Figure 5B). Quantification of viral internalization showed that in RhoA CA transfected cells the virus uptake was clearly decreased (ratio of internalized vs. surface baculovirus; Figure 5B).

To study further the role of RhoA in baculovirus entry, the endogenous expression of RhoA was reduced by siRNA (Figure 5C). In SiGlo positive cells, where the siRNA transfection was effective, viral internalization into 293 cell nuclei was clearly decreased at 6 h p.t. compared to the entry into cells treated with the scramble siRNA (Figure 5C). Western blotting confirmed a 70% knock-down of RhoA expression in siRNA transfected cells.

Since RhoA seemed to have an effect on baculovirus entry, we also tested the role of dynamin- and RhoA-dependent interleukin-2 (IL2)-receptor pathways [34]. After transfection with the Ntb-domain of IL2-receptor, the localization of internalized receptor was studied with respect to that of baculovirus. The confocal microscopy results showed that the surface-bound Ntb-detecting antibody did not colocalize with baculovirus in 293 cells between 5 and 60 min p.t. (Figure S3H), allowing us to conclude that baculovirus does not use the IL2 receptor pathway.

Next, we evaluated the influence of Rac1, another RhoGTPase family protein, on baculovirus transduction. In siRNA experiments against Rac1, nuclear entry of baculovirus at 6 h p.t. showed no statistically significant difference between Rac1 or scramble siRNA transfected cells (Figure 5C). Western blotting confirmed a 65% knock-down of Rac1 expression in siRNA transfected 293 cells. Due to the incomplete knock down, the negative results are merely suggestive, not conclusive. Further, at 2 h p.t. baculovirus showed normal internalization to both CA and DN Rac1-EGFP transfected 293 cells as detected by confocal microscopy (Figure S3F, S3G). In control studies, internalization

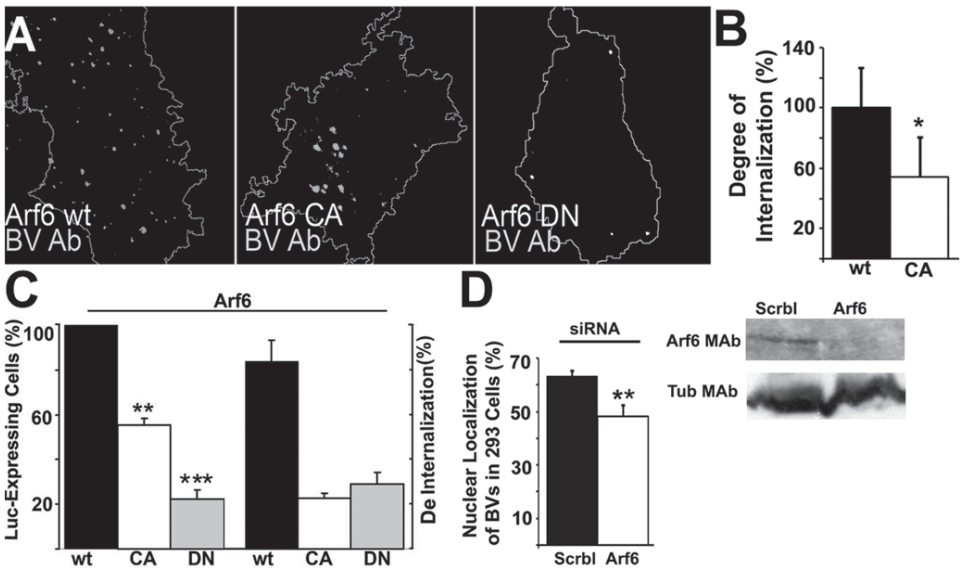

Figure 4. Arf6 is involved in baculovirus internalization and transgene expression. (A) 293 cells were transfected with Arf6 constructs (wt, CA, DN) for 48 h and then transduced with baculovirus (*Ac*VP39 MOI 200). The virus uptake after 30 min was detected by baculovirus Ab and Alexa-555-conjugated secondary antibody. The cell boundaries were determined from DIC image by ImageJ. The ratio of internalized vs. surface baculovirus in WT or Arf6DN transfected cells (30 cells from three separate experiments) was calculated from confocal sections using a differential labeling of baculovirus before and after permeabilization and using an internalization algorithm in BioimageXD software (see Materials and Methods section). Mean values and standard errors are shown. (B) Baculovirus-mediated luciferase (Luc; *Ac*-luc, MOI 200) expression in 293 cells was measured after 24 h in the presence Arf6 plasmids (left columns). Dextran internalization (2 h p.t.) in Arf6 expressing cells was measured to verify the efficacy of the mutant plasmids (right columns). The results show the proportional amount of cells which showed significant amount of dextran entry (cells with at least 10 dextran-positive vesicles in the cytoplasm). Mean values and standard deviations are calculated from three separate experiments. (C) Arf6 was knocked down in 293 cells using siRNAs. After siRNA treatments nuclear localization of baculovirus was calculated after 6 h baculovirus transduction. Western blotting was used to monitor the knock down effect with Arf6 antibodies. Statistical significance was determined by using the unpaired Student *t* test with a two-tailed *P* value. *$P<0.05$, **$P<0.01$, ***$P<0.001$.
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of TRITC-De into transfected DN cells was inhibited, in contrast to CA transfected cells, where TRITC-De was efficiently internalized. The internalization of TF was efficient with both constructs (data not shown). Altogether, the results suggest that RhoA regulates baculovirus entry in contrast to Rac1.

