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195

Anna Mäkelä

Towards Therapeutic Gene Delivery to Human Cancer Cells

Targeting and Entry of Baculovirus

JYVÄSKYLÄN YLIOPISTO

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2008

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"One can´t believe impossible things," said Alice. "I dare say you haven´t had much practice," said the Queen. "Why sometimes I´ve believed as many as six impossible things before breakfast."

Lewis Carroll (Alice in Wonderland)

ABSTRACT

Mäkelä, Anna R.
Towards therapeutic gene delivery to human cancer cells: targeting and entry of baculovirus
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Yhteenveto: Kohti terapeuttista geeninsiirtoa: bakuloviruksen kohdennus ja sisäänmeno ihmisen syöpäsoluihin
Diss.

Targeting of viral vectors to specific cells by vector engineering has become a major focus of cancer therapy research. Recently, peptides that recognize molecular markers expressed by tumor-associated cells and vasculature have shown promise in mediating site-specific vector targeting. The budded virion (BV) of baculovirus represents a multifunctional biotechnological tool and an auspicious new vector candidate for gene therapy and other biomedical applications. An exceptional advantage of this insect pathogen is its molecular flexibility, facilitating modification of the vector phenotype for tissue and cell targeting. To attain tumor-selective tropism, vectors displaying the tumor-homing peptides LyP-1, F3, and CGKRK were engineered. Each of these peptides significantly enhanced baculoviral binding and gene delivery to target cells *in vitro*, and the systemically administered vector displaying the lymphatic homing peptide LyP-1 also accumulated to xenografted human tumors *in vivo* in a mouse model. Furthermore, to develop complementary baculovirus-based tools, the interaction of occlusion-derived baculovirus (ODV) with human cancer cells, and the functionality of the P74 ODV envelope protein as a display platform were evaluated. Although capable of cellular binding and limited internalization, ODV was incapable of mediating successful transduction in human cells, rendering the BV more applicable for gene delivery and display technologies. Finally, baculovirus was shown to enter highly permissive human cancer cells via a clathrin-independent and raft-dependent pathway that was regulated by dynamin, the actin mediators Arf6 and RhoA, and induced the uptake of the phagocytic tracer *E. coli.* The mechanism thus shared features of phagocytosis. This clarification of the nature and regulation of baculovirus entry together with the first demonstration of *in vivo* tumor targeting of a tropism-modified baculoviral vector benefits future design and highlights the potential of baculovirus-mediated targeted therapies.

Keywords: Baculovirus; display; gene delivery; gene therapy; peptide; targeting; viral entry.

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LIST OF ORIGINAL PUBLICATIONS

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

I Mäkelä A.R.*, Matilainen, H.*, White, D.J., Ruoslahti, E., & Oker-Blom, C. 2006. Enhanced baculovirus-mediated transduction of human cancer cells by tumor-homing peptides. Journal of Virology 80: 6603-6611.

II Mäkelä A.R., Enbäck, J., Laakkonen, J.P., Vihinen-Ranta, M., Laakkonen, P., & Oker-Blom, C. 2008. Tumor targeting of baculovirus displaying a lymphatic homing peptide. Journal of Gene Medicine 10: 1019-1031.

III Mäkelä A.R., Närvänen, A., & Oker-Blom, C. 2008. Peptide-mediated interference with baculovirus transduction. Journal of Biotechnology 134: 20-32.

IV Mäkelä A.R., Tuusa J.E., Volkman L.E., & Oker-Blom, C. 2008. Occlusion-derived baculovirus: interaction with human cells and evaluation of the envelope protein P74 as a surface display platform. Journal of Biotechnology 135: 145–156.

V Laakkonen, J.P., Mäkelä, A.R., Kakkonen, E., Turkki, P., Kukkonen, S., Peränen, J., Ylä-Herttuala, S., Airenne, K.J., Oker-Blom, C., Vihinen-Ranta, M., & Marjomäki, V. 2008. Arf6 and RhoA regulate phagocytosis-like entry of baculovirus in human cells. Manuscript submitted.

*Equal contribution

RESPONSIBILITIES OF ANNA R. MÄKELÄ IN THE ARTICLES OF THIS THESIS

Article I — I designed and performed the majority of the experiments. The binding inhibition studies were conducted together with Heli Matilainen and Daniel White. I wrote the article and all authors participated in finalizing it.

Article II — I was responsible for the planning of the article. I performed the *in vitro* experiments of viral binding, internalization, as well as transduction together with Johanna Laakkonen. Juulia Enbäck and Pirjo Laakkonen conducted the *in vivo* targeting assays. I wrote the article and all authors participated in finalizing it.

Article III — I was responsible for the design and implementation of the study and performed the majority of the experiments. I wrote the article and all authors participated in finalizing it.

Article IV — I was responsible for the concept and experimental design of the article, and performed the majority of the experiments. Jenni Tuusa participated in the experimental work under my supervision. I wrote the article and all authors participated in finalizing it.

Article V — The experiments were designed and conducted together with Varpu Marjomäki, Johanna Laakkonen, and Elina Kakkonen. I was responsible for the baculovirus transduction studies, in which the effects of different chemical inhibitors were studied. I also constructed the expression reporter viruses used in these experiments. I participated to the quantification experiments of baculovirus colocalization with GPI-EGFP and the phagocytotic uptake of *E. coli* bioparticles upon viral entry. Varpu Marjomäki and Johanna Laakkonen wrote the article and I participated in finalizing it.

The studies were performed under the supervision of Professor Christian Oker-Blom (I-V) and Docent Varpu Marjomäki (V).

ABBREVIATIONS

293	human embryonic kidney cell line
A549	human lung carcinoma cell line
*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
AAV	adeno-associated virus
Ad	adenovirus
Arf6	ADP ribosylation factor 6
BAP	biotin acceptor peptide
BEVS	baculovirus expression vector system
BDVS	baculovirus display vector system
BV	baculovirus budded virion
CA	constitutively active
Cdc42	Cdc42 GTPase
CGKRK	cysteine-glycine-lysine-arginine-lysine
CME	clathrin-mediated endocytosis
CMV	cytomegalovirus
CT	cytoplasmic
DAF	decay accelerating factor
DMEM	Dulbecco's modified eagle medium
DN	dominant negative
E. coli	*Escherichia coli*
EEA-1	early endosomal antigen 1
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EIPA	5-(N-ethyl-N-isopropyl)-amiloride
ER	endoplasmic reticulum
F3	tumor-homing peptide
FBS	fetal bovine serum
FIV	feline immunodeficiency virus
GFP	green fluorescent protein
GP64	baculovirus group 1 major envelope glycoprotein 64
GPI	glycosyl-phosphatidylinositol
GTPase	guanosine triphosphate hydrolyzing enzyme
HepG2	human hepatocarcinoma cell line
HSV	herpes simplex virus
HIV	human immunodeficiency virus
IE	immediate early
Ig	immunoglobulin
ITR	inverted terminal repeat
LTR	long terminal repeat
luc	firefly luciferase
LyP-1	tumor-homing peptide
LYVE-1	lymphatic endothelial hyaluronan protein
MDA-MB-231	human breast carcinoma cell line

MDA-MB-435	human breast/melanoma carcinoma cell line
MEM	minimal essential medium
MNPV	multiple nucleopolyhedrovirus
MOI	multiplicity of infection
NDV	Newcastle disease virus
NPV	nucleopolyhedrovirus
ODV	baculovirus occlusion-derived virion
P10	baculovirus protein 10
P74	baculovirus protein 74
PAGE	polyacrylamide gel electrophoresis
Pak1	p21-activated kinase-1
PBS	phosphate buffered saline
PFA	paraformaldehyde
PFU	plaque forming unit
p.i.	post infection
PI	phosphoinositol
PLC	phospholipase C
polyAla	poly-alanine
PI3K	phosphoinositide 3-kinase
polh	baculovirus protein polyhedrin
p.t.	post transduction
Rac1	Ras-related C3 botulinum toxin substrate 1
RGD	arginine-glysine-aspartic acid
RhoA	Ras homolog gene family member A
RKK	arginine-lysine-lysine
RNAi	RNA interference
RT	room temperature
scFv	single–chain antibody
sCR1	soluble complement receptor type 1
SDS	sodium docedyl sulphate
Sf	*Spodoptera frugiperda*
SFV	Semliki Forest virus
siRNA	small interfering RNA
SNPV	single nucleopolyhedroviruses
SV40	simian virus 40
TM	transmembrane
VP39	baculovirus capsid protein 39
VSV	vesicular stomatitis virus
VSVG	vesicular stomatitis virus G-protein
WT	wild-type
Z domain	synthetic IgG-binding domain of protein A

1 INTRODUCTION

Cancer is a multigenic disorder involving mutations in both tumor suppressor genes and oncogenes. A large body of preclinical and recent clinical data has suggested that cancer growth can be arrested or even reversed by treatment with tumor targeted transfer vectors carrying a growth inhibitory or pro-apoptotic gene. Therefore, targeting of therapeutic entities to preselected cells by vector engineering has recently become a major focus of cancer therapy research. Viruses are currently regarded as the most efficient gene delivery vehicles. Since each viral vector system has unique advantages and shortcomings, each type of vector has applications for which it is best suitable. The use of human virus vectors is inherently problematic due to their pathogenic nature and requirement of secondary helper functions.

Baculovirus, an insect pathogen, holds great potential to successfully address many critical issues concerning safety and efficacy in gene therapy. Traditionally, baculoviruses have been applied as targeted biocontrol agents and for heterologous gene expression in insect cells and larvae. Since the discovery that baculovirus is able to transduce cells of mammalian origin, this viral vector system has found versatile applications also in biomedicine including vaccination as well as cancer and immunotherapy. Baculovirus is unique among other virus families in having two distinct viral phenotypes of an identical viral genotype: occlusion-derived (ODV) and budded (BV) virion, of which the BV has played a fundamental role in the evolution of baculovirus-based applications in biotechnology. The intriguing studies conducted with BV and the increasing understanding of baculovirology have generated optimism that the ODV could become a complementary tool for analogous use.

The focus of the present study was to engineer surface-modified baculoviral vectors for selective gene delivery to human cancer cells and to understand the molecular events underlying the mode and regulation of baculovirus uptake in human cancer cells. A detailed knowledge of baculovirus entry mechanisms and controlled modification of the vector tropism would significantly promote the evolution of this viral vector system into an efficient tool for gene therapy as well as for novel unforeseen applications.

2 REVIEW OF THE LITERATURE

2.1 Viral vectors for gene therapy

The concept of gene therapy refers to the delivery of genetic material to the cells of an individual for therapeutic benefit (Mulligan, 1993). It thus holds the potential of mediating the highest possible level of therapeutic specificity. Numerous approaches are currently under development to apply therapeutic gene delivery to a variety of disorders. Furthermore, the recently discovered RNA interference (RNAi) has offered a novel, powerful class of cargos for a number of disease applications (McCaffrey et al., 2002; van Rij, 2008). Since the first clinical trial using retroviral gene transduction for human gene therapy in 1989 (Rosenberg et al., 1990), more than 1340 gene therapy clinical trials have been completed, are in progress, or have been approved worldwide (Edelstein et al., 2007; http://www.wiley.co.uk/genmed/clinical). The past few years have witnessed the first hints of success in clinical trials, for example, the treatment of human severe combined immunodeficiency (SCID)-X1 disease in infants with a retrovirus vector (Cavazzana-Calvo et al., 2000), the partial cure of hemophilia B through the adeno-associated virus (AAV)-mediated delivery of a gene encoding Factor IX to several patients (Kay et al., 2000), the use of an oncolytic adenovirus (Ad) vector for the treatment of cancer (Khuri et al., 2000), and initial signs of therapeutic efficacy in a cystic fibrosis clinical trial involving AAV (Moss et al., 2004). Although the field holds great promise, it has also experienced intense criticism and skepticism during recent years because of the unfortunate occurrence of a few serious adverse events (Couzin and Kaiser, 2005; Edelstein et al., 2007). Since then, signifigant advancement has been gained in vector engineering and targeting, although the adverse events emphasize the need of further progress before gene therapy strategies become therapeutic realities.

Gene delivery vehicles can be roughly divided into two classes, viral and non-viral, with complementary advantages and disadvantages. Viruses have evolved numerous strategies to successfully internalize cells (see Chapter 2.3),

and thus are more efficient than their non-viral counterparts (Gao et al., 2007; Lundstrom and Boulikas, 2003). As viruses are highly complex and semi-optimized products of millions of years of evolution, modification of their intrinsic properties is required to regulate their safety, stability, and efficiency in serving as optimized human therapeutics. Vectors derived from Ad, retro- and lentiviruses, AAV, herpesvirus, vaccinia, and poxvirus are used in more than 70% of the clinical gene therapy trials worldwide (Edelstein et al., 2007). Because of the various properties of each viral vector, the definition of their application range depends on factors such as packaging capacity, host range, cell- or tissue-specific targeting, replication competency, genome integration, and duration of transgene expression. Recent engineering of modified viral vectors has contributed to improved specificity and efficiency of gene delivery (see Chapter 2.2). The properties of the most commonly applied viral gene delivery vectors are introduced below and summarized in Table 1.

TABLE 1 Characteristics of the most commonly applied viral gene delivery vectors. Modified from Hu, 2006; Waehler et al., 2007.

Features	**Adenovirus**	**Retro-/lentivirus**	**AAV**	**Baculovirus**
Ease of propagation	Yes	No	No	Yes
Vector size	70–130 nm	80-100 nm	20-25 nm	40x250 nm
Vector yield (transducing units/ml)	High (10^{12})	Moderate (10^{10})	High (10^{12})	High (10^{12})
Insertion capacity (kb)	Up to 36 kb	8-9 kb	4.9 kb*	> 100 kb
Route of administration	*Ex/in vivo*	*Ex/in vivo*	*Ex/in vivo*	*Ex/in vivo*
Nature of vector genome	Episomal	Integrated	Integrated	Episomal
Duration of gene expression	Short	Long	Long	Short
Tropism	Broad	Dividing cell/Broad	Broad	Broad
Emergence of replication competent vector *in vivo*	Low risk	High risk	Low risk	Negligible
Transduction of non-dividing cells	Yes	No/Yes	Yes	Yes
Immune response	High	Low	Medium	Medium
Pre-existing immunity	Yes	Unlikely	Yes	Unlikely
Safety	Toxicity, inflammatory response	Integration may induce oncogenesis	Toxicity, inflammatory response	High

*10 kb after heterodimerization of two AAV virions

2.1.1 Baculoviruses

2.1.1.1 Biology and structure of baculovirus

Baculoviruses comprise a family, *Baculoviridae,* of arthropod-specific viruses ubiquitously found in the environment, of which members have been isolated from more than 600 host insect species (Slack and Arif, 2007). 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 of Lepidoptera, and consequently are the best characterized isolates. The circular double-stranded DNA genome ranging from 80 to 180 kb in size is packed into bacillus-shaped nucleocapsids (Figure 1), hence the name "baculovirus". The family has been traditionally divided into two subgroups Granuloviruses and Nucleopolyhedroviruses, depending on the virion structure and hosts. The genera Nucleopolyhedrovirus is divided into two groups based on the number of nucleocapsids. The single nucleopolyhedroviruses (SNPV) contain one nucleocapsid per virion, whereas the multiple nucleoplyhedroviruses (MNPV) contain many. Both the SNPVs and MNPVs enclose numerous virions per occlusion body, ranging from 500 to 2000 nm in diameter (Figure 1). While the occlusion bodies of granuloviruses are capsule-shaped and contain only a single virion, those of NPVs are multisided crystals or polyhedra (Slack and Arif, 2007). This distinctive structure led to baculoviruses being the earliest described virus particles. At present, the genomes of at least 29 baculovirus species have been sequenced, providing a database of more than 4000 genes (Slack and Arif, 2007).

The *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), a prototype of *Baculoviridae*, was isolated from the *Ac* alfalfa looper in 1971, and by far has become the best characterized member of the family. The 134 kb genome of *Ac*MNPV is associated with the DNA binding protein p6.9, contains 154 open reading frames, and has been completely sequenced (Ayres et al., 1994). The nucleoprotein core is enclosed by the major capsid protein VP39 in addition to a few minor proteins forming a flexible nucleocapsid of 30-60 nm in diameter and 250-300 nm in length (Jehle et al., 2006; Slack and Arif, 2007).

The infection of *Ac*MNPV in insect cells is characterized by the production of two structurally and functionally distinct types of virions: the occluded or polyhedra derived virion (ODV) and the budded virion (BV) (Figure 1). These two virion phenotypes are produced at different locations in the cell and at different times of the life cycle. Consequently, they serve distinctly specialized functional roles and enter target cells by different mechanisms (see Chapter 2.3.6.1). BVs are produced in the late phase of the infection cycle by budding from the insect cell surface. Thus, the envelope of BV is derived from the modified plasma membrane of the host cell. The ODVs, on the other hand, are assembled within the nucleus in the very late phase of the infection by an unusual intranuclear envelopment of the nucleocapsids (Braunagel and Summers, 1994; Summers and Volkman, 1976). Since the nucleocapsids of both BV and ODV are assembled in the nucleus, the capsid structure together with the viral DNA appears to be identical in both phenotypes. Thus, the distinctive features of BV and ODV are the differences in the composition of the envelope and associated structures, resulting in differential roles in the infection cycle (see Chapter 2.1.1.2). For example, GP64, the major N-glycosylated envelope (phospho)protein of BV is absent from ODV, whereas the ODV envelope protein P74 is not present in BV (Braunagel and Summers, 1994; Braunagel et al., 2003; Faulkner et al., 1997; Haas-Stapleton et al., 2004; Kuzio et al., 1989; Oomens et al., 1995; Slack et al., 2001; Zhou et al., 2005). P74 mediates specific

receptor binding of ODV to the primary target cells within the larval midgut (Haas-Stapleton et al., 2004; Ohkawa et al., 2005), whereas GP64 mediates budding, attachment, and entry of BV in a variety of cell types (see Chapter 2.3.6; Hefferon et al., 1999; Monsma et al., 1996; Oomens and Blissard, 1999; Zhou and Blissard, 2008a; Zhou and Blissard, 2008b). The BV of *Ac*MNPV has played a fundamental role in the evolution of baculovirus-based applications in biotechnology, and recently also in biomedicine. These topics are introduced in Chapters 2.1.1.3, 2.1.1.4, and 2.2.5.