Phagocytosis-like uptake of baculovirus

Due to the large size of baculovirus, the induced ruffle formation, and the involvement of actin [34], Arf6, RhoA, as well as rafts in baculovirus transduction, the possible involvement of phagocytosis-like mechanisms in baculovirus entry was studied. For these experiments, we used heat-inactivated, Alexa-488-labelled *E. coli* (K12 strain, >1 μm) bioparticles, widely used as a marker of phagocytosis. First, we internalized *E. coli* particles and fluorescent baculovirus together for brief periods of time. The confocal results showed clear colocalization at 5 and 10 min p.t. in HepG2 cells (Figure 6A and 6B). Only a few *E. coli* particles were observed outside baculovirus-filled endosomes. We then monitored the intensity of fluorescent intracellular *E. coli* particles in baculovirus transduced cells by confocal microscopy. As a control, *E. coli* alone was fed to HepG2 cells for 1 h (Figure 6C). To separate the fluorescence of internalized and non-internalized particles, the cells were treated with trypan blue in order to quench the extracellular fluorescence. Untransduced control cells contained no apparent fluorescence after 1 h treatment with *E. coli* particles suggesting that bacteria did not enter the cells without baculovirus. Similarly, in 293 cells *E.coli* alone did not enter the cells and gave only low background fluorescence (data not shown). In contrast, virus-transduced HepG2 and 293 cells contained high amounts (49-fold and 10-fold more, respectively) of internalized *E. coli* particles ($P<0.001$), indicating that virus could induce entry of bacteria in non-phagocytic human cells. Interestingly, when baculovirus was first fed to HepG2 cells for 15 min and then *E. coli* for the next 60 min, bacteria could no longer enter cells efficiently. These results suggest that baculovirus is able to induce transient bacterial entry when administered simultaneously.

We also performed an *E. coli* internalization assay with dynasore and filipin in order to test whether the baculovirus stimulated uptake of *E. coli* occurs in a similar manner in drug-treated cells (Figure 6D). The assay showed that both filipin and dynasore caused a significant decrease of the stimulated uptake, suggesting that the entry of *E. coli* is also dynamin-dependent and originates from the raft membranes.

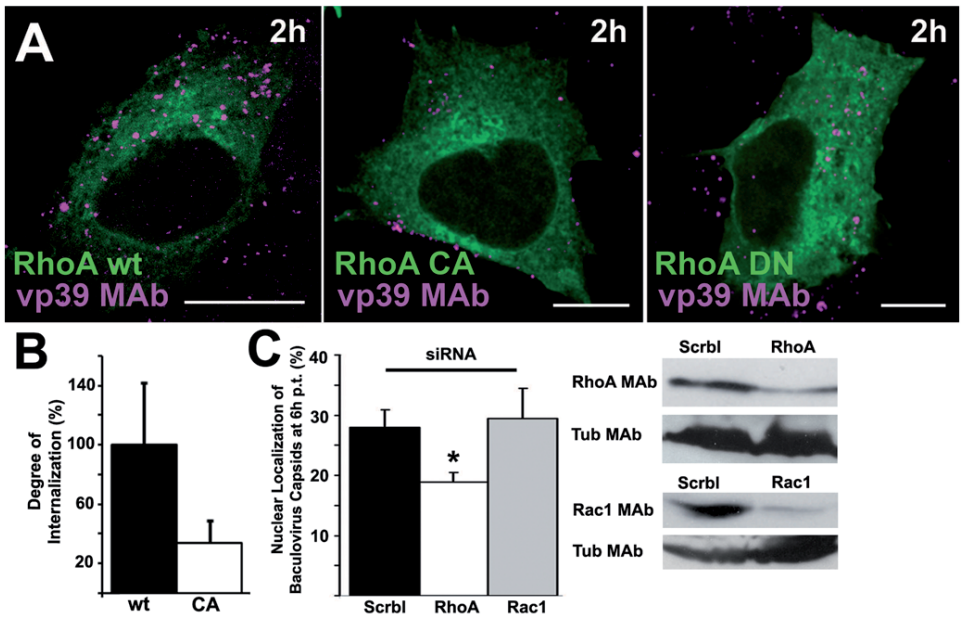

Figure 5. RhoA is involved in BV internalization and transgene expression. (A) BV internalization (wt, MOI 200) in 293 cells transfected for 24 h with wt, CA or DN RhoA-EGFP. The virus uptake (2 h) was detected by BV capsid antibody vp39 MAb (red) and A555-conjugated secondary antibody. Scale bars 10 μm. (B) Ratio of internalized vs. surface BV after RhoA wt and CA construct transfections was calculated from confocal sections after 30 min p.t. using a differential labeling of BV before and after cellular permeabilization (see Materials and Methods sections). (C) The effect of control (scrbl), RhoA or Rac1 siRNA treatments on BV nuclear entry was monitored in 293 cells (6 h p.t., wt, MOI 200). After immunolabeling, the proportion of SiGlo-positive nuclei positive for BV was measured from three separate samples (50–100 cells/each) by confocal microscopy. SiRNA knock down effects were monitored by western blotting with specific antibodies against RhoA (24 kDa) and Rac1 (26 kDa). In all quantitative images, mean values and standard errors are shown. Statistical significance was determined by using the unpaired Student's *t* test with a two-tailed *P* value, $^{*}P<0.05$.

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Discussion

We demonstrated previously that baculovirus accumulates into EEA1-positive endosomes in HepG2 cells starting at 30 min p.t. [12,13]. High frequency of ruffles and the presence of large smooth-surfaced endosomes full of baculovirus indicated the involvement of a more efficient entry pathway than the previously suggested clathrin-dependent uptake [12,15]. Since clathrin-coated vesicles (100–150 nm) and caveolae (50-80 nm) are thought to have a rather rigid coat structure and invariable, uniform size in different cell lines [35], the size of the virus itself (approx. 30-60 nm diameter×250–300 nm length [36]), may limit its internalization into human cells. However, recent results revealed, strikingly, that even large bacteria are able to recruit clathrin and utilize the pathway for their entry [37,38]. Therefore, clathrin structures may serve as one real alternative route for large virus particles. In this study, careful colocalization measurement by confocal microscopy showed no association of baculovirus with the clathrin heavy chain during viral transduction. Moreover, baculovirus does not associate with transferrin or recycling endosomal marker Rab11 [12]. Additionally, baculovirus containing clathrin-coated vesicles were not observed in our EM studies. Our results also showed that the early entry is sensitive to the cholesterol aggregating drug filipin suggesting that the raft domains are important for the first uptake step. Several viruses using the CME have been shown to be unaffected by the filipin-treatment, including Sindbis virus, West Nile virus and papillomavirus [39,40,41].

Recently, Long et al. [15] suggested a role for CME in the baculovirus transgene expression in BHK21 cells based on the effects of chlorpromazine and on the DN mutant form of Eps15. However, the initial entry step was not studied in detail [15]. In this study, we observed that the expression of DN Eps15 did not inhibit the actual viral entry step in 293 cells, indicating an inhibiting step later in the pathway leading to decreased transgene expression (Figure S1C, S1D). In addition, chlorpromazine did not have a significant effect on baculovirus entry in our assays. If the formation of clathrin coated pit (that is inhibited by chlorpromazine) would be crucial for baculovirus entry, it should have been also obvious in our clathrin colocalization studies as well as in our EM observations. Our results altogether suggest that baculovirus does not use the clathrin-mediated entry to efficiently express its transgenes in the human cells used in this study, but rather a clathrin-independent entry mechanism. We cannot, however, exclude the possible differences between the cell types.