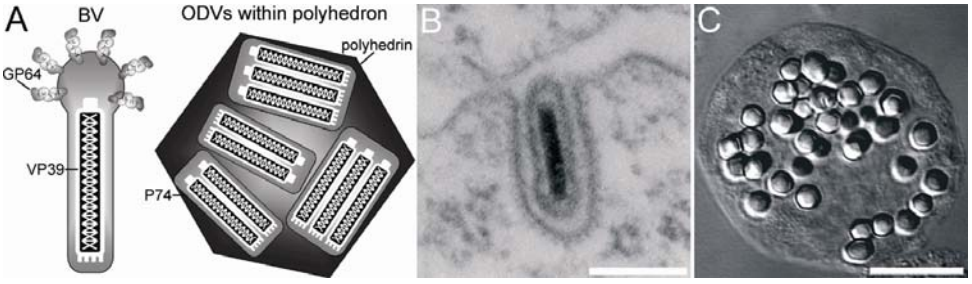

FIGURE 1 Baculovirus virion phenotypes. A) Schematic representation of a budded virion (BV; left) and occlusion-derived virion (ODV; right) embedded within an occlusion body (polyhedron). B) Electron micrograph of *Ac*MNPV BV bound to the *Sf*9 (*Spodoptera frugiperda*) insect cell surface in a pit with an electron-dense coating resembling clathrin. Scale bar 200 nm. The image was kindly provided by David Mottershead and Carl-Henrik von Bonsdorff. C) Differential interference contrast image of *Ac*MNPV-infected *Sf*9 cell at 72 h post infection. The cell nucleus is crowded with polyhedra. Scale bar 10 µm.

2.1.1.2 Baculovirus life cycle

In a natural infection of baculovirus, the two virions are functionally differentiated: ODV mediates interhost spreading through oral transmission and infection of the midgut epithelial cells (primary infection), whereas BV is required for the systemic infection of an individual host (secondary infection) (Figure 2). Upon ingestion by feeding lepidopteran larvae, the polyhedra encounter the highly alkaline midgut fluids (pH 9.2 to 11) that induce their rapid dissolution. The liberated ODV must survive the harsh alkaline digestive fluids of the host, penetrate the protective peritrophic membrane bordering the midgut epithelium, and attach to the apical microvilli of columnar cells in order to establish infection by direct fusion with the membrane of microvilli (Faulkner et al., 1997; Granados, 1978; Granados and Lawler, 1981; Horton and Burand, 1993; Kawanishi et al., 1972; Keddie et al., 1989; Tanada et al., 1975). The ODV, subjected to caustic pH and digestive proteases, is thus specialized to exploit one of the most extreme biological environments. The entry is followed by transportation of the nucleocapsids through the cytoplasm into the nucleus via an actin filament-mediated mechanism for uncoating of the viral DNA

(Charlton and Volkman, 1993; Lanier and Volkman, 1998). The transcription of viral genes and replication initiates immediately after the uncoating.

The infection cycle can be divided into three basic phases: early, late, and very late. The synthesis of viral proteins is regulated in a cascade-like manner, and host cell factors are required for the expression of the transactivating immediate-early (*ie*) genes. The expression of the delayed-early genes is required for the initiation of replication of the viral genome, while the late genes encode primarily structural proteins, and the very late genes participate in the formation of occlusion bodies (Slack and Arif, 2007).

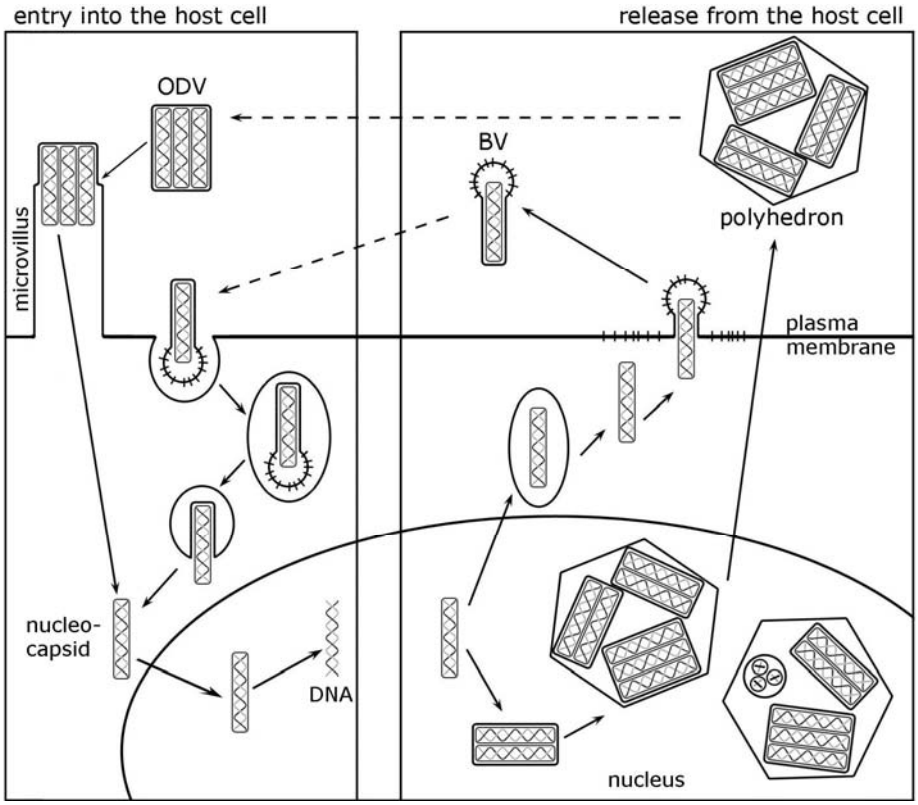

FIGURE 2 Baculovirus infection cycle. Occlusion-derived virion (ODV); budded virion (BV). The figure was kindly provided by Jenni E. Tuusa.

Subsequent to the primary round of replication, the resultant progeny nucleocapsids, assembled in the nucleus, bud through the nuclear membrane, and are transported to the cell membrane of the epithelial cells. The BV acquires its envelope containing the virus-encoded glycoprotein GP64 in one end of the virion by budding through the plasma membrane of the host cell. The BV mediates the systemic spread of the infection from the midgut to other tissues via the hemolymph within the host and is also responsible for the infection in cultures (Granados and Lawler, 1981; Keddie et al., 1989; Monsma and Blissard,

1995; Monsma et al., 1996; Volkman et al., 1984; Volkman and Goldsmith, 1984). An additional collection of progeny ODVs become enveloped within the nucleus by *de novo* assembled envelope. Subsequently, these virions are occluded within the occlusion matrix protein, polyhedrin. The addition of the polyhedral envelope around the periphery of forming occlusion bodies completes the maturation of polyhedra that are released into the environment subsequent to cell lysis and death of the insect host (Slack and Arif, 2007; Volkman, 1997). The infection endures from five to seven days until the host larvae eventually liquefy, and ODVs occluded within polyhedra are released into the environment to commence a new infection cycle.

2.1.1.3 Biotechnological applications of baculovirus

2.1.1.3.1 Control of insect pests

Baculoviruses have a distinguished history regarding their experimental exploitation as environmentally safe natural agents to control agricultural pests. The first studies reporting their potential as biological pesticides date back to the years 1925 in Europe and 1944 in North America. While baculoviruses as an alternative for chemical pest control gathered popularity, the mass production and intentional delivery of infectious virus into the ecosystems became a concern during the 1970s. This led to a careful scrutiny of baculovirus as a pest control agent by an international community of virologists, which comprehensively evaluated and examined the concerns regarding the environment and human health. These contributions stimulated a revolution in studies of the molecular biology and genetics of baculovirus, and the virus became generally accepted as safe for insect control. The milestones of these and other ground-breaking studies have recently been reviewed by Professor Max Summers (2006), one of the distinguished pioneers of baculovirology. Genetically engineered baculoviral pesticides alone and in combination with wild-type (WT) baculovirus have become valuable tools for insect control, albeit their full potential will not be realized until the public awareness and acceptance of genetically modified organisms increases (Inceoglu et al., 2006).

2.1.1.3.2 Production of heterologous proteins in insect cells

Extensive research by several laboratories was fundamental to discover the identity, role, and function of the baculoviral late genes encoding the major occlusion protein, polyhedrin, and another highly expressed protein, P10. These findings provided the foundation for the establishment of the baculovirus expression vector system (BEVS) that has facilitated the routine expression of countless heterologous proteins in high quantities in insect larvae and cultured cells with the aid of *polyhedrin* and *P10* promoters (Summers, 2006). Smith and colleagues (1983) were the first to demonstrate the cloning and expression of biologically active human β-interferon. Further knowledge of baculoviral genetics has also provided the option of expressing foreign genes within a

different temporal context than with the late promoters. For example, the promoter of the baculovirus *ie1* and the role of the protein as a transactivator can be used to supplement, replace, or add to the combinatorial effects of recombinant protein expression (Murges et al., 1997; Summers, 2006). Similar to other eukaryotic expression systems, baculovirus-mediated expression of heterologous genes allows proper folding, post-translational modification, oligomerization, and secretion essentially indistinguishable from those of mammalian cells (Harrison and Jarvis, 2006). To improve the therapeutic use of products, transgenic insect cell lines stably expressing mammalian glycosyltransferases are available to circumvent the limitations associated with the modification of complex N-linked oligosaccharides (Harrison and Jarvis, 2006). The BEVS also enables the co-expression of protein-modifying enzymes, and the assembly of multi-subunit protein complexes e.g. virus-like particles for vaccination and diagnostics (Kost et al., 2005). Other great attributes include the usage of insect cell culture medium devoid of serum components, the ease of generating recombinant viruses, high insertion capacity of foreign DNA (>100 kb), and the scalable production of the expression vectors and proteins. Also, stable insect cell lines and non-lytic expression systems have been developed to complement the technology (Douris et al., 2006; Ho et al., 2004; Ikonomou et al., 2003).

2.1.1.3.3 Baculovirus-mammalian cell technology

The first evidence indicating that baculovirus is able to internalize and transduce mammalian cells was attained during the 1980s (Carbonell et al., 1985; Volkman and Goldsmith, 1983). No particular attention was paid to the outcome of these studies until a decade later when two pioneering groups demonstrated baculovirus-mediated transgene expression in high level in human, rabbit, and rat hepatocytes using recombinant viruses equipped with mammalian expression cassettes (Boyce and Bucher, 1996; Hofmann et al., 1995). Subsequent to these preliminary discoveries, the diversity of permissive cells for baculovirus transduction has substantially grown to include multiple dividing and non-dividing transformed and primary cells of human, non-human primate, porcine, bovine, rodent, rabbit, or even fish origin (Hu, 2006; Kost et al., 2005). However, cells of hematopoietic origin are unpermissive for baculovirus transduction (Cheng et al., 2004; Condreay et al., 1999). The gene expression in transduced populations can be modulated by variation of viral multiplicity and transduction conditions, or by the use of inhibitors of histone deacetylases (Condreay et al., 1999; Hsu et al., 2004). The virus does not replicate in mammalian cells and is rapidly inactivated by serum complement (Hofmann and Strauss, 1998; Hofmann et al., 1999; Huser et al., 2001), granting it a favorable biosafety profile compared to other recombinant viral vectors. The advantages and flexibility of this system render it an excellent tool for the development of mammalian cell-based assays, where gene delivery is accomplished with simple liquid addition steps being compatible with automated high-throughput screening platforms (Kost et al., 2005). Today, this

technology, often referred to as the BacMam System, is widely used in diverse applications including cell-based assays for drug screening (Figure 3) (Condreay et al., 2006), RNAi (Nicholson et al., 2005), studies on gene and protein functions (Makela and Oker-Blom, 2008), delivery of vaccine immunogens (Strauss et al., 2007; van Oers, 2006), production of foreign recombinant viruses (Lesch et al., 2008), as well as cancer and immunotherapy (Kitajima and Takaku, 2007; Kitajima and Takaku, 2008; Nishibe et al., 2008; Troadec et al., 2007; Wang et al., 2006), for example.

2.1.1.3.4 Baculovirus display

The recently established baculovirus display vector system (BDVS) represents a eukaryotic display platform that combines the positive attributes of both cell and virus-based display approaches, allowing presentation of complex polypeptides on cellular and viral surfaces (Figure 3). The technology thus piggybacks on the molecular flexibility of the virus, allocating the combination of viral genotype with phenotype and thereby surface display of heterologous (poly)peptides. Compared to the microbial display systems, the BDVS has the advantage of correct protein folding and post-translational modifications similar to those of mammalian cells. This facilitates the expression and analysis of proteins with a therapeutic interest.

Currently, a variety of baculovirus-based assays aiming at routine high-throughput identification of agents targeting cell surface receptors or studies on ligand-receptor interactions are under construction. Furthermore, modification of the vector phenotype has the potential to be adapted for studies such as complex virus–host cell interactions (see Chapter 2.3.6), cell and tissue targeting (see Chapter 2.2.5), eukaryotic library development, antibody production, and vaccination. My colleagues and I have recently reviewed the characteristics of the baculovirus display platforms and the latest developments of the system (Makela and Oker-Blom, 2006; Makela et al., 2007; Makela and Oker-Blom, 2008).

2.1.1.4 Baculovirus in gene therapy

The transient nature of baculovirus-mediated gene expression renders the virus an attractive vector for the treatment of cancer (Kim et al., 2007; Wang et al., 2006; Wang et al., 2008) and cardiovascular diseases (Airenne et al., 2000; Grassi et al., 2006). Several studies have highlighted the potential of the virus also for engineering of stem cells, as well as bone and cartilage tissue (Chen et al., 2006; Chen et al., 2008; Chuang et al., 2007; Sung et al., 2007), and for gene delivery into the cells of the nervous system (Lehtolainen et al., 2002; Sarkis et al., 2000; Tani et al., 2003; Wang et al., 2007). The virus is regarded as safe with negligible pathogenic potential in humans, has a huge transgene insertion capacity (>100 kb), induces no cytotoxic effects, is able to mediate transgene expression without replication, and facilitates large-scale vector production (Hu, 2006; Kost et al., 2005). Furthermore, partial integration of baculoviral DNA into the host

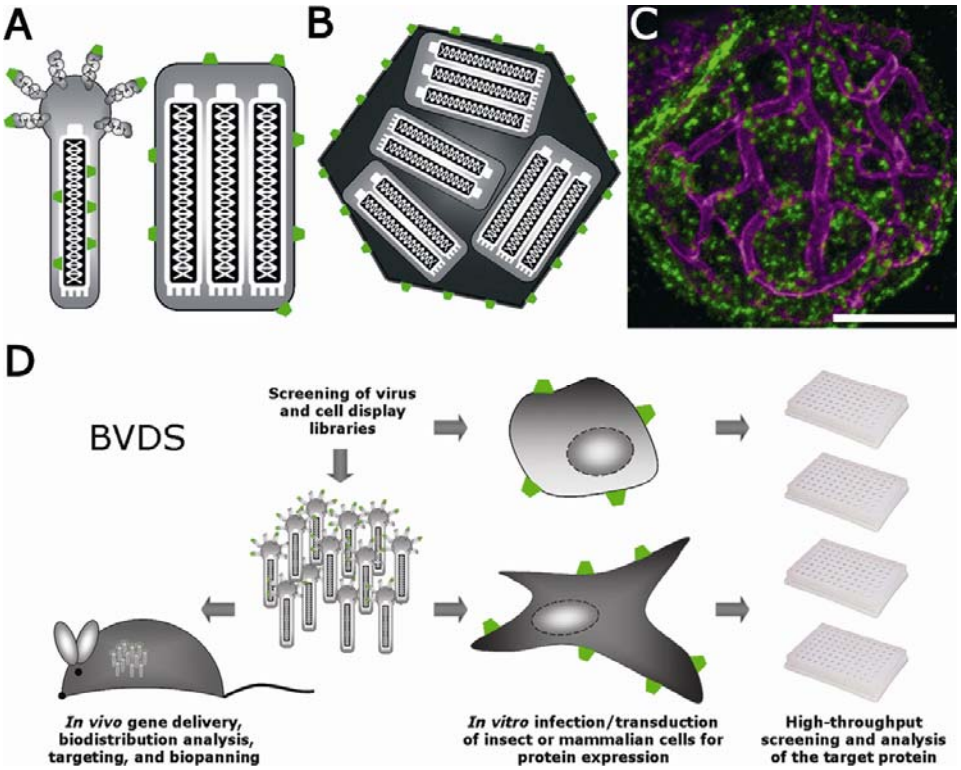

FIGURE 3 Baculovirus display platforms and applications of the baculovirus display vector system (BDVS). Baculovirus represents a versatile tool to display foreign peptides and proteins on the surface of budded virion (BV) and occlusion-derived virion (ODV) (A), polyhedron (B), as well as insect and mammalian cells (C). A) Schematic presentation of BV and ODV displaying a heterologous protein by fusion to GP64 as well as VP39, or an ODV-envelope protein (e.g. P74, E25, E66, or E56). B) Display of a foreign protein on the surface of a polyhdedron by fusion to polyhedrin. C) Baculovirus-mediated expression of a heterologous protein on the surface of an insect cell (GP64, green; P10, purple; scale bar 10 µm). D) The BDVS represents a multifunctional technique adaptable for diverse biomedical applications including molecular screening using virus surface display, insect and mammalian cell display, or combinations of these platforms.

genome has been observed only under selective pressure *in vitro* (Condreay et al., 1999; Merrihew et al., 2001), thus the risk of insertional mutagenesis is low *in vivo*. Baculovirus-mediated transgene expression is characteristically transient peaking at 3-5 days (Airenne et al., 2000; Lehtolainen et al., 2002).

However, transgene expression has been demonstrated to persist up to 200 days in the absence of the serum complement system (Pieroni et al., 2001). Prolonged or stable expression can be achieved using baculovirus hybrid vectors capable of integration into the host cell genome with the aid of the inverted terminal repeats (ITRs; see Chapter 2.1.4) of AAV (Palombo et al., 1998;

Wang and Wang, 2005; Zeng et al., 2007), or episomal replication using the genetic elements responsible for the episomal maintenance of Epstein-Barr virus during the latent infection (Shan et al., 2006; Wang et al., 2008).

The exploitation of baculovirus for systemic gene delivery, however, is challenged by vector immunogenicity (Saitoh et al., 2007; Strauss et al., 2007), transduction of non-target tissues (Kim et al., 2006; Kircheis et al., 2001; Raty et al., 2006; Raty et al., 2007), and activation of innate immune responses (Abe et al., 2003; Abe et al., 2005; Airenne et al., 2000; Beck et al., 2000; Gronowski et al., 1999), the major limitation being viral inactivation by the complement system. Partial protection has been accomplished *in vitro* in the presence of human or animal sera by applying inhibitory agents against the complement system (Hoare et al., 2005; Hofmann and Strauss, 1998; Hofmann et al., 1999; Tani et al., 2003), or *in vivo* using a baculovirus displaying human decay-accelerating factor as a GP64 fusion (Huser et al., 2001). An alternative approach is to avoid viral contact with the blood components (Airenne et al., 2000; Sandig et al., 1996), or to deliver the virus into immunopriviledged areas (Haeseleer et al., 2001; Lehtolainen et al., 2002; Sarkis et al., 2000). Baculovirus also provokes production of pro-inflammatory cytokines and interferons (Abe et al., 2003; Abe et al., 2005; Beck et al., 2000; Gronowski et al., 1999), and encompasses strong adjuvant properties in mice, promoting both humoral and T-cell responses against the coadministered or expressed antigen (Hervas-Stubbs et al., 2007). Also induction of antitumor immunity by baculovirus has been demonstrated (Kitajima et al., 2007; Kitajima and Takaku, 2008). Thus in addition to gene therapy, baculovirus shows promise in vaccination and immunotherapy.

2.1.2 Adenoviruses

Over 50 distinct serotypes of human adenoviruses (Ad) have been discovered (Davison et al., 2003). The human Ad consist of a ~36 kb linear double-stranded DNA genome enclosed within a non-enveloped icosahedral particle of 70-90 nm in diameter and composed of 12 distinct polypeptides (Chroboczek et al., 1992). Hexon, the major structural protein of the Ad capsid, plays a structural role, while the pentameric penton base and homo-trimeric fiber mediate cellular attachment and internalization. The initial binding is mediated by the fiber to cell surface coxsackievirus B and Ad receptor (CAR) (Bergelson et al., 1997; Campos and Barry, 2006). Additionally, heparan sulfate proteoglycans promote Ad attachment (Dechecchi et al., 2001). Secondary interactions between the RGD motifs present within the penton base and cell surface integrins stimulate the subsequent uptake primarily via clathrin-mediated endocytosis (CME) (Li et al., 1998a; Li et al., 1998b; Salone et al., 2003; Wickham et al., 1993).

By July 2007, Ad vectors were used in 24.8% of the 1,346 gene therapy clinical trials worldwide, thus overtaking retroviruses (Edelstein et al., 2007). Replication-defective vectors based on human Ad serotypes 2 and 5 (Chroboczek et al., 1992) possess a number of features that have favored their widespread employment for gene delivery both *in vitro* and *in vivo*. The major advantages of Ad vectors include the large insertion capacity of foreign DNA,

efficient transient transduction of a wide variety of quiescent and proliferating cells, and easy production to high titers. Moreover, the risk of insertional mutagenesis is low, as the viral DNA is not integrated into the host genome (Russell, 2000). However, the elicitation of both innate and acquired immune responses limits the current clinical applications to few areas (Hartman et al., 2008), for which is descriptive that the majority of Ad clinical trials involve the treatment of cancer and cardiovascular diseases (Edelstein et al., 2007). Moreover, the application of Ad (and other viruses) in cancer therapy is more straightforward than in indications where long-term and regulated transgene expression is required. This has also led to the establishment of conditionally replicating oncolytic Ad vectors applicable to cancer therapy (Jounaidi et al., 2007), and gutless helper-dependent Ad vectors to minimize vector toxicity and for retargeted vector tropism (see Chapter 2.2) (Brunetti-Pierri and Ng, 2008; Campos and Barry, 2007; Seiler et al., 2007).

2.1.3 Retro- and lentiviruses

Retroviruses are lipid-enveloped particles encompassing a homodimer of linear, positive-sense, single-stranded RNA genomes of 7 to 11 kb. Following entry into target cells, the RNA genome is reverse-transcribed into linear double-stranded DNA and randomly integrated into the host cell chromatin. The tandem *gag*, *pol* and *env* genes encoding the structural proteins, nucleic-acid polymerases/integrases, and surface glycoproteins, respectively, are framed by two *cis*-acting long terminal repeat (LTR) sequences. In addition to these, lentiviruses encode two regulatory genes, *tat* and *rev*, and a variable set of accessory genes (Biffi and Naldini, 2005; Naldini and Verma, 2000). Both retro- and lentiviruses characteristically establish a well-tolerated chronic infection, which may incite latent diseases extending from malignancy to immunodeficiency (Biffi and Naldini, 2005; Kay et al., 2001).

To date, retroviruses are the most widely used vector system along Ad in gene therapy clinical trials (Edelstein et al., 2007). The location of most *cis*-acting sequences in the terminal regions of the genome allows insertion of heterologous DNA of up to 8 kb in place of the viral genes, which are provided *in trans* (Biffi and Naldini, 2005). This split construct design improves the biosafety of the vector by reducing the risk of recombination to reconstitute a replication-competent genome (Chong et al., 1998; Otto et al., 1994). The retroviral vector development has been intense, for instance, self-inactivating and self-activating vectors have been engineered (Blesch, 2004). Furthermore, retroviral vectors are able to efficiently integrate into the genome of the target cells, facilitating stable expression and maintenance of the transduced gene in a self-renewing tissue and in the clonal outgrowth of a stem cell (Naldini and Verma, 2000). However, the nuclear entry and productive transduction strictly depend on the mitotic disruption of the nuclear membrane and target cell division (Miller et al., 1990; Roe et al., 1993), limiting their therapeutic applicability. Lentiviruses, on the other hand, rely on active nuclear transport (Bukrinsky and Haffar, 1999), which enables also the transduction of non-

dividing cells such as neurons (Naldini et al., 1996). Indeed, a lentivirus vector pseudotyped with vesicular stomatitis virus (VSV) G-protein (VSVG) with an expanded tropism originally spurred the application of lentiviruses for gene therapy (Naldini et al., 1996). Many of the current lentivirus vectors used in gene therapy are based on HIV-1 (Biffi and Naldini, 2005; Naldini and Verma, 2000; Vigna and Naldini, 2000). The inability of high-titer vector production and safety concerns limit the use of lentivirus vectors despite the engineering of packaging cell lines and replication-deficient viruses.

2.1.4 Adeno-associated viruses

AAVs belong to the family of *Parvoviridae* and the genus Dependovirus as the virus is dependent on the coinfection of an unrelated helper virus (e.g. Ad or herpesvirus) for productive infection. AAV is emerging as one of the leading gene therapy vectors owing to its non-pathogenicity and low immunogenicity, stability, and the potential to integrate site-specifically without known side-effects (Buning et al., 2008; Smith, 2008). To date, 14 serotypes and multiple variants have been described, each of which contain a single-stranded DNA genome of approximately 5 kb, which is packaged into an icosahedral, non-enveloped capsid (Buning et al., 2008; Van Vliet et al., 2008). The genome can be divided into three functional regions: two open reading frames (ORF; *rep* and *cap*) and the ITRs. The *rep* ORF encodes a family of multifunctional nonstructural proteins, while the *cap* ORF codes for the three capsid proteins VP1, VP2 and VP3, which share most of their amino acid sequences except for the N-terminus (Gigout et al., 2005; Van Vliet et al., 2008). The ITR sequences are the solely required *cis* elements for viral genome replication and its packaging into viral particles, whereas the structural and non-structural proteins can be deleted from the recombinant AAVs and provided *in trans* (Buning et al., 2008). Differences of the capsid protein sequence of the various AAV serotypes result in the use of different cell surface receptors for cell entry by receptor-mediated endocytosis (Asokan et al., 2006; Sanlioglu et al., 2000).

A portfolio of recombinant AAV vector types has been developed with the aim of optimizing efficiency, specificity and thereby also the safety of *in vitro* and *in vivo* gene transfer (Alexander et al., 2008; Buch et al., 2008; Buning et al., 2008; Maheshri et al., 2006; McCarty, 2008; Mueller and Flotte, 2008). Recent clinical trials have also shown promising results (Mueller and Flotte, 2008). The vast majority of past and current clinical trials employ AAV vectors based on serotype 2. Most of these applications have focused on the treatment of monogenic disorders and cancer using local vector application or AAV-modified cells, respectively (Mueller and Flotte, 2008). Despite the impressive longevity of transgene expression obtained with AAV-2, its application has been limited because of low levels of transgene expression. Blocks at the level of vector entry and post entry processing contribute to these inefficiencies. Progress in overcoming these barriers as well as vector readminstration has been made through the development of vectors based on other serotypes, which have shown superior transduction efficiencies for various tissues *in vivo*

(Buning et al., 2008). Recently, the large scale production of AAV-2 in insect cells using suspension cell cultures and baculovirus expression vectors has been described (Aucoin et al., 2006; Huang et al., 2007). The particles produced in the insect cells are indistinguishable from those produced in mammalian cells, and particle yields are significantly higher.

2.1.5 Other viruses

In addition to the most extensively exploited viral vectors described above, vectors derived from e.g. herpesviruses, alphaviruses, and poxviruses with distinct benefits and shortcomings are being employed in, or developed for diverse gene therapeutic applications. For example, the herpes simplex virus type 1 (HSV-1) is a human neurotropic virus, and therefore interest has largely focused on using HSV-1 as a vector for gene transfer to the nervous system. Furthermore, because of latency, HSV vectors are highly attractive for application areas where life-long sustained transgene expression is desirable (Burton et al., 2005). Also the high preference of expression in neuronal cells by alphaviruses such as Semliki Forest virus (SFV) has led to application of these vectors in neuroscience, and the broad host range has additionally facilitated studies on gene expression and function (Lundstrom, 2005). The attractive properties of Simian Virus (SV40), a polyomavirus, include high-titer replication, infectivity of most dividing or resting cell types, potential for integration into the genome of the host cell, a peculiar pathway for entering cells that bypasses the antigen processing apparatus, high stability, and the ability to activate the expression of its own capsid genes *in trans* (Strayer et al., 2005). SV40-derived vectors have been applied to inhibit HIV, hepatitis C virus and other viruses, correction of inherited hepatic and other protein deficiencies, immunizing against lentiviral and other antigens, treatment of inherited and acquired diseases of the central nervous system, protecting the lung and other organs from free radical-induced injury, for example (Strayer et al., 2005).

The prototype of poxvirus family, vaccinia, possesses many features necessary for an ideal viral backbone for use in oncolytic virotherapy of cancer. In addition to the non-pathogenic nature, these include a short lifecycle with rapid cell-to-cell spread, strong lytic ability, a large cloning capacity, and well-defined molecular biology (Shen and Nemunaitis, 2005). The inherent ability of certain RNA viruses, such as VSV, measles virus, Newcastle disease virus (NDV), influenza virus A, and reovirus, to replicate selectively in tumor cells has also been exploited for cancer therapy (Guo et al., 2008). The defective interferon signaling pathway in tumor cells allows the tumor-specific replication of both NDV and VSV. Naturally attenuated strains of NDV have given impressive antitumor effects in a range of cancers including glioma, colorectal cancer, and breast cancer. Reovirus replication targets tumor cells as a consequence of constitutive activation of the Ras oncogene pathway. Data from early clinical trials suggest that intratumoral injection of reovirus in patients with glioma or prostate cancer is safe, and a number of clinical trials are currently underway (www.oncolyticsbiotech.com).

2.2 Targeted gene delivery by viral vectors

Targeted gene therapy of malignancies can be achieved through targeted gene delivery (transductional targeting) or targeted gene expression (transcriptional targeting) (Galanis et al., 2001; Sadeghi and Hitt, 2005; Waehler et al., 2007). The latest advancements of transcriptional targeting have highlighted the potential of exploiting the differential expression profiles of microRNAs in the target cells to exclude transgene expression (Brown et al., 2006; Brown et al., 2007a; Brown et al., 2007b) or vector replication (Chang et al., 2008; Edge et al., 2008; Ylosmaki et al., 2008) in non-target cells. The properties of such microRNA-regulated conditionally acting vectors may prove particularly useful for example in optimizing the gene expression of oncolytic viruses for maximal antitumor activity and safety. Although transcriptional targeting may reduce or even eliminate potential toxic side effects of the transgene or the vector, it does not address the need to avoid those resulting from the mislocalization of the vector particles. This chapter will therefore focus on the transductional targeting in the context of viral vectors.

Since viruses did not evolve to serve human therapeutic needs, many of their properties such as safety, efficiency, and capacity for targeted gene delivery require considerable improvement. Vector engineering at the molecular level is generally challenging since viruses are highly complex biological entities, and the capsid and/or envelope proteins that mediate their gene delivery play also a critical role in their life cycle (Galanis et al., 2001; Waehler et al., 2007). The natural tropism of some viruses is compatible with their utility as vectors. In most cases, engineering of the vector is required in order to acquire a new tropism especially for systemic targeting. Promising targeting methodologies have been developed for some viral vector systems, particularly Ad and AAV vectors (Campos and Barry, 2007; Nicklin and Baker, 2002), but progress towards targeting of retrovirus vectors has been slower and more challenging, despite enticing results in some lentivirus systems (Sandrin et al., 2003; Strizki, 2008). Transductional targeting methodologies developed for viral vector systems are described below and summarized in Table 2.

2.2.1 Pseudotyping

Pseudotyping (Table 2) refers to phenotypic mixing, in which the natural envelope or capsid proteins of the virus are modified, replaced, or expressed with the surface proteins from a donor virus. In this way the host range of virus vectors can be expanded or altered. This approach has been most extensively used to modulate the host-cell tropisms of retro- and lentiviral vectors (Cronin et al., 2005; Schnierle et al., 1997). VSVG is among the first and still most widely used glycoproteins for pseudotyping due to the very extensive tropism and stability of the resulting pseudotypes (Burns et al., 1993). In addition to retro- and lentiviruses (Croyle et al., 2004; Emi et al., 1991; Guibinga et al., 2004; Schnitzer et al., 1977), both native and modified VSVG have extensively been

applied for Ad (Yun et al., 2003), herpesvirus (Anderson et al., 2000; Tang et al., 2001), as well as baculovirus (Barsoum et al., 1997; Kitagawa et al., 2005; Mangor et al., 2001; Park et al., 2001; Pieroni et al., 2001; Tani et al., 2001).

While the VSVG–pseudotyped vectors are valuable for many diverse studies, their wide tropism may contribute to toxicity and serious adverse effects through transduction of non-target cells (Cronin et al., 2005). Structurally stable lentiviral vectors pseudotyped with baculovirus envelope glycoprotein GP64 can be produced at similar titers to VSVG with no associated cytotoxicity (Kumar et al., 2003). These vectors can mediate efficient transduction of several cell types except the cells of hematopoietic origin (Schauber et al., 2004). Furthermore, the utility of feline immunodeficiency virus (FIV) pseudotyped with GP64 has been demonstrated for targeting of hepatocytes and nasal epithelium (Kang et al., 2005; Sinn et al., 2005; Sinn et al., 2007). The concept of pseudotyping has also been extended to the incorporation of host-cell viral receptors into viral envelopes for targeted entry into cells expressing viral envelope glycoproteins (Endres et al., 1997; Kitagawa et al., 2005; Schnell et al., 1997; Somia et al., 2000). Pseudotyping of non-enveloped vectors, including AAV and Ad, has mainly been achieved by substituting coat proteins with homologous proteins of other related serotypes (Waehler et al., 2007). Furthermore, the usage of prokaryotic-eukaryotic hybrid vectors piggybacks onto a prokaryotic vector displaying a eukaryotic ligand, which facilitates the binding and entry into mammalian cells. One such example is a tumor-targetable phage, which displayed the RGD peptide on its surface and comprised the ITRs of AAV (Hajitou et al., 2006).

2.2.2 Adaptor-based targeting strategies

In adaptor-based targeting (Table 2), a bifunctional bridging agent recognizing both the virus and a specific cell surface molecule interacts with the viral vector and directs the vector to the targeted cell population. This approach is highly flexible and can be applied even with an incomplete knowledge of the viral structure. Since the molecular bridge is not covalently linked to the vector particle, the complex is prone to dissociation in the blood stream following intravenous administration, thus limiting its applicability. Furthermore, transduction efficiencies are relatively low with this strategy (Waehler et al., 2007). Therefore, the adaptor-based systems have proved particularly useful for proof-of-principle preclinical studies, facilitating simple testing of several target receptors (Parrott et al., 2003). Most adaptors can achieve the central objectives of targeted delivery: ablating the native tropism and conferring a novel tropism towards the desired target. Molecules successfully used for this strategy include receptor-ligand complexes (Pereboev et al., 2004; Verheije et al., 2006), polymers such as polyethylene glycol (PEG), bispecific antibodies (Duval et al., 2008), and high-affinity (strept)avidin-biotin molecular bridges.