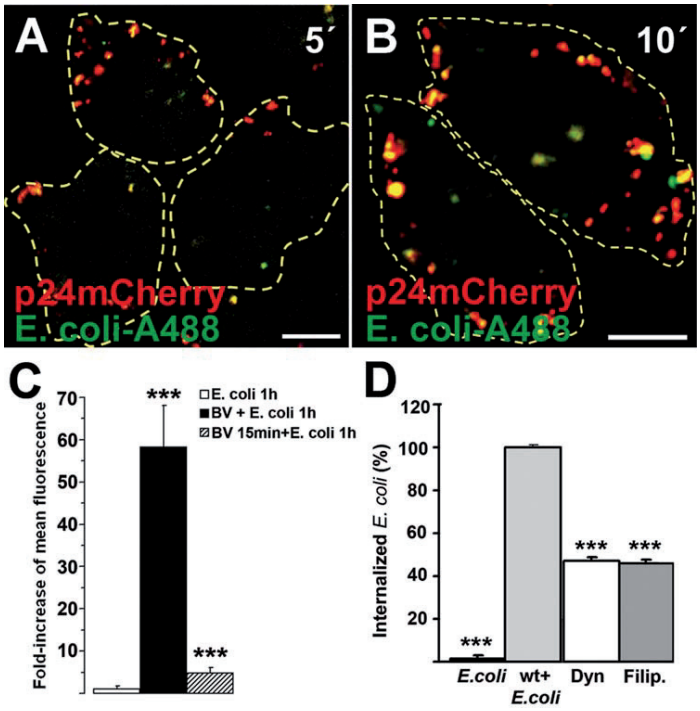

Figure 6. Phagocytosis-like uptake of baculovirus. (A,B) Baculovirus (p24mCherry, MOI 200; red) internalized together with A488-labeled *E. coli* bioparticles (green) in HepG2 cells at 5 (A) and 10 min (B) p.t. Scale bars, 10 µm. (C) Induction of phagocytic uptake of *E. coli* during baculovirus transduction. Fluorescent *E. coli* were fed simultaneously with baculovirus (MOI 200; baculovirus *+E. coli*) into cells and fixed at 60 min p.t. As a control, *E. coli* particles were fed into cells without baculovirus (*E. coli*) or after virus transduction for 15 min (baculovirus 15′*+E. coli*). To separate the fluorescence of internalized and non-internalized particles, the cells were treated with trypan blue. Normalized mean fluorescence values (Ctrl = 1) and standard deviation from 250–300 cells are shown. Fluorescence intensity was measured from confocal microscopy images (see Materials and Methods). (D) Dynasore and filipin were tested for their effects on stimulated *E. coli* uptake during baculovirus transduction. Co-internalization of baculovirus and E. Coli for 1 h without drugs was set to 100%. Fluorescence intensitites were calculated from three separate experiments (30–40 cells) using segmentation tools embedded in the BioimageXD software. Dyn (dynasore, 80 µM) and Filip. (filipin, 1 µg/ml) were added 30 min before the experiment and they were present during the whole internalization assay. Statistical significance was determined by using the unpaired Student's *t* test with a two-tailed *P* value, ***$P<0.001$.
doi:10.1371/journal.pone.0005093.g006

As more data accumulates on ligands using clathrin- and caveolin independent carriers [23,42] there is increasing evidence that these pathways show differential usage of cellular regulators such as dynamin and RhoGTPases. Clathrin- and caveolin independent pathways include e.g. the dynamin-dependent IL-2 receptor pathway and the dynamin-independent GEEC and flotillin pathways. The GEEC pathway carrying GPI-anchored proteins is dependent on Cdc42 for early endosomal targeting [26]. Dynamin-independent flotillin, on the other hand, defines its own pathway and does not seem to share any similarities with the GEEC pathway [27]. Our data on RhoGTPases in 293 cells suggest that baculovirus entry is independent of Rac1 and Cdc42. Moreover, only RhoA had an effect on baculovirus uptake. RhoA has been linked with clathrin- and caveolin-independent but dynamin-dependent entry, such as is the IL2-receptor pathway [34]. However, the observed lack of colocalization with the IL2-receptor implies that the entry of baculovirus does not involve the IL2-receptor pathway. Moreover, baculovirus did not colocalize with internalized flotillin or with GPI-EGFP, suggesting that functional baculovirus entry is independent of GEEC and flotillin pathways as well. Interestingly though, overexpression of GPI-EGFP, Rac1 and Cdc42 did inhibit uptake of baculovirus to some extent (Figure S5). It may be possible that their expression boosts "competing entry pathways" e.g. the GEEC pathway, which may down-regulate other pathways, such as that used by baculovirus. This effect has previously been observed between raft-derived and clathrin-dependent pathways when siRNAs against effectors of clathrin route boosted raft-derived pathway and vice versa [43].

Macropinocytosis is a form of clathrin- and caveolin-independent uptake, which has very little in common with other caveolin- and clathrin-independent pathways but shows many similarities with phagocytosis [44]. Macropinosomes may form spontaneously or they may be elicited by growth factors and phorbol esters. Macropinocytosis relies on molecules such as RhoGTPase Rac1,

p21-kinase Pak1, CtBP1/BARS, PI3K, PLC, GTPase Rab34 and actin for entry [30,45]. Macropinocytosis has also been associated with frequent actin-driven ruffles on the plasma membrane. A few bacteria and viruses, such as *Shigella flexneri*, *Salmonella typhimurium*, *Haemophilus influenzae*, HIV-1 and adenovirus 3 have been shown to rely on macropinocytosis for their entry [46,47,48,49]. In contrast to our expectations, the transduction of baculovirus was not regulated by Rac1, Pak1, CtBP1/BARS, Rab34 proteins or EIPA. Furthermore, baculovirus treatment did not induce fluid-phase uptake which, altogether, suggest that baculovirus is not internalized by a macropinocytic process.