For example, fusion of the ectodomain of CAR to a single-chain antibody (scFv) against human carcinoembryonic antigen allowed vector targeting to subcutaneous tumors as well as hepatic metastases of colon cancer in nude

mice, while simultaneously ablating liver tropism (Li et al., 2007). PEG-derived polymers, in parallel, have been used to couple non-enveloped vectors such as Ad (Lanciotti et al., 2003), and enveloped viruses such as VSV and baculovirus (Croyle et al., 2004; Kim et al., 2007) to ligands targeting cancer cells. Importantly, PEGylation has the potential to shield the vector from the innate immune system *in vivo* (Mok et al., 2005), and may allow transduction in the presence of vector antibodies (Eto et al., 2005). A combined strategy of genetic (see Chapter 2.2.3) and chemical modification of Ad capsid enabled flexible and efficient de- and retargeting of the vectors (Kreppel et al., 2005). The earliest application of the avidin-biotin technology (Laitinen et al., 2007) in viral targeting was demonstrated with an ecotropic retroviral vector in 1989 (Roux et al., 1989). Since then, the methodology has been further expanded to other vector systems by display of a biotin-acceptor peptide (BAP) on AAV, adeno-, and baculoviral vectors (Arnold et al., 2006; Kaikkonen et al., 2008; Parrott et al., 2003). The BAP is metabolically biotinylated during vector production by an endogenous or expressed biotin ligase, and can therefore be coupled to a biotinylated targeting ligand via avidin or -related proteins. In addition to targeting, this strategy is convenient for vector purification (Campos et al., 2004; Campos and Barry, 2006; Kaikkonen et al., 2008; Parrott et al., 2003). A combined strategy is to display an immunoglobulin (Ig) binding domain, such as the Z-domain of *Staphylococcus aureus* protein A, on the viral surface as a genetic fusion to the coat protein, and then to utilize monoclonal antibody to crosslink the vector with the target cell (Gigout et al., 2005; Korokhov et al., 2003; Ohno et al., 1997; Tai et al., 2003). Albeit potentially sensitive to competition by the polyclonal Igs present in the blood serum, this approach has been successfully used *in vitro* and in SCID mice (Morizono et al., 2005). Finally, artificially enveloped Ad vectors prepared by self-assembly of lipid bilayers around the Ad capsid have recently been described. Using cationic, neutral, fusogenic, and PEGylated lipids and maintaining a particle size of less than 200 nm, this strategy blocked the native tropism of Ad, extended blood residence time, and enhanced tumor targeting. Moreover, the PEGylated lipid-enveloped Ad was capable of specifically delivering genes via the systemic circulation to subcutaneously implanted solid tumors (Singh et al., 2008).

2.2.3 Genetic targeting strategies

To circumvent the potential complexities of adaptor systems, incorporation of targeting ligands into viral vectors by genetic engineering provides homogenous retargeted vector particles and facilitates high-titer production by eliminating the need to create a separate adaptor molecule (Table 2). Despite being technically more challenging, such single component systems have the additional advantage of overcoming the regulatory issues of two-component systems (Waehler et al., 2007). This approach was pioneered in 1993 by the display of a single-chain hapten antibody on the surface of murine leukemia virus (MLV) (Russell et al., 1993). Since then, retargeting of viral transduction by display of single-chain antibodies has been demonstrated with several

TABLE 2 Targeting methodologies of viral vectors. Modified from Waehler et al., 2007.

Approach	Principle	Advantages	Disadvantages
Pseudotyping	Use of a heterologous viral binding protein	Easy if the biology is supportive or compatible	Limited availability of pseudotypes
Adaptor systems	Use of a molecule that binds both the vector and target cell receptor	Limited knowledge of vector structure is sufficient; flexibility; minimal/no change in vector structure easy testing of ligands	Two-component system; adaptor may dissociate *in vivo*; separate production of the two molecules; stoichiometry
- Receptor–ligand	A native viral receptor is fused with the targeting ligand	Easy testing	Testing of the correct folding of each receptor-ligand
- Bispecific antibody	Use of an antibody with a specificity for the vector and the target	Antibody easy to engineer; screening for different targets is readily possible	Variation in the binding affinity of the targeting complex to the vector
- Chemical linkage	Targeting moiety is chemically bound to the vector	No adaptor dissociation from the vector; covalent linkage	Technically demanding
- Avidin–biotin	Avidin or biotin is coupled to the vector then bound to the biotin /avidin-ligand complex	High-affinity binding; easy vector purification	Possible toxicity
- Antibody binding	Antibody binds to a genetically incorporated Ig binding domain of the vector	Vast pool of available antibodies; easy coupling	Interference by the antibodies present in serum
Genetic systems	Genetic engineering of the vector for incorporation of the targeting (poly)peptide	Single-component system; clinical application; high-titer vector production	Can be detrimental to vector or ligand structure
- Serotype switching	Use of a different serotype	Biological compatibility	Limited availability of serotypes
- Small targeting motifs	Insertion of small peptides into virions	Minimal disturbance of vector structure	Broadens tropism without ablating native tropism
- Single-chain antibody	Incorporation of a single-chain antibody into the vector	Vast pool of targeting antibodies available	Adaptation to the pathway of viral protein production
- Mosaic viral attachment proteins	Two viral proteins are combined, allowing targeting, production or imaging in parallel	Multifunctionality of the virion	Optimal stoichiometry difficult to achieve
- Ablation of native tropism	Mutation of the amino acids responsible for the native tropism	Compatible with other approaches	May interfere with vector production in a packaging cell line

vectors including AAV (Yang et al., 1998), Ad (Hedley et al., 2006), retrovirus (Chowdhury et al., 2004), measles virus (Nakamura et al., 2005), and HSV, highlighting the versatility and flexibility of this approach. As the glycoproteins of enveloped vectors are routed through the endoplasmic reticulum (ER), which supports the folding and post-translational modification of complex proteins fused to the envelope, polypeptide ligands with multiple disulphide bonds, stringent glycosylation requirements or oligomeric structures can be more readily displayed on enveloped viruses than on non-enveloped viruses. Numerous complex polypeptide ligands, including growth factors and cytokines, have hence been successfully displayed on various enveloped viruses (Waehler et al., 2007). Furthermore, as targeted virus attachment does not necessarily lead to targeted entry, inverse targeting strategies have been developed, in which the viral envelope glycoprotein is modified to selectively destroy its binding and internalization into cells expressing a targeted receptor (Fielding et al., 1998). Alternatively, viral infectivity can be engineered to depend on the proteolytic maturation of a viral surface protein, thus selectively reactivating the inhibition imposed by inverse targeting (Sandrin et al., 2003; Szecsi et al., 2006). The feasibility of transductional targeting *in vivo* using retro- and lentiviral vectors displaying genetically incorporated polypeptide ligands has been demonstrated in several studies. Rexin-G, a pathotropic retroviral vector displaying a von Willebrand factor-targeting motif and expressing a dominant negative cyclin G1 gene, is targeted to the extracellular matrix of tumor tissue (Gordon et al., 2001), and represents the first and the only targeted vector that has been tested for its antitumor activities in three clinical studies (Gordon et al., 2006).

The introduction of large targeting proteins may impede the correct folding of the incorporated polypeptide or the viral protein, into which they are inserted, while the display of short peptide motifs are less likely to perturb the structure of the display scaffold. The targeting characteristics of a vector can be dramatically altered despite the small size of the peptide ligand (see Chapter 2.2.4). Finally, a significant progress has been gained in approaches that mimic the mechanisms, by which viruses arose in the first place. Accordingly, library-based selection and directed evolution, based on the multiple rounds of library construction and screening for iterative improvement of function, have strong potential in introducing novel properties into virus vectors (Jang et al., 2007; Maheshri et al., 2006). Collectively, the development of targetable and injectable vector will determine the success of a number of different gene therapy systems. To date, major clinical experience has been gained with viral vectors (Edelstein et al., 2007), but the biosafety of the modification is still disputable. Importantly, the ongoing and initiating clinical studies with targeted vectors can gain regulatory approval from the Food and Drug Administration (FDA) in the USA (Gordon et al., 2006), and as most western nations are connected to FDA procedures, this approval represents an encouraging sign for targeted therapies worldwide.

2.2.4 Tumor targeting by homing peptides

Tumor vasculature expresses a number of biochemically distinct molecular markers that are absent or differ from those in the blood or lymphatic vessels of normal tissues (Adams and Alitalo, 2007; Ruoslahti, 2002; Ruoslahti, 2005). Several peptides and antibodies that recognize tumor-specific vascular signatures have been provided by novel methods such as *in vivo* screening of phage libraries (Arap et al., 1998; Pasqualini and Ruoslahti, 1996), revealing extensive heterogeneity in tumor blood vessels and lymphatics. Only a few receptors, although representing a diverse population, have been characterized for these peptides. Also the presence of specialized lymphatic vessels in tumors has recently been discovered, providing an alternative object for targeted therapies (Adams and Alitalo, 2007; Laakkonen et al., 2008). Examples of recently identified homing peptides and their exploitation for targeting of viral vectors are presented below.

Small molecular weight peptides have the potential for enhancing the targeting of compounds, and they may also have therapeutic effects by themselves. Target cell-specific delivery of viral vectors can be improved by peptides that penetrate the cell membrane or alternatively induce receptor-mediated endocytosis. Proof for the vasculature-targeted delivery principle has been obtained in studies with experimental tumors. Several viral gene therapy vectors have been genetically modified to display homing peptides on their surface. The tumor homing-peptide RGD, targeting αVβ3 integrin (Arap et al., 1998), represents today the most often applied peptide for targeting purposes. RGD-mediated tumor targeting has been achieved *in vitro* for AAV (Stachler and Bartlett, 2006), retrovirus (Gollan and Green, 2002), and baculovirus (Ernst et al., 2006; Matilainen et al., 2006), and *in vivo* for Ad (Haviv et al., 2002) as well as phage–AAV hybrid vector (Hajitou et al., 2006). Of note, an RGD-modified conditionally replicating Ad (Bauerschmitz et al., 2002) is soon to be used clinically for local applications in ovarian carcinoma at the University of Alabama at Birmingham (USA) following the recent completion of animal safety tests (Page et al., 2007). In an non-viral approach, RGD and CNGRC targeting peptides coupled with doxorubicin, a compound that inhibits angiogenesis in addition to being toxic to tumor cells, yielded compounds that were more effective and less toxic than doxorubicin alone (Arap et al., 1998). Moreover, compounds that use peptides for targeting and pro-apoptotic peptides as drug components have been developed (Arap et al., 2002; Ellerby et al., 1999). A lung-homing peptide has been linked to a neutralizing Ad antibody adaptor molecule to target Ad vectors to membrane dipeptidase, a receptor specifically expressed in the lung endothelium and epithelium (Trepel et al., 2000). Additionally, Ad vectors have been specifically targeted to endothelial cells using an endothelium-specific peptide linked to a neutralizing monoclonal Ad antibody. The same peptide was also directly incorporated into the capsid of AAV, resulting in enhanced transduction of endothelial cells *in vitro* (Nicklin et al., 2001). Moreover, *in vitro* retargeting and transduction of moloney MLV to stimulated human endothelial cells was accomplished by integrating tumor-

vasculature targeting peptides into the viral envelope (Liu et al., 2000). In conclusion, promising results have been obtained from the preliminary attempts to direct drugs and viral vectors using targeting peptides that home to tumor vasculature and tumor cells. However, improved versions of these peptides and their viral or non-viral conjugates could be developed as the identity of the receptors for the peptides will be uncovered.

LyP-1, a cyclic nonapeptide (CGNKRTRGC) with a targeting specificity to lymphatic vessels in certain tumors, was identified in a combined *ex vivo* and *in vivo* phage display screen (Laakkonen et al., 2002). Intravenously injected phage displaying the LyP-1 was shown to home to human MDA-MB-435 breast carcinoma and KRIB osteosarcoma tumors *in vivo*. Both the LyP-1 phage and fluorescein-conjugated LyP-1 peptide colocalized with markers expressed in the lymphatic endothelia, but not with the markers of blood vessels. In addition to the primary tumors, the peptide recognized their metastases, was internalized by tumor cells and tumor lymphatic endothelial cells, and induced cell death both *in vitro* and *in vivo* (Laakkonen et al., 2002; Laakkonen et al., 2004). The mitochondrial/cell-surface protein p32 or gC1q receptor was recently identified as the receptor for LyP-1 (Fogal et al., 2008).

F3, a linear, highly basic 31-amino acid fragment (KDEPQRRSAR LSAKPAPPKP EPKPKKAPAKK) of the high mobility group protein 2 (HMGN2), was originally identified by *in vivo* screening of phage displayed cDNA libraries for homing to human leukemia cell xenograft tumors (Porkka et al., 2002). The peptide is encoded by exons 3 and 4 of HMGN2 and corresponds to the nucleosomal binding domain of the protein (Porkka et al., 2002). F3 recognizes a variety of tumor types by homing to tumor vasculature followed by binding to and accumulation within both tumor endothelial cells and tumor cells, and final transport to the nuclei of these target cells (Porkka et al., 2002). The binding and internalization of the peptide is mediated by cell surface nucleolin present on the surface of actively growing cells (Christian et al., 2003). As both F3 and intravenously injected nucleolin antibodies specifically homed to tumor vessels and angiogenic vessels, nucleolin was identified as a marker of endothelial cells in angiogenic blood vessels (Christian et al., 2003).

CGKRK, a basic linear peptide identified in a combined *ex vivo* and *in vivo* phage display screen, homes to tumor neovasculature and to dysplastic skin vasculature (Hoffman et al., 2003). The CGKRK peptide coupled to fluorescein was shown to home to endothelial cells in various transplant tumors localizing in the cytoplasm and nuclei of the target cells, and the homing appeared to be specific for a distinct state of the dysplasia. The peptide was reported to bind a range of tumor cells *in vitro*, and by the virtue of the overall positive charge (+3) of the peptide, the recognition of heparan sulphate or phosphatidylserine on the cell surface by CGKRK was suggested (Hoffman et al., 2003).

2.2.5 Transductional targeting of baculovirus

Potential gene therapeutic applications of baculovirus would significantly benefit from selective vector homing and entry to target cells *in vivo* as opposed

TABLE 3 Summary of baculovirus (*Ac*MNPV) displayed targeting motifs.

Strategy	Display platform	Reference
Pseudotyping		
GP120 of HIV-1	second copy of GP64	Boublik et al., 1995
Ectodomain of HIV-1 gp41	full-length and truncated GP64	Grabherr et al., 1997
E1 and E2 of rubella virus	second copy of GP64	Mottershead et al., 1997
GP64	GP64-positive baculovirus	Tani et al., 2001
Full-length VSVG	GP64-negative baculovirus	Mangor et al., 2001; Lung et al., 2002
Full-length VSVG	GP64-positive baculovirus	Barsoum et al., 1997; Facciabene et al., 2004; Kitagawa et al., 2005; Park et al., 2001; Pieroni et al., 2001; Tani et al., 2001, 2003
Truncated VSVG	GP64-positive baculovirus	Kaikkonen et al., 2006
F protein of *Lymantria dispar* and *Spodoptera exigua* MNPV	GP64-negative baculovirus	Lung et al., 2002
Measles virus receptors, CD46 and SLAM	GP64-negative baculovirus	Kitagawa et al. 2005
Hemagglutinin of influenza virus	truncated VSVG on GP64-negative baculovirus	Zhou and Blissard, 2008
Conjugate-based strategies		
Biotin mimic streptagII	native GP64	Ernst et al., 2000
Murine scFv for hapten 2-phenyloxazolone	second copy of GP64	Mottershead et al., 2000
Human scFv for carcinoembryonic antigen	second copy of GP64	Mottershead et al., 2000 Ojala et al., 2001
IgG binding Z/ZZ domains of protein A	second copy of GP64	Mottershead et al., 2000 Ojala et al., 2001
Avidin	second copy of GP64	Raty et al., 2004
IgG binding Z/ZZ domains of protein A	truncated VSVG	Ojala et al., 2004
Biotin acceptor peptide	native and second copy of GP64, truncated VSVG	Kaikkonen et al., 2008
Poly(ethylene glycol) in combination with folate	GP64-positive baculovirus	Kim et al., 2007
Display of targeting peptides		
ELDKVA of HIV-1 GP41	native GP64	Ernst et al., 2000 Spenger et al., 2002
RKK	green fluorescent protein fused to the second copy of GP64	Riikonen et al. 2005
RGD of coxsackie virus A9 and parechovirus VP1	second copy of GP64	Matilainen et al., 2006
RGD of foot-and-mouth disease virus VP1	native and second copy of GP64	Ernst et al., 2006
EDPGFFNVEI of Epstein-Barr virus GP 350/220	second copy of GP64	Ge et al., 2007

to the broad tropism that baculovirus naturally possesses. To restrict vector entry and transgene expression only to the cells of interest, effort has been devoted to improve the specificity of baculoviral vectors via incorporation of specific targeting mechanisms, both transductional (Makela and Oker-Blom, 2006) and transcriptional (Luz-Madrigal et al., 2007; Mahonen et al., 2007; Wang et al., 2006). The transductional targeting of baculovirus is challenged by the complexity and the large size of the virus, and the presence of the major envelope glycoprotein GP64, which determines the viral receptor preference and defines the transduction efficiency in mammalian cells (Duisit et al., 1999; Tani et al., 2001; Zhou and Blissard, 2008b). Albeit modification of the vector tropism has increased the specificity and efficacy of viral binding to target cells, only limited improvement in the internalization and gene transduction of the vectors has been achieved *in vitro*, and baculoviral targeting *in vivo* has not been demonstrated (Makela and Oker-Blom, 2006). Recent studies have also focused on elucidating the general behavior and biodistribution of baculovirus *in vivo* to better comprehend the kinetics of viral administration and to optimize the viral dose (Airenne et al., 2000; Kircheis et al., 2001; Lehtolainen et al., 2002; Raty et al., 2006; Raty et al., 2007; Tani et al., 2003).