Recent data from bacterial pathogens suggests that various particles may be ingested together with fluid by a macropinosome-like phagocytic process [44]. Ruffles form a loose fitting phagosome, which close up to form a phagocytic cup. Regulators of movement and actin polymerization show several shared features among macropinocytosis and phagocytosis and they include members of RhoGTPases, such as Rac1, Cdc42, Arf6 and RhoA. It was recently shown that CtBP1/BARS phosphorylated by Pak1 is used for the closure of the macropinocytic cup [30]. In the case of baculovirus, we found no connection to CtBP1/BARS or Pak1, which further supported our conclusion that baculovirus entry does not follow macropinocytosis. In contrast, for functional baculovirus transduction, two regulators were identified, Arf6 and RhoA. Previously, Arf6 GTPase was shown to facilitate the phagocytic uptake of red blood cells in macrophages and entry of a small bacterium Chlamydia by reorganizing actin [50]. In our study, baculovirus uptake and transgene expression were affected by DN and CA mutants or specific siRNA of Arf6. Inhibition of entry by the CA mutant may have, however, occurred through inactivation of RhoA. This effect has previously been shown by Boshans et al. [51], who demonstrated that activation of Arf6 downregulates RhoA signaling and depletes stress fibers in CHO cells.

A recent study on mimivirus showed that professional phagocytes may engulf large viruses using a phagocytic mechanism [52]. In addition to professional phagocytes, such as macrophages, dendritic cells and polymorphonuclear leucocytes, many other cell types are able to engulf material by a phagocytic mechanism [53]. In a recent interesting study, Herpes Simplex virus 1 was demonstrated to use phagocytosis-like uptake regulated by RhoA but not Cdc42 or Rac1 in professional and non-professional phagocytes [54]. Although phagocytosis-mediated engulfment is expected to be induced only by particles larger than 0.5 μm [55,56], the study showed that Herpes Simplex virus (0.17–0.2 μm), close to the diameter of baculovirus, activated the entry of phagocytic tracer *E. coli* bioparticles [54]. Previous work has linked RhoA, but not Cdc42 or Rac1, with complement-activated phagocytosis [57]. Here, we show that baculovirus entry enhances ruffle formation on the cell surface and the uptake of *E. coli* bioparticles in non-phagocytotic epithelial mammalian cells. Phagocytosis is suggested to be a dynamin-dependent and raft-derived process on the plasma membrane [58]. Similarly, baculovirus early uptake was sensitive to dynasore and to drugs that affected raft domains. Filipin also showed similar inhibition in stimulated *E. coli* uptake. The stimulated *E. coli* uptake followed similar regulation as baculovirus uptake.

To conclude, in human cells, the cellular binding of baculovirus induces ruffle formation and engulfment of several baculovirus in large cellular invaginations mainly in the raft areas. The functional entry of baculovirus occurs via clathrin-independent smooth-surfaced vesicles and does not involve raft-derived IL2-receptor, flotillin or GEEC pathways. The entry mechanism is reminiscent of phagocytosis, as it is regulated by dynamin, RhoA and Arf6 and as it induces the uptake of *E. coli* in non-phagocytic human cells.

Materials and Methods

Cells

Human hepatocarcinoma (HepG2) and human embryonic kidney (293) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in monolayer in Minimum Essential Medium (MEM) supplemented with 10% inactivated fetal calf serum (FCS), L-glutamine and penicillin-streptomycin (Gibco BRL, Paisley, UK) at 37°C, in 5% CO_2. For HepG2 cells non-essential amino acids (Gibco BRL) and Na-pyruvate (Merck & Co. Inc., Whitehouse Station, NJ) were also used. *Spodoptera frugiperda* (*Sf*9; GibcoBRL, Grand Island, NY/CRL 1711, ATCC) insect cells were maintained in monolayer and suspension cultures at 28°C using serum-free Insect-XPRESS culture medium (Cambrex, Walkersville, MD) or HyQ®SFX-Insect medium (HyClone Inc, Logan, UT) without antibiotics.

Viruses

Wild type (wt), *Autographa californica* nucleopolyhedrovirus (*Ac*MNPV; E2 strain), recombinant baculovirus vp39EGFP (Kukkonen et al. 2003), *Ac*-luc [59], CAG-BV-EGFP [60], *Ac*-EGFP and *Ac*VP39 [61] and p24mCherry were used. Shortly, p24mCherry virus was prepared by cloning baculovirus capsid protein p24-RFP (mCherry; [62] into pBACcap-1 vector [13]. The concentrated batches of viruses were prepared and the virus titers were gained as described previously [63]. As the virus dose, MOI 200 was used in all mammalian cell transductions unless otherwise stated.