The modification of baculovirus envelope for targeted or enhanced gene delivery, and the characteristics of baculovirus display platforms have recently been reviewed by me and my colleagues (Makela and Oker-Blom, 2006; Makela et al., 2007; Makela and Oker-Blom, 2008). The molecules used for pseudotyping and targeting of baculoviral vectors are summarized in Table 3 above. Briefly, the insertion of short tumor-targeting peptides and larger polypeptide-binding domains into the viral envelope has generally been conducted using the native GP64, an additional copy of GP64, or truncated VSVG as the display platform. Examples of molecules successfully used for adaptor-based targeting strategies of baculovirus include the high-affinity avidin-biotin molecular bridge, the IgG-binding domain of *Staphylococcus aureus* protein A, and PEGylation in combination with folate.

2.3 Viral entry by endocytosis

Endocytosis occurs via mechanistically diverse and highly regulated endocytic pathways, which can be roughly divided into two categories phagocytosis or "cell eating" and pinocytosis or "cell drinking". Phagocytosis mediates the internalization of large particles and is typically restricted to specialized mammalian cells, whereas pinocytosis involves the uptake of fluid and solutes in all cell types. At least four basic mechanisms including macropinocytosis, CME, caveolae-mediated endocytosis, and clathrin- and caveolae independent endocytosis have been distinguished for pinocytosis (Conner and Schmid, 2003; Polo and Di Fiore, 2006). Yet, novel endocytic pathways differing from the traditional classifications await their discovery.

Viruses have evolved a variety of means to deliver their genes and accessory proteins into their host cells (Figure 4), and have emerged as powerful tools to study membrane transport pathways (Le Blanc et al., 2005; Marsh and Helenius, 2006; Smith and Helenius, 2004). Several mechanisms of viral internalization, differing in the size of the endocytic vesicles, the nature of the cargo, and the mechanism of vesicle formation, has been described (Marsh and Helenius, 2006; Smith and Helenius, 2004). The great diversity of endocytosis pathways have been uncovered using increasingly sophisticated imaging techniques, high-resolution electron microscopy and systems biology (Pelkmans et al., 2005; Pelkmans, 2005). The notion that viruses of similar families can enter via completely different pathways, or that viruses from different families can enter via similar pathways, can only be understood and predicted by systematic and comprehensive studies on the cell biology underlying viral entry (Damm and Pelkmans, 2006).

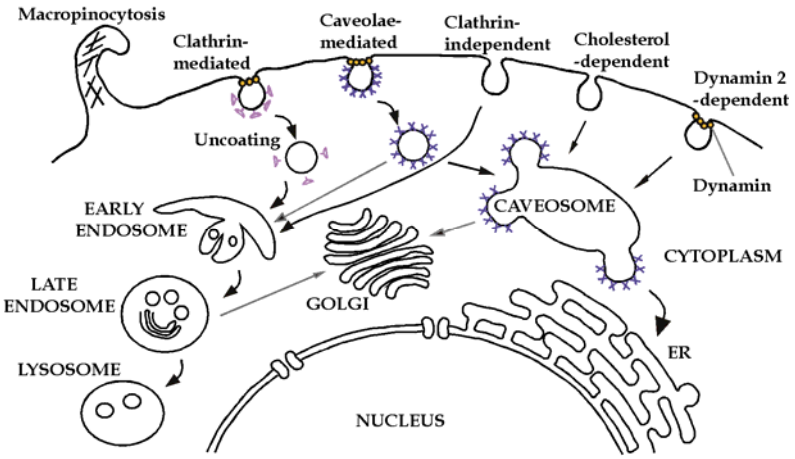

FIGURE 4 Endocytic pathways utilized by viruses. The pathways may differ in their dependence on clathrin, caveolin and dynamin, but may share the intracellular machinery. Reproduced with permission from Upla, 2008.

2.3.1 Phagocytosis

Phagocytosis of pathogens, such as bacteria, yeast and parasites, is primarily conducted by specialized cell types including macrophages, monocytes, dendritic cells, and neutrophils. This initiates the innate immune response, which in turn orchestrates the adaptive response (Aderem and Underhill, 1999). Phagocytosis is also crucial for clearing apoptotic cells and cell debris, both at the sites of inflammation and tissue damage, as well as during development (Aderem and Underhill, 1999). The phagocytic process comprises several sequential and complex events initiated by the recognition of the surface antigens of the pathogen by specific receptors on the phagocytic cells (Aderem and Underhill, 1999). The pathway is active and highly regulated by the Rho family GTPases, their activators, and downstream effectors to control the local

reorganization of the actin cytoskeleton and subsequent formation of membrane extensions for particle engulfment (Chimini and Chavrier, 2000; Hall and Nobes, 2000; Niedergang and Chavrier, 2004; Niedergang and Chavrier, 2005). Similar cellular protrusions have been described to be associated also with macropinocytotic events (Aderem and Underhill, 1999; Schnatwinkel et al., 2004). Of the Rho family GTPases, RhoA is generally required for complement-mediated phagocytosis, and Rac1 and/or Cdc42 typically are implicated in triggered phagocytosis (Caron and Hall, 1998). In contrast to macropinocytosis (see Chapter 2.3.2), phagocytosis requires dynamin-2, a ubiquitously expressed GTPase that has a critical role in the scission of forming clathrin-coated vesicles from the plasma membrane and in the formation of phagosomes (Conner and Schmid, 2003). Phagocytosis can be inhibited by over-expression of a DN dynamin-2 mutant (Gold et al., 1999), although it is not clear whether this reflects the role of dynamin in vesicle formation or in the regulation of the actin cytoskeleton (Conner and Schmid, 2003). In addition to professional phagocytes, a number of other cell types are able to engulf material by a phagocytic mechanism (Niedergang and Chavrier, 2005).

In addition to bacteria and parasites, phagocytosis or a phagocytosis-like mechanism has recently been identified as a novel pathway for viral entry. The mimivirus, a giant (750 nm), double-stranded DNA virus that grows in amoeba, was demonstrated to enter macrophages, but not non-phagocytic cells, by phagocytosis (Ghigo et al., 2008). The process involved the formation of cellular protrusions around the entering virus, reorganization of the actin cytoskeleton, dependence on dynamin-2, and the activation of phosphatidylinositol 3-kinases (PI3K) (Araki et al., 1996; Greenberg, 1999). Furthermore, HSV-1 was shown to enter non-phagocytic cells by a phagocytosis-like mechanism following association with plasma membrane protrusions. This process was regulated by RhoA, and involved rearrangement of actin cytoskeleton and trafficking of the virions in large phagosome-like vesicles (Clement et al., 2006).

2.3.2 Macropinocytosis

Macropinocytosis (Figure 4), which traps high quantities of macromolecules and extracellular fluid, encompasses a series of events initiated by extensive actin-driven plasma membrane reorganization or ruffling. Cellular protrusions at the site of membrane ruffling collapse onto and fuse again with the plasma membrane to form large, morphologically heterogeneous, non-coated vesicles called macropinosomes (Jones, 2007; Swanson and Watts, 1995). Macropinosomes are readily labeled with fluid-phase markers such as horse radish peroxidase (HRP), and are characterized by the presence of rabankyrin-5 (Schnatwinkel et al., 2004). The process is constitutive in some cell types, such as macrophages and dendritic cells, and the behavior of the forming macropinosomes resemble early endosomes by progressively maturing into late endosomes and finally fusing with the lysosomes (Racoosin and Swanson, 1993; West et al., 2000). In other cell types, macropinocytosis is pronounced following activation by growth factors that stimulates the formation of recycling

macropinosomes (Bryant et al., 2007; Nobes and Hall, 1995a; Nobes and Hall, 1995b; West et al., 1989). Maturation of macropinosomes occurs through the requirement of sorting nexins, a group of hydrophobic proteins regulating cargo trafficking in the endosomal system (Kerr et al., 2006). Like phagocytosis, macropinocytosis is highly dependent on the remodeling of subcortical actin filaments by the Rho family GTPases Rac1 and Cdc42 (Anton et al., 2003; West et al., 2000). Membrane localization of activated Rac1, reorganization of actin, as well as the activity of protein kinase C has been shown to be dependent on cholesterol (Grimmer et al., 2002). Other regulators include the p21-activated kinase-1 (Pak1), that controls the process downstream of Rac1 and Cdc42 (Dharmawardhane et al., 2000), PI3K for the closure of macropinosomes as well as phagosomes (Araki et al., 1996; Araki et al., 2007), and Na^+/H^+-exchangers (Meier et al., 2002). This pathway is independent of receptors and dynamin-2 (Swanson and Watts, 1995), and can be inhibited by amiloride analogs (Kee et al., 2004; Meier et al., 2002; West et al., 1989). The actin-driven formation of large macropinosomes and phagosomes differs mechanistically from the involution of more selective plasma-membrane domains that give rise to smaller pinocytic vesicles (see below).

Interestingly, Ad serotype 2, vaccinia virus, and HIV-1 have been shown to utilize macropinocytosis in certain conditions to invade host cells (Liu et al., 2002; Locker et al., 2000; Marechal et al., 2001; Meier et al., 2002; Meier and Greber, 2004; Mercer and Helenius, 2008).

2.3.3 Clathrin-mediated endocytosis

CME (Figure 4) is a constitutively active process that occurs in all mammalian cell types. It is responsible for the continuous uptake of essential nutrients and their integral membrane receptors (Benmerah and Lamaze, 2007). For viral entry, it is the most frequently exploited of the endocytic pathways (Marsh and Helenius, 1989; Marsh and Helenius, 2006). Clathrin coated pits, of approximately 120 nm in diameter, are composed of a lattice-like assembly of clathrin. The pits form in response to an internalization signal, present in the cytoplasmic tail of the receptor, by clathrin assembly on the cytoplasmic face of the plasma membrane. The cytoplasmic domains of the receptor are recognized by the adapter protein complex AP-2 and by several other adapter molecules and accessory factors that operate in the assembly of clathrin-coated pits and the sorting of cargo molecules (Conner and Schmid, 2003). In addition to phagocytosis, caveolae-mediated endocytosis, and some clathrin- and caveolae-independent endocytic pathways (Figure 4), the GTPase dynamin mediates, in conjunction with actin polymerization, the invagination and pinching-off of the maturing clathrin-coated vesicle from the plasma membrane (Marsh and McMahon, 1999). This renders dynamin a master regulator of membrane trafficking events at the cell surface (Conner and Schmid, 2003). The release of the clathrin-coated vesicle is rapidly followed by uncoating and fusion of the vesicle with the early endosomes (pH 6.2), which are tubulo-vesicular structures responsible for the major sorting of the cargo either back to the

plasma membrane (the recycling pathway) or further to the late endosomes via multivesicular intermediates. Downregulated receptors and some pathogenic invaders are transported from the late endosomes to lysosomes for degradation by acid hydrolases (Gruenberg, 2003). There is increasing evidence that this targeting of viruses and other endocytosed cargo to specific populations of endosomes is a selective and highly regulated process (Kirkham et al., 2005). Late endosomes contain cisternal and vesicular regions, often have the appearance of multivesicular bodies, and differ from the early endosomes by their lower luminal pH (5.5-5.0) and discrete protein composition (Piper and Luzio, 2001). Distinct Rab GTPases coordinate the vesicular traffic typical to the individual endosomal compartments (Seabra and Wasmeier, 2004).

Viruses from diverse families including adenoviruses, rhabdoviruses (e.g. VSV), alphaviruses (e.g. SFV), orthomyxoviruses (e.g. influenza virus), SARS coronavirus, certain picornaviruses, and the non-enveloped mammalian reoviruses use CME for their entry (Marsh and Pelchen-Matthews, 2000; Marsh and Helenius, 2006; Pelkmans and Helenius, 2003). The internalization of virus-receptor complexes by CME is a rapid and efficient process leading to exposure of the virions to the acidic milieu of early and late endosomes within minutes after internalization. The acidic surroundings induce conformational changes in the surface architecture of the virion leading to penetration in a location that is dependent on the pH threshold of the virus. In certain cases the acidic pH alone is not sufficient to induce fusion, and proteolytic cleavage of viral proteins by acid-dependent endosomal proteases is required for the penetration (Marsh and Helenius, 2006; Pelkmans and Helenius, 2003).

2.3.4 Caveolae-mediated endocytosis

In recent years, various clathrin-independent pathways have been identified (Figure 4). Caveolae (50–80 nm) are flask-shaped, static invaginations of the plasma membrane that demarcate specifically ordered microdomains rich in cholesterol and sphingolipids (Anderson, 1998), also referred to as lipid rafts (Simons and Ikonen, 1997). The term lipid raft itself, however, does not specify a particular endocytic route (Pelkmans, 2005), and whether rafts are pre-existing structures in membranes that are self-organized by the aggregation of sphingolipids and cholesterol (Simons and Ikonen, 1997), or induced by the clustering of membrane proteins (Gaus et al., 2006), is controversial. The assembly of caveolar domains occurs in the Golgi complex, from which they are transported to the cell surface (Anderson, 1998). The lateral movement of caveolae at the cell surface is inhibited by a tight association with the cortical actin cytoskeleton (Tagawa et al., 2005). Caveolae and lipid rafts internalize cargo upon stimulation of signaling events by multivalent ligands such as non-enveloped viruses. For example, stimulation by the simian virus 40 (SV40) triggers a transient disintegration of actin filaments, granting space for the caveolar vesicles to internalize (Pelkmans et al., 2001; Pelkmans and Helenius, 2002; Pelkmans, 2005). Caveolins are integral membrane proteins with both N- and C-termini facing the cytoplasm. They are able to bind cholesterol and fatty

acids, and confer to the shape and structural organization of caveolae by self-association to form a striated coat on the membrane invaginations (Anderson, 1998). The formation of caveolae can be disrupted by the depletion of plasma-membrane cholesterol, the overexpression of DN caveolin mutants, and the knockout of caveolin expression (Drab et al., 2001; Razani et al., 2002).

Numerous signalling molecules are associated with caveolae, highlighting their role in the compartmentalization and regulation of specific signalling cascades (Anderson, 1998; Razani et al., 2002). The caveolae-mediated endocytosis is a slow (half-time ~20 min), cargo-triggered, dynamin- and actin-dependent process of low capacity. Internalized caveolae accumulate to intracellular structures called caveosomes (50–60 nm) with neutral pH and multiple flask-shaped domains enriched in caveolin-1. As exemplified with SV40, the virions are transported after a second activation step by caveolin-free, microtubule-dependent vesicles to the ER, where penetration occurs (Pelkmans et al., 2001). Caveosomes, in general, are poorly characterized, and differ from the classical endocytic and biosynthetic organelles by lacking appropriate markers such as Rab GTPases (Pelkmans et al., 2001; Pelkmans and Helenius, 2002; Pelkmans et al., 2005). In addition to SV40 (Pelkmans et al., 2001), certain coronaviruses, filoviruses, influenza viruses, and polyomaviruses utilize caveolae for functional entry (Marsh and Helenius, 2006). Recent evidence has also suggested the existence of caveolin-independent transport mechanisms from rafts to caveosomes (Figure 4) (Damm et al., 2005; Kirkham et al., 2005).

2.3.5 Clathrin- and caveolin-independent endocytosis

Caveolae represent just one type of cholesterol-rich microdomain of the plasma membrane. The mechanisms that govern other caveolae- and clathrin-independent endocytosis remain poorly understood, but domains rich in glycosylphosphatidylinositol (GPI)-anchored proteins are often associated with these processes. These pathways differ according to the cell type and in their dependence on regulators such as host-cell kinases, dynamin, Rac, Rab, and Arf-family GTPases, actin and tubulin, as well as cholesterol (Marsh and Helenius, 2006). For example, the internalization of GPI-anchored proteins into GPI-anchored protein-enriched early endosomal compartments (GEECs) and recycling endosomes is independent on dynamin, Arf6, and RhoA, but requires Cdc42 (Kalia et al., 2006; Sabharanjak et al., 2002). The interleukin-2 (IL-2) receptor pathway, on the other hand, is sensitive to cholesterol, is independent on dynamin, but is specifically regulated by RhoA (Lamaze et al., 2001).

In addition to caveolae-mediated entry (Pelkmans et al., 2001), SV40 has been shown to use a lipid raft-mediated pathway independent on dynamin and Arf6 for internalization into cells devoid of caveolin-1 (Damm et al., 2005). The viruses were internalized into small, tight-fitting vesicles and transported to membrane-bound, pH-neutral organelles resembling caveosomes but devoid of caveolin-1 and -2, and the pathway could be activated also in the presence of caveolin-1. Thus, in combination with bacterial toxins such as cholera, anthrax,

and Shiga toxin, viruses are emerging as valuable tools for charting the various ligand-inducible pathways of endocytosis (Marsh and Helenius, 2006).

2.3.6 Mechanism of baculovirus entry

2.3.6.1 Entry into insect cells

Baculoviruses have evolved a remarkable variety of invasion and infection strategies in their insect hosts. Cellular entry by BV of *Ac*MNPV occurs via receptor-mediated endocytosis (Volkman et al., 1984; Zhou and Blissard, 2008b) possibly through clathrin-coated pits (Long et al., 2006), while the ODV fuses directly with the plasma membrane at the cell surface (Granados and Lawler, 1981; Horton and Burand, 1993). For ODV, P74 (*i.e. per os* infectivity factor 0, PIF0), in addition to PIFs 1 and 2, plays an essential role in the primary infection, and mediates specific receptor binding of ODV to the primary target cells within the larval midgut (Haas-Stapleton et al., 2004; Ohkawa et al., 2005). For BV, the envelope glycoprotein GP64 serves two major roles during virus entry. Firstly, GP64 is involved in host cell receptor binding, and secondly, the protein mediates the low-pH-triggered membrane fusion activity necessary for release of the nucleocapsid into the cytosol during entry (Blissard and Wenz, 1992; Hefferon et al., 1999; Kingsley et al., 1999; Zhou and Blissard, 2008a). The *Ac*MNPV, a member of group I NPVs, also encodes and expresses a baculovirus F protein called *Ac*23, a functional homolog of GP64 present in group II NPVs (Lung et al., 2003). First data suggesting a role for the *Ac*23 protein in host cell binding was recently published (Zhou and Blissard, 2008b).

BV binds to insect cells in a saturable and competitive manner (Wickham et al., 1992). The receptor for BV attachment is yet to be identified, although prior studies have proposed the involvement of a specific cellular protein (Wang et al., 1997; Wickham et al., 1992). Additionally, bivalent cations (Wang et al., 1997), phospholipids (Tani et al., 2001), and the glycosylation state of GP64 (Jarvis et al., 1998) may have a role in the binding process. Monoclonal antibodies raised against GP64 capable of neutralizing viral entry at a step following viral binding have been generated (Hohmann and Faulkner, 1983; Keddie et al., 1989; Volkman and Goldsmith, 1985). Recently, antiserum raised against the N-terminal region of GP64 was able to inhibit also virion binding (Zhou and Blissard, 2008b). The region of amino acids 121 to 160 was identified to be important, and substitutions at amino acid positions 153 and 156 substantially reduced viral binding to insect cells, confirming an essential role for this N-terminal domain in receptor binding (Zhou and Blissard, 2008b).

After endosomal release, baculovirus induces actin polymerization. The transport involves a myosin-like motor, and the multiplicity of infection directly reflects the number of actin cables formed (Charlton and Volkman, 1993; Lanier et al., 1996; Lanier and Volkman, 1998). In contrast, microtubule depolymerization may be a necessary event in viral infection (Volkman and Zaal, 1990). The uncoating mechanism of the viral DNA is unknown, but the removal of stabilizing zinc ions is needed (van Loo et al., 2001).

2.3.6.2 Entry into mammalian cells

The binding of baculovirus and its entry into mammalian cells have been considered as universal phenomena. The principles of these interactions have been characterized, while the exact endocytic mechanism, route, and participating molecules enabling functional transduction are largely unidentified. As the virus is able to enter a vast variety of cell types, the receptor molecules for baculovirus attachment and uptake have been suggested to comprise common constituents of the cell membrane such as negatively charged phospholipids, heparan sulfate proteoglycans, or asialoglycoprotein receptor (Duisit et al., 1999; Hofmann et al., 1995; Tani et al., 2001). In parallel with insect cells (Zhou and Blissard, 2006), GP64 appears to play an essential role during baculovirus entry in mammalian cells as the monoclonal GP64 antibody, *Ac*V1, neutralizes virus-mediated transgene expression but not the cellular binding (Hofmann and Strauss, 1998; Tani et al., 2001). In support, the overexpression of GP64 on the viral envelope improves transduction and broadens the tropism of the virus (Tani et al., 2001), whereas a mutant virus lacking GP64 is unable to mediate successful transduction (Abe et al., 2005). Furthermore, GP64 of *Ac*MNPV is able to rescue the transduction of *Helicoverpa armigera* SNPV, a baculovirus inherently incapable of transducing mammalian cells (Liang et al., 2005). As GP64 is negatively charged under physiological conditions (Volkman and Goldsmith, 1984), additional proteins and/or phospholipids on the viral envelope may participate in the binding process (Duisit et al., 1999; Tani et al., 2001). Furthermore, viral entry may be conditionally dependent on the contact with the basolateral cell surface (Bilello et al., 2001; Bilello et al., 2003).

Baculovirus enters mammalian cells by endocytosis, which is followed by low endosomal pH-induced fusion of the viral envelope with the endosomal membrane and release of the nucleocapsids into the cytoplasm (Boyce and Bucher, 1996; Hofmann et al., 1995; Kukkonen et al., 2003; van Loo et al., 2001). The viruses accumulate into endosomes positive for the early endosomal antigen (EEA-1) in hepatocarcinoma cells starting at 30 min post transduction (p.t.) (Kukkonen et al., 2003; Matilainen et al., 2005). Consequently, drugs that interfere with the endosomal maturation such as bafilomycin A1, chloroquine, ammonium chloride, and monensin confine baculovirus into early endosomes inhibiting the cytoplasmic release (Boyce and Bucher, 1996; Condreay et al., 1999; Hofmann et al., 1995; Kukkonen et al., 2003; van Loo et al., 2001). The half-time of endosomal escape was estimated to be roughly 50 min in pig kidney cells (van Loo et al., 2001).

The mechanism of uptake has been suggested to involve CME as inhibition of viral transduction was observed in baby hamster kidney cells (BHK21) treated with chlorpromazine, which acts by shifting clathrin and the AP-2 complex to the late endosomal compartment (Long et al., 2006). In support, a DN form of Eps15 (epidermal growth factor receptor pathway substrate clone 15), which interferes with the formation of clathrin-coated vesicles, partially inhibits virus-mediated transgene expression (Long et al.,

2006). Despite early endosomal targeting and occasional virus attachment with plasma membrane-bound coated pits (Matilainen et al., 2005), no internalization of baculovirus into budded clathrin-coated vesicles has been documented. Moreover, the virus does not associate with transferrin or the recycling endosomal marker Rab11 (Matilainen et al., 2005). Instead, the high frequency of ruffles and the presence of large smooth-surfaced endosomes full of baculovirus have reflected the involvement of a more efficient entry pathway such as macropinocytosis (Matilainen et al., 2005). Also, a possible contribution of caveolae has been proposed as genistein, a drug that interferes with the formation of caveosomes, enhances baculovirus transduction (Long et al., 2006).

Until recently, the endosomal escape was generally assumed to confer the transductional block of baculovirus in certain cell lines (Boublik et al., 1995; Boyce and Bucher, 1996). The results obtained with VSVG-pseudotyped baculoviruses have further endorsed this assumption as both full-length and truncated VSVG improve baculovirus transduction by augmenting viral release from the endosomes (Barsoum et al., 1997; Kaikkonen et al., 2006). Alternatively, cytoplasmic trafficking or nuclear import of the nucleocapsids may compromise viral transduction (Kukkonen et al., 2003). As is evident with insect cells, viral nucleocapsids may stimulate the formation of actin filaments also in mammalian cells (van Loo et al., 2001). Disruption of the cytoskeleton with microtubule depolymerizing agents increases viral transduction and localization in the nuclei subsequent to viral inoculation or microinjection of the purified capsids into the cytoplasm, respectively. This suggests that intact microtubules constitute an obstacle for intracellular trafficking of baculovirus towards the nucleus, and that no modification of the viral capsid is required for successful nuclear entry (Salminen et al., 2005; van Loo et al., 2001).

Since the virus is able to transduce both dividing and non-dividing mammalian cells, the mode of baculoviral nuclear entry appears to be somewhat different from that of other DNA viruses in that the cigar-shaped nucleocapsids are apparently transported through the nuclear pore together with the viral genome (van Loo et al., 2001). The nuclear localization of the virus capsids is detectable starting at 4 h p.t. (Laakkonen et al., 2007; van Loo et al., 2001) in tandem with the transgene expression (Matilainen et al., 2005), and is independent of mitotic disintegration (Laakkonen et al., 2007). Baculovirus-mediated transduction efficiency can be notably improved by taking advantage of the histone deacetylase inhibitors such as sodium butyrate, trichostatin A, and valproic acid (Condreay et al., 1999; Hu et al., 2003), emphasizing the impact of hyperacetylation of baculovirus genome for activation of transcription and expression of foreign genes. Although no viral replication occurs, certain baculoviral immediate early genes, including *ie-0, ie-1, ie-2, pe38, gp64* and *p38*, are expressed at least at the mRNA level (Fujita et al., 2006; Kitajima et al., 2006; Liu et al., 2007; Laakkonen et al., 2007). This was shown to alter the expression profiles of mammalian genes but not the cellular physiology, while no evidence for the functional expression of viral genes exists at present (Fujita et al., 2006; Kenoutis et al., 2006; Liu et al., 2007).

3 AIMS OF THE STUDY

The BV of *Ac*MNPV has provided the foundation for the establishment and widespread utilization of baculovirus-based gene delivery vectors and the baculovirus display technology in multiple applications. The objectives of the present study were to extend the knowledge of the applicability of surface-modified baculoviral vectors for targeted gene delivery to human cancer cells, and to understand the molecular events underlying the mode and regulation of baculovirus uptake in human cancer cells.

The specific aims of the study were:

1. To engineer surface-modified baculovirus vectors for specific and enhanced gene delivery to human cancer cells by displaying an array of tumor-homing peptides on the viral envelope, and to analyze their targeting potential both *in vitro* and *in vivo*.

2. To characterize the interaction and gene delivery of ODV in human cancer cells, and to analyze the functionality of the ODV-specific P74 envelope protein as a platform for the display of heterologous peptides on the surface of ODV.

3. To elucidate the nature and regulation of baculovirus entry mechanism in human cancer cells to facilitate further development of viral targeting and gene delivery strategies.

4 SUMMARY OF MATERIALS AND METHODS

4.1 Cells and tumors (I-V)

E. coli strains JM109 and DH10Bac (Invitrogen, Carlsbad, CA) were grown in suspension cultures in Luria-Bertani medium supplemented with appropriate antibiotics at 37°C. *Spodoptera frugiperda* (*Sf*9; American Type Culture Collection, ATCC CRL-1711, Manassas, VA; GibcoBRL, Grand Island, NY) insect cells were maintained in monolayer and suspension cultures at 28°C using serum-free Insect-XPRESS culture medium (Cambrex, Walkersville, MD) without antibiotics. Human MDA-MB-435 (breast/melanoma) carcinoma (provided by Pirjo Laakkonen, University of Helsinki, Finland), and A549 lung carcinoma (ATCC CCL-185) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (all from Invitrogen). Human MDA-MB-231 breast carcinoma cells (ATCC HTB-26) were maintained in RPMI-1640 Medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin (all from Invitrogen). Human HepG2 hepatocarcinoma (ATCC HB-8065) and 293 embryonic kidney (ATCC CRL-1573) cells were maintained in Minimum Essential Medium (MEM) supplemented with 1% penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% FBS (all from Invitrogen). All human cell lines were grown in monolayer cultures in a humidified 5% CO_2 atmosphere at 37ºC.

Athymic, 4-6-weeks old female BALB/c *nu/nu* mice (Taconic Europe A/S, Ejby, Denmark) were maintained under specific pathogen-free conditions in a temperature- and humidity-controlled environment. Mice under anesthesia were injected with 2.5 x 10^6 tumor cells into the mammary fat pad to induce tumors and were used for viral targeting experiments at 8-12 weeks post injection. All animal studies were conducted according to the guidelines of the Provincial Government of Southern Finland and the protocol was approved by the Experimental Animal Committee of the University of Helsinki, Finland.

4.2 Antibodies (I-V)

Primary and secondary antibodies used in this thesis are described in Table 4.

TABLE 4 Primary and secondary antibodies used in this thesis.

PRIMARY ANTIBODIES				
Antibody	**Target**	**Source**	**Provider/Reference**	**Article**
B12D5	GP64 envelope protein	mouse	Dr. Loy Volkman; Keddie et al., 1989	I-V
p10C6	VP39 capsid protein	mouse	Dr. Loy Volkman; Whitt and Manning, 1988	I-V
N25-8C	P74 envelope protein	mouse	Dr. Loy Volkman; Faulkner et al., 1997	IV
*Ac*MNPV BV antiserum	*Ac*MNPV budded virus	rabbit	Dr. Max Summers; Braunagel and Summers, 1994	II-V
*Ac*MNPV ODV antiserum	*Ac*MNPV occlusion-derived virus	rabbit	Dr. Loy Volkman; Volkman, 1983	IV
P10	P10 protein	rabbit	M.Sc. Kirsi Pakkanen	IV
IgG	Z-domain of protein A	rabbit	Sigma Aldrich	IV
VSVGtag	VSVG, aa 497-511	rabbit	Sigma Aldrich	I
LAMP-2	lysosomal-associated membrane protein 2	mouse	Developmental Studies Hybridoma Bank	II, IV-V
LYVE-1	endothelial hyaluronic acid receptor-1	rabbit	Laakkonen et al., 2002	II
VEGFR-3	vascular endothelial growth factor receptor-3	goat	R&D Systems	II
CD31	blood vessels	rat	BD Biosciences Pharmingen	II
His-tag	His peptide	rabbit	Immunol. Cons. Laboratory	II
NCL3	nucleolin	rabbit	Christian et al., 2003	III-IV
TfR	transferring receptor	rabbit	Cymbus Biotechnology	III
Streptavidin	streptavidin	rabbit	Dr. Edward Bayer	III
Luciferase	Photinus pyralis luciferase	mouse	Serotec	V
9E10	myc peptide	mouse	American Type Culture Collection	V
EEA-1	early endosomal antigen-1	mouse	Transduction Laboratories	V
EEA-1	early endosomal antigen-1	mouse	Abcam	IV
Lamin A/C	nuclear lamin A/C	mouse	Novocastra laboratories	V
Flotillin-1	flotillin-1	mouse	BD Biosciences Pharmingen	V
Arf6	ADP-ribosylation factor 6	mouse	Thermo Fisher Scientific	V
RhoA	Ras homolog gene family member A	mouse	SantaCruz Biotechnology Inc.	V
Rac1	Ras-related C3 botulinum toxin substrate 1	mouse	Millipore	V
Tubulin	tubulin	mouse	Sigma Aldrich	V
M2	FLAG epitope	mouse	Sigma Aldrich	V
Dynamin-2	dynamin-2	rabbit	Dr. Mark McNiven	V
NTB	(IL-2):Cy3-561	rabbit	Dr. Alice Dautry-Varsat	V
Actin	actin	rabbit	Sigma Aldrich	V

SECONDARY ANTIBODIES				
Antibody	**Conjugate**	**Source**	**Provider/Reference**	**Article**
anti-mouse IgG	alkaline phosphatase	goat	Promega	I-IV
anti-rabbit IgG	alkaline phosphatase	goat	Promega	I, III-IV
anti-mouse IgG	horseradish-peroxidase	goat	Promega	V
anti-rabbit IgG	horseradish-peroxidase	goat	Promega	V
anti-mouse IgG	Alexa Fluor® 488 nm	goat	Molecular Probes	II-V
anti-rabbit IgG	Alexa Fluor® 488 nm	goat	Molecular Probes	II-V
anti-mouse IgG	Alexa Fluor® 546 nm	goat	Molecular Probes	V
anti-rabbit IgG	Alexa Fluor® 546 nm	goat	Molecular Probes	II-V
anti-rabbit IgG	Alexa Fluor® 555 nm	goat	Molecular Probes	I, IV
anti-rabbit IgG	Alexa Fluor® 594 nm	goat	Molecular Probes	I

4.3 Viruses (I-V)

The transfer vectors and recombinant baculoviruses used in this thesis are summarized in Tables 5 and 6, respectively. The viruses were produced according to the Bac-to-Bac® Baculovirus Expression System (Invitrogen) and propagated in *Sf*9 cells at a multiplicity of infection (MOI) of 0.5-1. Viral titers were determined by end-point dilution assay either from unconcentrated, concentrated (in PBS; 7000 x g, 7 h, 4°C), or concentrated and sucrose gradient purified (20-50% w/w, 100 000 x g, 1 h, 4°C) baculovirus stocks (I-V). The isolation of ODV from cell culture was conducted with standard protocols (Haas-Stapleton et al., 2004), and total protein concentrations were determined with NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at A280 nm using bovine serum albumin (BSA) as a reference (IV). The ODV preparations used were a mixture of ODVs containing single and multiple nucleocapsids per virion.

The pFastBac Dual (Invitrogen) and pBACcap-1 (Kukkonen et al., 2003) -based transfer plasmids were used as vector backbones to generate recombinant viruses. The cloning procedures are explained in detail in the original publications (I-V). Briefly, the LyP-1 (Laakkonen et al., 2002), F3 (Porkka et al., 2002), and CGKRK (Hoffman et al., 2003) tumor-homing peptides were displayed on the baculovirus envelope by fusion to the transmembrane (TM; residues 463-482) and cytoplasmic (CT; residues 483-511) domains of VSVG through a linker encoding twenty alanine residues (polyAla). The signal sequence of the baculovirus major envelope protein GP64 (GP64ss; residues 1-20) (Whitford et al., 1989) was utilized to direct the fusion proteins to the cellular and viral membranes upon expression. In addition, the F3 peptide and enhanced green fluorescent protein (EGFP) (Kukkonen et al., 2003) were displayed on the baculovirus capsid as N- and C-terminal fusions with the baculovirus major capsid protein VP39 (Thiem and Miller, 1989), respectively. The display of the red fluorescent protein (RFP, mCherry) was conducted by fusion to the baculovirus capsid protein P24 (Ayres et al., 1994). Two IgG-

binding Z domains (ZZ) of *Staphylococcus aureus* protein A (Nilsson et al., 1987) were N-terminally fused to an extra copy of the ODV-specific P74 envelope protein (Kuzio et al., 1989). The recombinant viruses were further equipped with expression cassettes encoding either firefly luciferase (luc) and/or EGFP, enabling the detection of transgene expression in insect and/or mammalian cells. The expression of the displayed fusion proteins and reporter genes was regulated either by the polyhedrin (polh) or P10 promoter, and the SV40 or cytomegalovirus (CMV) promoter, respectively.

TABLE 5 Donor vectors used for the generation of the recombinant viruses. See text for details.