Antibodies and chemicals

Monoclonal antibodies (MAb) against the following proteins were used: Photinus pyralis luciferase MAb (Serotec, Oxford, UK), early endosome antigen 1 MAb (eea-1; Transduction Laboratories, Lexington, KY, UK), myc MAb (9E10, ATCC), flotillin-1 MAb (BD Biosciences, San Jose, CA), nuclear lamin A/C (Novocastra laboratories, Newcastle upon Tyne, UK), Arf6 MAb (Thermo Fisher Scientific, Fremont, CA), RhoA MAb (SantaCruz Biotechnology Inc., Santa Cruz, CA), Rac-1 MAb (Millipore, Billerica, MA), FLAG M2 MAb, tubulin MA (Sigma Aldrich, St Louis, MO) and *Ac*MNPV vp39 capsid protein MAb (Dr. L. Volkman, University of California, Berkeley, CA). Polyclonal antibodies (Ab) against the following proteins were used: Dynamin-2 Ab (Dr. M. McNiven, Mayo Clinic College of Medicine, Rochester, MN), NTb (IL-2):Cy3-561 (Dr. A. Dautry-Varsat, Pasteur Institut, Paris, France), actin Ab (Sigma Aldrich), clathrin heavy chain (Abcam) and *Ac*MNPV Ab (baculovirus Ab; Drs. S. Braunagel and M. Summers, Texas A&M University, TX). HRP (type II), filipin, amiloride (EIPA), chlorpromazine, TRITC-phalloidin and cycloheximide were from Sigma. TRITC-labeled dextran (TRITC-De, 10 kDa), Alexa-546- and Alexa-488-labeled transferrin (A546-TF, A488-TF) and A488-labeled *E. coli* (K-12 strain) bioparticles were from Molecular Probes (Eugene, OR). In the double-labeling studies, A488, A555 and A633-conjugated anti-mouse and anti-rabbit antibodies (Molecular Probes) were used. Dynasore ($C_{18}H_{14}N_2O_4$); synthesized by Dr. Henry E. Pelish, (Kirchhausen Lab, Immune Disease Institute, Boston, MA), was used at final concentration of 80 μM in serum free medium.

Virus transduction

The cells were grown to subconfluency and transduced in MEM containing 1% FCS for 1 h at 4°C or 37°C followed by incubation in MEM containing 10% FCS at 37°C. For co-internalization studies, the cells were first transduced with virus (wt/vp39EGFP MOI 200–1000) for 15 min, washed, and then fed with TRITC-

De (250 μg/ml, in 1% culture medium) for 5–45 min. A546-TF (200–250 μg/ml), FITC-De (1000 μg/ml) or A488-labeled *E. coli* bioparticles (60 particles/cell) were fed simultaneously with virus. In experiments with *E. coli*, virus (wt MOI 200) was also fed to cells for 15 min, followed by Alexa-488-labeled *E. coli* particles for 60 min. All samples were treated with trypan blue in order to separate the fluorescence of internalized and non-internalized *E. coli* particles. Additionally, in experiments with the GPI-EGFP construct, the cells were treated continuously with cycloheximide (100 μg/ml, diluted in 1% medium) for 4 h prior to virus transduction, in order to chase the expressed GPI-EGFP to the plasma membrane. To get rid of excess plasma membrane stain, the cells were further washed with 0.5 M NaCl, 0.2 M sodium acetate buffer (pH 4.5) and finally immunolabeled and detected with confocal microscopy. In inhibition experiments detected by confocal microscopy, the cells were preincubated with filipin (1 μg/ml) for 30–60 min, followed by baculovirus binding on ice for 30–60 min and transduction with or without drugs for 6–24 h at 37°C. The drug treatments did not affect cell viability as determined by CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTT assay; Promega) according to the manufacturer's protocol.

Electron microscopy

For HRP labeling experiments, the cells were first incubated for 1 h on ice and/or 5–30 min at 37°C in complete culture medium containing 10 mg/ml HRP (Sigma, type II). After viral transduction (wt, MOI 500), the cells were fixed in 4% paraformaldehyde (PFA) containing 0.1% glutaraldehyde (in 50 mM Tris buffer, pH 7.6) for 1 h at RT. HRP was detected with 0.1% diaminobenzidine (Sigma) for 30 min, followed by another 30 min in 0.1% diaminobenzidine supplemented with 0.1% hydrogen peroxide. The cells were then washed, post-fixed and processed for EM.

HRP uptake assay

HRP (2 mg/ml in DMEM containing 1% serum) was administered with or without various amounts (200, 500 and 1000 MOI) of wild type baculovirus on HepG2 cells. After a 30 min-internalization period cells were put on ice and extensively washed with PBS supplemented with BSA (0.5%) to remove of plasma membrane-bound HRP. EM samples with HRP labeling showed that such washes were sufficient. Cells were scraped from the dishes, centrifuged to give a cell pellet and treated with 1% Triton X-100 in PBS for 30 min on ice. Cell debris were centrifuged (5 min at 15 000 g) and discarded, whereas the supernatant was evaluated for its content of HRP activity [64] and protein (BIO-RAD Protein assay).