Plasmid	**Promoter and insert**	**Restriction sites (5´/3´)**	**Article**
pP10SV40-luc	polh: –	–	I
	p10: SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
pP10CMV-EGFP	polh: –	–	II-III
	p10: CMV-EGFP	*Kpn*I/*Kpn*I	
pLyP-1-VSVG/luc	polh: GP64ss-LyP-1-polyAla-VSVGTM/CT	*Eco*RI/*Pst*I	I
	p10: SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
pLyP-1-VSVG/EGFP	polh: GP64ss-LyP-1-polyAla-VSVGTM/CT	*Eco*RI/*Pst*I	II
	p10: CMV-EGFP	*Kpn*I/*Kpn*I	
pF3-VSVG/luc	polh: GP64ss-F3-polyAla-VSVGTM/CT	*Eco*RI/*Pst*I	I
	p10: SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
pCGKRK-VSVG/luc	polh: GP64ss-CGKRK-polyAla-VSVGTM/CT	*Eco*RI/*Pst*I	I
	p10: SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
pVP39/luc/EGFP	polh: VP39-His	*Eco*RI/*Pst*I	III
	p10: SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
	CMV-EGFP	*Kpn*I/*Kpn*I	
pF3-VP39/luc/EGFP	polh: F3-VP39-His	*Eco*RI/*Pst*I	III
	p10: SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
	CMV-EGFP	*Kpn*I/*Kpn*I	
pF3-VSVG/F3-VP39/	polh: F3-VP39-His	*Eco*RI/*Pst*I	III
luc/EGFP	p10: gp64ss-F3-polyAla-VSVGTM/CTpolyA	*Xho*I/*Xho*I	
	SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
	CMV-EGFP	*Kpn*I/*Kpn*I	
pSV40-luc/CMV-EGFP/	polh: polyhedrin	*Eco*RI/*Pst*I	IV
polh	p10: SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
	CMV-EGFP	*Kpn*I/*Kpn*I	
pSV40-luc/CMV-EGFP/	polh: polyhedrin	*Eco*RI/*Pst*I	IV
polh/ZZP74	p10: ZZP74	*Sma*I/*Sma*I	
	SV40-luc-polyA-enhancer	*Xho*I/*Kpn*I	
	CMV-EGFP	*Kpn*I/*Kpn*I	
p24-RFP in pBACcap-1	polh: p24-RFP		V
	CMV-dsRed		

TABLE 6 Recombinant baculoviruses used in this thesis.

Virus	Displayed fusion protein	Fusion partner	Expression cassette	Article
Ac-luc	−	−	SV40-luc	I-II, V
Ac-EGFP	−	−	CMV-EGFP	II-III, V
*Ac*LyP-1-luc	LyP-1-VSVG	VSVG TM/CT	SV40-luc	I-II
*Ac*LyP-1-EGFP	LyP-1-VSVG	VSVG TM/CT	CMV-EGFP	II
*Ac*F3-luc	F3-VSVG	VSVG TM/CT	SV40-luc	I-II
*Ac*CGKRK-luc	CGKRK-VSVG	VSVG TM/CT	SV40-luc	I-II
*Ac*VP39	VP39-His	VP39	SV40-luc and CMV-EGFP	III, V
*Ac*F3-VP39	F3-VP39-His	VP39	SV40-luc and CMV-EGFP	III
*Ac*F3-VSVG/F3-VP39	F3-VSVG and F3-VP39-His	VSVG TM/CT VP39	SV40-luc and CMV-EGFP	III
*Ac*WT	−	−	SV40-luc and CMV-EGFP	IV
*Ac*ZZP74	ZZP74	P74	SV40-luc and CMV-EGFP	IV
*Ac*ZZVSVG*	ZZVSVG	VSVG TM/CT	−	IV
vp39EGFP	vp39-EGFP	VP39	−	Kukkonen et al., 2003; V
p24mCherry	p24-RFP	p24	CMV-dsRed	V
*Ac*MNPV strain E2 (wt)	−	−	−	II, V

* An expression marker gene-deficient derivative of *Ac*ZZVSVgTM-EGFP (Ojala et al., 2004)

4.4 Characterization of recombinant viruses

4.4.1 Detection of protein expression in insect cells (I, IV)

*Sf*9 cells were infected with recombinant baculoviruses (Table 6) at an MOI of 3 or 10 for set times (20-72 h) at 28°C. The cells were fixed with 4% paraformaldehyde in PBS (PFA-PBS) for 20 min at room temperature (RT), permeabilized with 0.1% Triton X-100, and immunolabeled with primary antibodies raised against baculovirus-specific proteins or the displayed fusion protein (Table 6) and Alexa Fluor® -conjugated secondary antibodies (Table 4). Alternatively, the cells were labeled without fixation at 4ºC. The samples were mounted in Mowiol (Calbiochem, Darmstadt, Germany) supplemented with DABCO (25 mg/ml; Sigma-Aldrich, St. Louis, MO) for further analysis by confocal microscopy (see Chapter 4.5.1). Quantification of the protein expression levels was performed with a FACSCalibur flow cytometer (Beckton Dickinson, Heidelberg, Germany) using CellQuest software.

4.4.2 SDS-PAGE and western blotting (I-IV)

Viral incorporation of the displayed fusion proteins (Table 6), and the ratio of total versus infectious viral particles were evaluated by exposing equal quantities of BV (0.25 or 1 x 10^8 PFU) or ODV (1 µg) of the recombinant viruses along with appropriate controls to analysis by western blotting. The samples were solubilized in SDS-PAGE sample buffer containing 5% β–mercaptoethanol, heat-denatured (5 min, 100°C), separated by reducing 12-15% SDS-PAGE, and analyzed using appropriate primary antibodies (Table 4). Detection was performed using alkaline phosphatase-conjugated secondary antibodies (Table 4), NBT (Nitro-Blue Tetrazolium Chloride) and BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) according to the manufacturer's instructions (Sigma-Aldrich, St. Luis, MO). The separation of BV and ODV nucleocapsid and envelope fractions was essentially performed as previously described (McCarthy et al., 2007). Total protein was visualized by staining with the PageBlue Protein Staining Solution (MBI Fermentas, Vilnius, Lithuania).

4.5 Viral binding, internalization and transduction studies

4.5.1 Immunofluorescence and confocal microscopy (I-V)

For confocal microscopic analysis of viral binding and internalization, cells were grown to subconfluency on cover slips for 1-2 days at 37°C. The viruses were allowed to adsorb to cells for 1 h at 4°C in PBS to avoid viral internalization, or for 1 h at 37ºC in culture medium containing 1% FBS in cases where simultaneous viral internalization was pursued. The cells were further incubated for set times at 37°C in culture medium containing 10% FBS. For binding experiments, unbound virus was removed by extensive washes with PBS at 4°C. The cells were fixed with 4% PFA-PBS for 20 min at RT, permeabilized with 0.1% Triton X-100, immunolabeled using appropriate primary and secondary antibodies (Table 4), embedded in ProLong®Gold antifade reagent supplemented with DAPI (Molecular Probes, Eugene, OR) or in Mowiol-DABCO, and analyzed by laser scanning confocal microscopy (Zeiss LSM 510, Carl Zeiss AG, Jena, Germany or Olympus Fluo-View 1000, Olympus Optical Co., Tokyo, Japan). The images were acquired using appropriate excitation and emission settings (488 nm argon laser, 543 nm and 633 nm HeNe-lasers) together with the multitracking mode to avoid false colocalization signals of different excitation wavelengths. Serial sections were obtained using 60x Apo (numerical aperture, NA=1.35), 63x Plan-Neofluor (NA=1.25), or 63x Plan-Apochromat (NA=1.40) oil immersion objectives with a resolution of 512 x 512 pixels/image. For live cell microscopy (Zeiss LSM 510), the objective and the sample holder were pre-heated to 37°C. The quantification of fluorescence intensity or colocalization was performed by ImageJ and BioimageXD software

(http://rsbweb.nih.gov/ij/; Kankaanpaa et al., 2006). The image series were processed and edited using Adobe Photoshop 8.0.

4.5.2 Plasmid transfection and protein depletion by siRNA (V)

The transient plasmid transfections were performed for 48 h with Fugene transfection reagent (Roche, Basel, Switzerland) according to the manufacturer´s instructions. The plasmid constructs (WT, wild-type; DN, dominant negative; CA, constitutively active) used in this thesis are described in Table 7. The cells transfected with GPI-EGFP were treated continuously with cycloheximide (Table 8; 100 µg/ml in culture medium supplemented with 1% FBS) for 4 h prior to virus transduction in order to chase the expressed GPI-EGFP to the plasma membrane. To remove the excess staining from the plasma membrane, the cells were washed with an acidic 0.5 M NaCl, 0.2 M sodium acetate buffer (pH 4.5).

TABLE 7 Plasmid constructs used for expression of the indicated protein mutants (V).

Plasmid	Expressed protein/tag	Provider and affiliation
Pak1 T423E	p21-activated kinase-1/c-Myc (DN)	Dr. Jonathan Chernoff, Fox Chase Cancer Center, Philadelphia, PA
Pak1 AID	p21-activated kinase-1/FLAG (CA)	Dr. Ed Manser , Institute of Molecular and Cell Biology, Singapore
GPI-EGFP	Glycosylphosphatidylinositol (GPI)-anchored protein /EGFP	Dr. Lucas Pelkmans, Institute of Molecular Systems Biology, ETH Zürich, Zürich
Cdc42	Cdc42 GTPase/EGFP (WT)	Dr. Lucas Pelkmans
Cdc42 17N	Cdc42 GTPase/EGFP (DN)	Dr. Johan Peränen, Institute of Biotechnology, Viikki Biocenter, University of Helsinki, Finland
Cdc42 12V	Cdc42 GTPase/EGFP (CA)	Dr. Johan Peränen
Rac1 17N	Rac1 GTPase /EGFP (DN)	Dr. Johan Peränen
Rac1 12V	Rac1 GTPase /EGFP (CA)	Dr. Johan Peränen
Arf6	ADP-ribosylation factor 6/EGFP (WT)	Dr. Johan Peränen
Arf6 T27N	ADP-ribosylation factor 6/- (DN)	Dr. Johan Peränen
Arf6 Q67L	ADP-ribosylation factor 6/- (DN)	Dr. Johan Peränen
RhoA	RhoA GTPase/EGFP (WT)	Dr. Johan Peränen
RhoA 14V	RhoA GTPase/EGFP (DN)	Dr. Johan Peränen
RhoA 19N	RhoA GTPase/EGFP (CA)	Dr. Johan Peränen
Rab34	Rab34/EGFP (WT, DN, CA)	Dr. Wanjin Hong, Institute of Molecular and Cell Biology, Singapore
Clathrin	Clathrin light chain/tomato (WT)	Dr. Tom Kirchhausen, Harvard Medical School, Boston, MA
IL-2R beta-chain	NTB-domain of IL2-receptor/-	Dr. Alice Dautry-Varsat, Pasteur Institut, Paris, France

The following siRNA oligonucleotides (20 µM; Dharmacon, Thermo Fisher Scientific, Fremont, CA) were used for protein depletion by RNA interference: RhoA (5´-GAAGUCAAGCAUUUCUGUC-3´), Rac1 (5´-GAUAACUCAC CACUGUCCA-3´), Arf6 (5´-GCACCGCAUUAUCAAUGACCGUU-3´), and dynamin-2 (SMARTpool). Non-specific siRNA (scramble) was used as a negative control, while siGLO (Dharmacon) acted as a transfection marker. The cells were transfected for 48 h with Oligofectamine (Invitrogen) according to the manufacturer´s instructions. Mock-transfected cells were treated with Oligofectamine alone. The functionality of the siRNAs was confirmed by western blotting of the respective proteins, and tubulin served as a loading control. The intensities of the protein bands from cells treated with scramble and target siRNAs were analyzed with the ImageJ software.

4.5.3 Quantification of viral binding (I-IV)

Viral binding was quantified by incubating cells grown in complete or serum-free medium with different concentrations of BV (100 - 10 000 PFU/cell) or ODV (0.1 - 10 ng of total virus protein/cell) for 1-2 h at 4°C. The cells were detached by trypzinization prior to viral binding, and all the steps throughout the experiment were performed in PBS at 4°C. Unbound virus particles were removed by extensive washes with 1.5-3% BSA in PBS (BSA-PBS). The bound BV and ODV were detected by incubation with mouse monoclonal B12D5 GP64 and rabbit polyclonal *Ac*MNPV ODV antibodies (Table 4), respectively, for 1 h followed by washes with 1.5-3% BSA-PBS and incubation with Alexa Fluor® -conjugated secondary antibodies (Table 4) for 30 min. The fluorescence was analyzed with FACSCalibur flow cytometer (Beckton Dickinson) using CellQuest software.

Binding inhibition experiments were similarly performed by preincubating the cells for 15 min at 4°C with synthetic LyP-1, F3, or CGKRK peptides (Table 8; 50, 200, 500, and/or 1,000 µM), or for 1 h at 4ºC with rabbit polyclonal NCL3 nucleolin antibody (100 µg/ml) and rabbit IgG (50 µg/ml; Table 4) prior to virus exposure.

4.5.4 Quantification of viral internalization (II-V)

BV (200 PFU/cell in PBS) or ODV (0.5 ng of total virus protein/cell) were bound to cells for 1 h at 4°C to avoid virus internalization. Unbound virus was removed by washes with PBS at 4°C followed by incubation at 37°C in complete culture medium for set times and fixation with 3% PFA-PBS for 15 min at RT. The viruses were labeled without permeabilization with rabbit polyclonal *Ac*MNPV BV or *Ac*MNPV ODV primary antibodies (Table 4) for 1 h and with goat anti-rabbit Alexa Fluor® 546 or 488 secondary antibody conjugates, respectively (Table 4), for 30 min at RT. Subsequent to washes with 1.5% BSA-PBS and permeabilization with 0.1% Triton X-100 in PBS, the virus was similarly labeled but now using goat anti-rabbit Alexa Fluor® 488 or 546 secondary antibody conjugates, respectively. Thus, the cell membrane-

associated viruses were labeled with both Alexa Fluor® -conjugates, and the internalized/cytoplasmic only with the other conjugate. For quantification of viral internalization kinetics, 25 images (z-stacks) representing a minimum of 40 cells were analyzed for each virus and time point with ImageJ software using the Colocalization Threshold plug-in (Tony Collins, Wright Cell Imaging Facility, WCIF, Toronto, Canada) based on the Costes Colocalization Quantification algorithm (Costes et al., 2004). Percentage values of colocalized fluorescence intensity above threshold for each channel were used to determine the relative quantity of the internalized virus. In addition, the internalization algorithm and segmentation tools of the BioimageXD software was used (Kankaanpaa et al. 2006).

For cointernalization studies, the cells were first transduced with baculovirus for 15 min and then fed with TRITC-Dextran (10 kDa, 250 µg/ml; Molecular Probes, Eugene, OR) for 5-45 min. Alexa Fluor® 546-Transferrin (200-250 µg/ml; Molecular Probes), FITC-Dextran (1000 µg/ml; Molecular Probes) or Alexa Fluor® 488-labelled *E. coli* bioparticles (60 particles/cell; K12 strain; Molecular Probes) were fed simultaneously with the virus. In the experiments with *E. coli*, the virus was allowed to internalize for 15 min, followed by Alexa Fluor® 488-labelled *E. coli* particles for 60 min. Samples were treated with trypan blue in order to separate the fluorescence of internalized and non-internalized *E. coli* particles.

4.5.5 Quantification of viral transduction efficiency (I-V)

Viral transduction was generally performed for 6-48 h at 37°C using 100–1,000 PFU/cell of baculovirus diluted in appropriate cell culture medium. Cells were harvested by scraping, concentrated by centrifugation, and resuspended in PBS. The enzymatic activity of luciferase was measured with a Wallac 1420 Victor multilabel counter (Wallac Oy, Turku, Finland) immediately after the addition of the substrate 1 mM D-luciferin (Sigma-Aldrich, St. Louis, MO; in 0.1 M Na citrate buffer, pH 5). The expression of EGFP was monitored with a FACSCalibur flow cytometer (Beckton Dickinson, Heidelberg, Germany) using CellQuest software. Viral transduction efficiency was alternatively monitored by confocal microscopy.

Transduction competition/inhibition experiments were conducted in the presence or absence of synthetic LyP-1, F3, CGKRK or RKK peptides (Table 8; I, III), various chemical inhibitors (Table 8; I-V), or antibodies (Table 4; III). The experiments are described in detail in the original publications (I-V).

4.5.6 Electron microscopy (IV-V)

For morphological studies of the recombinant viruses, samples of the concentrated viruses were deposited onto 100-mesh carbon-coated copper grids for 1 h followed by washes with H_2O, staining of the grids with 1.5% methyl cellulose/ 0.4% uranyl acetate and air-drying. Cointernalization of baculovirus with horseradish peroxidase (HRP) was performed by incubating the cells in

complete culture medium supplemented with 10 mg/ml of HRP (type II; Sigma Aldrich, St. Louis, MO) for 15 min at 37ºC. Following viral transduction (wt, 500 PFU/cell), the cells were fixed with 4% PFA-PBS 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 Aldrich) for 30 min, followed by an additional incubation of 30 min in 0.1% diaminobenzidine supplemented with 0.1% hydrogen peroxide. The samples were then washed, post-fixed in 1% osmium tetroxide, dehydrated, stained with uranyl acetate, and embedded in LX-122 Epon (Ladd Research Industries, Williston, VT). The samples were analyzed at 60 kV with a JEOL JEM-1200 EX transmission electron microscope (Jeol Ltd., Tokyo, Japan).

TABLE 8 Peptides and chemicals used in viral transduction experiments.