Transfection experiments

293 cells were transfected for one to two days according to manufacturer's protocol (Fugene, Roche, Basel, Switzerland). In the experiments, the following constructs were used: myc-tagged Pak1T423E (Dr. J. Chernoff, Fox Chase Cancer Center, Philadelphia, PA), flag-tagged Pak1AID (Dr. E. Manser, GSK-IMCB Laboratory, Singapore), Cdc42 WT and GPI-EGFP (Dr. L. Pelkmans (ETH Zürich, Switzerland), Cdc42 and Rac1 (17N, 12V), Arf6-EGFP WT, Arf6-T27N, Arf6-Q67L, RhoA (WT, 14V, 19N; Dr. J. Peränen, University of Helsinki, Finland), Rab34 (WT, CA and DN; Dr. W. Hong, GSK-IMCB Laboratory, Singapore), clathrin-chain-tomato (Dr. T. Kirchhausen, Harvard Medical School, Boston, MA) and IL-2R beta-chain (NTb; Dr. A. Dautry-Varsat, Pasteur Institut, Paris, France). The effects of expression of the WT constructs of different proteins on virus uptake were compared to each other, as well as to virus upatake after mock transfection, and without any transfection (Figure S5). No major changes were revealed in the virus uptake due to most plasmid transfections. Interestingly though, overexpression of GPI-GFP, Rac1 and Cdc42 did inhibit uptake of baculovirus to some extent possibly by boosting competing entry pathways.

Immunofluorescence labeling, confocal microscopy, and data analysis

Cells were fixed with 4% PFA for 20 min, permeabilized with 0.2% Triton X-100, immunolabeled according to standard protocols, and subjected to confocal microscopy (Zeiss LSM 510, Carl Zeiss AG, Jena, Germany or Olympus Fluo-View 1000, Olympus Optical Co., Tokyo, Japan). In the imaging, appropriate excitation and emission settings were used (488-nm argon laser, 543-nm and 633-nm HeNe-lasers). In live cell microscopy (Zeiss LSM 510) the objective and sample holder were heated to 37°C. Serial sections were obtained by using 60× APO oil immersion objective (NA = 1.35) or 63× Plan-Neofluor oil immersion objective (NA = 1.25) with a resolution of 512×512 pixels/image.

Quantification of internalization and colocalization was determined with a free, open source software package, BioImageXD [24] as described before [33]. Briefly, to quantify the level of colocalization, 30 cells from three independent experiments, 10 cells from each experiment, were randomly selected and optically sectioned using a confocal microscope. Colocalization was evaluated from the center slice of the cell by examination of the merged images, and analysis was performed with BioImageXD.

BioImageXD contains a simple algorithm for calculating the ratio of internalized/surface virus. The formula is: Ch1/(Ch2 – Coloc), where Ch1 = number of voxels stained after permeabilization, Ch2 = number of voxels stained before permeabilization, Coloc = number of colocalized voxels. Only voxels with intensity values above thresholds were considered for each of the three values. The thresholds were determined as above.

The amount of internalized *E. Coli* in cells was determined by intensity threshold segmentation. The threshold was selected so that only clear intracellular structures were visible. A connected component labeling algorithm was used to eliminate structures less than three voxels.

Flow cytometry

The cells were first preincubated with or without drug-containing medium (EIPA 0.025–0.1 mM) for 30–60 min, followed by baculovirus binding on ice for 30–60 min and transduction with or without drugs for 24 h at 37°C. The possible interfering effect of cellular inhibitors on viral binding, or on the fluorescence signal were controlled. The samples were scraped or detached by trypsin-treatment and analyzed with FACSCalibur and CellQuest software (Beckton Dickinson, Heidelberg, Germany).

RNAi

The following siRNA sequences (20 μM; Dharmacon, Thermo Fisher Scientific, Fremont, CA) were used: RhoA (5′-GAAGUCAAGCAUUUCUGUC-3′), Rac1 (5′-GAUAACUCACCACUGUCCA-3′), Arf6 (5′-GCACCGCAUUAUCAAUGACCGUU-3′) and dynamin2 (SMARTpool). Non-specific siRNA was used as a negative control, wheras siGLO (Dharmacon) acted as a transfection marker. Cells were transfected using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Mock-transfected cells were treated with Oligofectamine alone. At 72 h transfection, the cells were transduced with baculovirus for 6 h, fixed and immunolabeled with virus capsid labeling antibody. After immunolabeling, virus capsid localization in SiGlo-positive nuclei was analyzed visually from three separate samples (50–100

cells/each) by confocal microscopy. The functionality of siRNAs was shown by SDS-PAGE and western blot analysis of the respective proteins. Tubulin was used as a loading control. Using ImageJ software, differential gel band intensities of scramble and target siRNAs were detected.

Statistical testing

T-test was used for pairwise statistical comparison between samples. For percentages or ratio figures, t-test was applied after arcsin $\sqrt{}$ or logarithmic transformation of the original variable to convert the binomial distribution of the data to a normal distribution.

Supporting Information

Figure S1 Clathrin-mediated endocytosis is not involved in baculovirus early uptake. (A,B) Effect of baculovirus transduction on clathrin localization pattern in 293 cells. By live confocal microscopy, the expressed, fluorescent light chain clathrin (clathrin light chain tomato) showed similar distribution after baculovirus transduction (C; wt, 1000 MOI, 60 min p.t.) as untransduced, transfected control cells (B). Pseudocolor images are presented for better visualization of the expressed clathrin. (C,D) The expression of wt (C) or DN (D) clathrin coat assembly regulator protein Eps15-EGFP (green) and internalization of baculovirus capsid (wt, vp39 MAb, 200 MOI; red) after 2 h p.t. in 293 cells. (E,F) Baculovirus (p24mCherry, 200 MOI; red) was internalized in untreated (E) or dynasore-treated (F) 293 cells for 30 min together with CME marker transferrin (A546-TF; red). (G) Baculovirus (p24mCherry, 200 MOI, red) localization in 293 cells with caveolin-1 MAb (green). Alexa-555 and -488 were used as secondary antibodies. In all images, scale bars 10 μm.
Found at: doi:10.1371/journal.pone.0005093.s001 (4.90 MB TIF)

Figure S2 Baculovirus induces ruffle formation. (A) Cellular ruffle formation in live baculovirus transduced HepG2 cells. Baculovirus (MOI 400) was directly fed onto cells in the confocal microscope, and details of the cell surface protrusions were followed immediately during 0–15 min p.t. DIC images reveal protrusions growing from the cell surface. Baculovirus is not visualized for clarity. (B) Baculovirus was internalized for 15 min into 293 cells and, after PFA fixation, baculovirus capsid and actin were labeled by antibodies (Ab, Alexa-488, green) and TRITC-phalloidin (red), respectively. Scale bars, 10 μm.
Found at: doi:10.1371/journal.pone.0005093.s002 (1.30 MB TIF)

Figure S3 Localication and entry of baculovirus after expression of various endocytic membrane traffic regulators. (A,B) Baculovirus (wt, MOI 200, red) localization with Flotillin-1 MAb at 15 min p.t. (A) or GPI-EGFP at 30 min p.t. (B) in 293 cells (green). Baculovirus (wt, MOI 200) entry into 293 cells transfected with the wt, CA and DN mutants of Cdc42-EGFP at 6 h p.t. (C–E), as well as DN and CA mutants of Rac1-EGFP at 2 h p.t. (F,G) (green). (H) 293 cells were transfected with the Ntb-domain of IL2-receptor visualizing the localization of internalized IL2-receptor after baculovirus (wt, MOI 200, red) transduction for 30 min. Ntb-detecting antibody conjugate (NtB(IL-2):Cy3-561; green) was added on the plasma membrane before baculovirus addition. (I–O) Baculovirus (p24mCherry, MOI 200) entry after 6 h p.t. in 293 cells transfected with the wt and DN mutant of CtBP1/BARS (BARS; I–J), CA and DN mutants of Pak1 (K,L) as well as wt, DN and CA mutants of Rab34 (M–O; green). In images A,B and F–H, baculovirus was labeled with capsid labeling antibodies vp39 MAb or BV Ab together with Alexa-555 secondary antibody (red). In images C-E and I-O, p24mCherry construct was used. In all images, scale bars 10 μm.
Found at: doi:10.1371/journal.pone.0005093.s003 (9.89 MB TIF)

Figure S4 The effect of EIPA on fluid-phase entry. The efficacy of EIPA was tested by internalizing TRITC-De for 30 min in untreated (A) or EIPA-treated (0.1 mM) 293 cells (B). A representative group of cells are shown with (right) or without (left) DIC image merged with TRITC-De. Scale bars, 10 μm.
Found at: doi:10.1371/journal.pone.0005093.s004 (2.63 MB TIF)

Figure S5 Putative effects on baculovirus entry due to expression of various endocytosis regulating WT constructs. Various plasmid constructs were transfected into 293 cells for 48 hours prior to baculovirus (p24mCherry, 200 MOI) uptake for 30 min. The plasma membrane-bound virus was extensively washed before the PFA fixation of the. Transfected cells (n = 300–400 cells) were scanned by confocal microscopy from three separate samples and analyzed for their p24mCherry fluorescence intensity. The results are shown as mean values ± SE. Untransfected cells (control) and cells expressing plain EGFP (GFP) were as controls.
Found at: doi:10.1371/journal.pone.0005093.s005 (1.64 MB TIF)

Video S1 Baculovirus internalization into living 293 cells was observed by confocal microscopy. In the imaging, fluorescent (p24mCherry, MOI 400) virus was fed onto cells and the attachment and internalization of the viruses were monitored thereafter. Differential contrast image (DIC), virus (red) and selected time frames (0–400 s) are shown.
Found at: doi:10.1371/journal.pone.0005093.s006 (3.92 MB MOV)

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Author Contributions

Conceived and designed the experiments: JPL ARM EK VM. Performed the experiments: JPL ARM EK PT VM. Analyzed the data: JPL ARM EK KA VM. Contributed reagents/materials/analysis tools: SK JP SYH KA COB MVR VM. Wrote the paper: JPL ARM EK VM.

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