PEPTIDES				
Peptide	**Sequence (concentration)**		**Provider/Reference**	**Article**
LyP-1	CGNKRTRGC (50-1,000 μM)		Erkki Ruoslahti; Laakkonen et al., 2002	I
F3	KDEPQRRSARLSAKPAPPKPEPKPKK APAKK (50-1,000 μM)		Erkki Ruoslahti; Ale Närvänen; Porkka et al., 2002	I, III
CGKRK	CGKRK (50-1,000 μM)		Erkki Ruoslahti; Christian et al., 2003	I
RKK	CTRKKHDNAQC (50-1,000 μM)		Jyrki Heino; Ivaska et al., 1999	I, III
CHEMICAL INHIBITORS				
Inhibitor	**Provider**	**Concentration**	**Effect**	**Article**
Ammonium chloride	Sigma Aldrich	1-10 mM	Inhibition of endosomal acidification	I-III
Sodium butyrate	Fluka Chemie	5 mM	Inhibition of histone deacetylase	I-II, IV
Nocodazole	Sigma Aldrich	30-60 μM	Depolymerization of microtubules	II, IV
Methyl-β-cyclodextrin	Sigma Aldrich	1.25 mM	Extraction of cholesterol	V
Nystatin	Sigma Aldrich	50 μg/ml	Sequestering of cholesterol	V
Filipin	Sigma Aldrich	1 μg/ml	Perturbation of lipid raft organization	V
EIPA (5-(N-ethyl-N-iso-propyl)-amiloride)	Sigma Aldrich	0.05-0.2 mM	Inhibition of fluid-phase endocytosis	V
U73122	Sigma Aldrich	1 μg/ml	Inhibition of phospho-lipase C (PLC)	V
LY294002	BioSource International	50 μM	Inhibition of phosphatidyl-inositol-3-kinase (PI3K)	V
Cycloheximide	Sigma Aldrich	100 μg/ml	Inhibition of protein biosynthesis	V
Dynasore ($C_{18}H_{14}N_2O_4$)	Dr. Henry E. Pelish	80 μM	Inhibition of dynamin	V

4.5.7 Cytotoxicity assay (II, V)

The cytotoxicity of the recombinant baculoviruses (Table 6) or chemical inhibitors (Table 8) was determined by CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTT assay; Promega, Madison, WI) according to the manufacturer's instructions. The measurements were performed with a multilabel counter Wallac 1420 Victor2 (Wallac Oy, Turku, Finland) at an absorbance of 492 nm using software version 2.00.

4.5.8 Statistical analysis (I-III, V)

The means and standard deviations of the raw data were calculated using the Microsoft Office Excel 2007 software. The data groups were compared using the unpaired Student's t test with a two-tailed *P* value. Statistical significance was determined relative to the positive control samples (** $P<0.05$ and *** $P<0.001$). The results were shown as means ± standard deviation or standard error. When required, the numerical values were normalized relative to the positive control samples.

4.6 *In vivo* targeting assays (II)

Nude mice bearing tumor xenografts derived from MDA-MB-435 or MDA-MB-231 cells were used as a preclinical model in viral targeting assays. Viruses (5 x 10^8 PFU in PBS) were intravenously injected via the tail vein and allowed to circulate for 24 h. The mice were perfused through the heart with PBS followed by fixation with 4% PFA-PBS. Tumors and organs were removed, fixed with PFA, soaked in 30% sucrose (w/v) overnight, and frozen in OCT (optimum cutting temperature) embedding medium (Tissue-Tek, Sakura Finetek USA, Torrance, CA). The distribution of the viruses in tumor and organ sections (5-10 µm) was analyzed by immunohistochemistry using rabbit polyclonal *Ac*MNPV antibody (Table 4). The nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, CA). Blood and lymphatic vessels were visualized by staining tissue sections with antibodies raised against CD31, VEGFR-3 or LYVE-1 (Table 4). The sections were examined under an inverted fluorescence microscope (Zeiss Axioplan 2, Jena, Germany), and the intensity of baculovirus-specific fluorescence was analyzed with ImageJ software.

5 REVIEW OF THE RESULTS

5.1 Characterization of recombinant baculoviruses

5.1.1 Expression and localization of baculovirus-encoded recombinant proteins in infected insect cells

The expression and subcellular localization of the baculovirus-encoded fusion and reporter proteins was studied in *Sf*9 cells by confocal microscopy. The LyP-1, F3, and CGKRK tumor-homing peptides fused to the transmembrane anchor of VSVG through a polyAla linker (I, Fig. 1) were highly expressed and transported to the surface of the infected cells (I, Fig. 2A). The expression of the corresponding fusion proteins was also confirmed by western blotting (I; not shown). In addition, strong luciferase (I, Fig. 2B) and EGFP (I-II, not shown) reporter gene expression was detectable in the infected cells.

The baculovirus ODV-specific P74 envelope protein displaying two synthetic IgG-binding Z domains (ZZ) of *Staphylococcus aureus* protein A (IV, Fig. 4A) was effectively expressed as a chimera (IV, Fig. 2C), localized on the nuclear membrane as well as in the intranuclear ring zone, and enriched in virus-induced microvesicles of the infected cells (IV, Figs. 2 and 3) similar to wild-type P74 (Faulkner et al., 1997; Slack et al., 2001). This intranuclear localization of the ZZP74 chimera altered as the infection proceeded with an initial vesicular accumulation in the intranuclear ring zone initiating at 24 h p.i. followed by condensation in the centre of the nucleus by 72 h p.i. (IV, Fig. 3). The enrichment and localization of the ZZP74 chimera did not correspond to those of the ZZVSVG fusion protein (IV, Fig. 2A; Ojala et al., 2004), the baculovirus major capsid protein VP39 (IV, Fig. 4C), or the tubular baculovirus protein P10 (IV, Fig. 2B). Furthermore, the expression of ZZVSVG was 3-fold higher compared to the ZZP74 as assessed by flow cytometry (IV, Fig. 2D), reflecting the trimeric and monomeric nature of VSVG and P74, respectively.

5.1.2 Incorporation of fusion proteins into viral particles

To verify successful incorporation of the expressed fusion proteins into virus particles, samples of the envelope- and capsid-modified baculoviruses were analyzed by SDS-PAGE and western blotting. Results of the viruses displaying tumor-homing peptides, *Ac*LyP-1-luc, *Ac*F3-luc, and *Ac*CGKRK-luc (I, Fig. 1), showed the presence of the LyP-1, F3, and CGKRK peptide-VSVG fusion proteins in the budded virions mainly in SDS-resistant dimeric and trimeric forms (I, Fig. 2C) (Jeetendra et al., 2002; Robison and Whitt, 2000). Additional bands likely representing post-translationally modified fusion protein were identified for the *Ac*F3-luc (I, Fig. 2C). Two bands positive for the His-tag antibody, representing the full-length and truncated F3-VP39-His fusion proteins, were detected for the capsid-modified baculoviruses *Ac*F3-VP39 and *Ac*F3-VSVG/F3-VP39 (III, Fig. 3A). The control virus, *Ac*VP39, produced a single band representing the VP39-His (III, Fig. 3B, left).

Bands positive for the ZZ (IV, Fig. 4B) and P74 (IV, not shown) antibodies representing monomeric ZZP74 fusion proteins were detected for the *Ac*ZZP74-infected cells, polyhedra and ODV (IV, Fig. 4A) but not for the corresponding samples of the control virus *Ac*WT. Unexpectedly, an identical band to *Ac*ZZP74 ODV was also distinguished for the *Ac*ZZP74 BV (IV, Fig. 4B). Subsequent separation and immunoblot analysis of the nucleocapsid and envelope fractions of both *Ac*ZZp74 phenotypes showed that the ZZP74 chimera was mainly present in the nucleocapsid fraction (IV, Fig. 5A), confirming the unpredicted association of the ZZP74 with *Ac*ZZP74 BV. Compared with the BV of *Ac*WT, the infectivity (IV, Fig. 5B), polypeptide profile (IV, Fig. 5C) and morphology (IV, Fig. 5D) of *Ac*ZZP74 BV were unaffected by the presence of the ZZP74.

With each modified virus (I-IV), the ratio of total particle number versus the amount of infectious virus particles was similar compared to that of the control virus (I, Fig. 2D; III, Fig. 3B; and IV, Fig. 5B).

5.2 Viral binding to human cancer cells

5.2.1 Efficiency and pattern of viral binding

Binding of WT baculovirus in human cells is nonsaturable, of low affinity, and mediated by ubiquitous cell surface molecules (Duisit et al., 1999; Tani et al., 2001). To evaluate whether the viral surface modifications affected the efficacy and mode of baculovirus binding, cellular attachment was quantitatively analyzed by flow cytometry or qualitatively by confocal microscopy. The viruses displaying the tumor-homing peptides, LyP-1, F3, or CGKRK, exhibited from 2- to 5-fold greater binding to both MDA-MB-435 and HepG2 cells compared to the control virus *Ac*-luc (I, Fig. 3; II, Fig. 1). In contrast to *Ac*-luc, the equilibrium binding of the virus displaying the LyP-1 peptide was saturable

(II, Fig. 1A) and exhibited a more scattered and uniform appearance in both MDA-MB-435 and HepG2 cells (II, Fig. 1B). This suggests the involvement of a specific, saturable receptor during the cellular attachment and internalization. In support, the *Ac*LyP-1-luc also showed stronger competitiveness against the transduction of wild-type baculovirus than the *Ac*-luc (II, Fig. 2). While the attachment of the fluorescein-conjugated LyP-1 peptide is increased to serum-starved MDA-MB-435 cells (Laakkonen et al., 2004), here the starvation had no effect on binding or gene delivery of the *Ac*LyP-1-luc to MDA-MB-435 or HepG2 cells (II, not shown). Despite higher efficiency (I, Fig. 3), the binding of viruses displaying the F3 and CGKRK peptides was similar to that of *Ac*-luc with respect to saturability and the general pattern of binding (I-II, not shown). Yet, the virus displaying the F3 peptide, but not the CGKRK, was able to compete against the transduction of WT baculovirus (II, not shown). As expected, the binding of the capsid modified viruses, *Ac*F3-VP39 and *Ac*F3-VSVG/F3-VP39, remained unaltered following display of the F3 peptide on the viral capsid (III, not shown).

The interaction of ODV with human cells was first evaluated by investigating the preliminary details of the mode and kinetics of viral binding to HepG2 and A549 cells (IV, Fig. 1). Examination by confocal microscopy revealed that the *Ac*WT ODV was able to efficiently bind to both cell types at 4ºC with a similar pattern but a lower efficiency compared to that of the *Ac*WT BV (IV, Fig. 1A). Subsequent flow cytometric analysis showed the binding to be concentration-dependent, reaching constant efficiency with virus concentrations above 5 and 2.5 ng of total viral protein/cell in HepG2 and A549 cells, respectively (IV, Fig. 1B). While the unexpected incorporation of the ODV-specific ZZP74 fusion protein into the *Ac*ZZP74 BV (IV, Figs. 4 and 5) impaired viral internalization and gene transduction in HepG2 cells (IV, Fig. 6A, C-E), the cellular binding was uncompromised (IV, Fig. 6B).

5.2.2 Inhibition of viral binding

Actively growing MDA-MB-435 and HepG2 cells both express the cell surface nucleolin (Christian et al., 2003; Deng et al., 1996), the receptor responsible for the internalization of the F3 peptide (Christian et al., 2003), and heparan sulfate proteoglycans, which represent the putative cellular attachment and/or internalization molecules for the F3 and CGKRK peptides, as well as baculovirus (Christian et al., 2003; Duisit et al., 1999; Hoffman et al., 2003). To elucidate the specificity of viral attachment to target cells, flow cytometry was used in binding competition and inhibition assays to analyze the effects of soluble LyP-1, F3, and CGKRK peptides, nucleolin antibody, as well as serum-starvation on the binding of the modified viruses. Attachment of the tumor-homing peptide displaying viruses to MDA-MB-435 cells was inhibited by the corresponding soluble peptides in a concentration-dependent fashion to a variable degree, the LyP-1 peptide being the strongest inhibitor in terms of both the magnitude and the specificity of inhibition (I, Fig. 6). In addition, each peptide cross-inhibited the binding of all the peptide displaying viruses and the

control virus, the F3 and CGKRK peptides to a lesser extent than the LyP-1 peptide (I, Fig. 6), reflecting the complexity of viral binding.

The nucleolin antibody failed to inhibit the binding of both the *Ac*F3-luc and the control virus *Ac*-luc to HepG2 (III, Fig. 6B) and MDA-MB-435 cells (I, III, not shown), indicating that nucleolin is not involved in the initial cellular attachment of the F3 displaying or unmodified baculovirus. Unexpectedly, the binding of all virus constructs to serum-starved trypsinized, but not to serum-starved attached cells, was markedly impaired (I, not shown; III, Fig. 6A), suggesting that the combination of serum depletion and trypsin cleavage reduces the cell surface receptor pool relevant for baculovirus binding.

5.3 Viral transduction of human cancer cells

5.3.1 Efficiency of viral transduction

To examine ligand-directed gene transduction or a general functionality of the envelope and capsid-modified baculoviruses, the efficiency of viral gene delivery was compared with the phenotypically unmodified control viruses using viral quantities ranging from 20 to 1,000 PFU/cell. The transduction efficiencies were assessed in terms of transgene expression level and/or the number of transduced cells by measuring SV40 or CMV promoter driven luciferase activity or EGFP fluorescence at 4-96 h p.t. Being the most prominent at low virus concentrations, from 7- to 24-fold increases in the efficiency of transduction were achieved for the viruses displaying the tumor-homing peptides, *Ac*LyP-1-luc, *Ac*F3-luc, and *Ac*CGKRK-luc, in MDA-MB-435 and HepG2 cells, respectively (I, Fig. 4A; II, 3E). The luciferase expression was detectable at 4 h p.t. with each virus and climaxed approximately at 48 h p.t. in MDA-MB-435 cells (I, not shown; IV, not shown) and at 34 h p.t. in HepG2 cells (I, Fig. 4B). Notably, the baculovirus-mediated luciferase expression levels were significantly lower in MDA-MB-435 cells than in HepG2 cells, rendering MDA-MB-435 cells relatively non-permissive for baculovirus transduction. No statistically significant cytotoxicity was associated with the transduction of the viruses displaying the peptides or the control virus as detected by an MTT assay (II, Table 1).

While the display of the F3 peptide in the viral envelope improved the efficacy of baculovirus binding and gene transduction (I, Figs. 3 and 4; III, Fig. 6A), the display of the peptide on the viral nucleocapsid diminished the transduction efficiency of both *Ac*F3-VP39 and *Ac*F3-VSVG/F3-VP39 up to 70% in HepG2 cells compared to the control viruses *Ac*VP39 (III, Fig. 3C) and *Ac*-EGFP (III, not shown). The decrease was less significant with the *Ac*F3-VSVG/F3-VP39 (III, Fig. 3C), indicating that the envelope displayed F3 was able to partially restore the reducing effect.

No *Ac*WT ODV-mediated expression of luciferase or EGFP was detected in HepG2 or A549 cells although various viral concentrations, extended

transduction periods, lowered temperatures (20-28°C), as well as alkaline pH were tested (IV, not shown). The transductional block was not rescued by the treatment of HepG2 cells either with nocodazole, a microtubule depolymerizing agent known to enhance baculovirus transduction following endosomal release (Salminen et al., 2005; van Loo et al., 2001), or with sodium butyrate, a histone deacetylase inhibitor mediating transcriptional activation of baculovirus-encoded transgenes (Condreay et al., 1999) (IV, not shown). While the transduction of *Ac*WT BV resulted in typical transgene expression levels in HepG2 cells (IV, Fig. 6A), the unexpected incorporation of the ODV-specific ZZP74 fusion protein into the *Ac*ZZP74 BV (IV, Figs. 4 and 5) completely abolished the internalization and gene transduction of the virus (IV, Fig. 6A, C-E). Similar to the *Ac*WT ODV, no *Ac*ZZP74 ODV-mediated transgene expression was detected with or without a targeting antibody for preselected cell surface receptors (IV, not shown).

5.3.2 Inhibition of viral transduction

To explore the specificity of the gene delivery by the viruses displaying the LyP-1, F3, and CGKRK tumor-homing peptides, the inhibitory effect of the corresponding soluble peptides on viral transduction was studied in HepG2 cells. The LyP-1 peptide reduced the transduction of *Ac*LyP-1-luc at the highest by 50%, but had no effect on the transduction of the control virus *Ac*-luc (I, Fig. 7A). Unexpectedly, the F3 peptide resulted in a practically complete inhibition of the gene delivery by both the *Ac*F3-luc and *Ac*-luc (I, Fig. 7B). By using the reporter baculovirus, *Ac*-EGFP, the F3 peptide was specified to establish its inhibitory effect following a coincubation with the virus, but not when the target cells were incubated with the peptide prior or subsequent to viral binding (III, Fig. 1). The F3-mediated reduction of transduction was concentration-dependent (I, Fig. 7B; III, not shown), and did not interfere with viral binding (I, Fig. 6; III, Fig. 2A) or early steps of internalization (III, Fig. 2A). Instead, the peptide impeded the progression of baculovirus internalization during the later steps of entry, as evidenced by viral confinement in vesicles at the cell periphery and the absence of capsids in the nuclei (III, Fig. 2B). These vesicles were negative for the early endosomal marker, EEA-1 (unpublished results). A restricted BLAST-search (NCBI BLAST2, Swiss Institute of Bioinformatics) to the taxonomic group of viruses showed that F3 shares sequence homology with a hypothetical, 12 kDa protein of the baculovirus *Choristoneura fumiferana* multiple nucleopolyhedrovirus (*Cf*MNPV), but, of note, not with any other protein from the virus taxon. Accordingly, eight out of nine amino acids of the F3 sequence, EPQRRSARL, matched with the residues 10-18 of the *Cf*MNPV hypothetical protein. Finally, the CGKRK peptide reduced the transduction efficiency of the *Ac*CGKRK-luc in a concentration-dependent fashion but to a considerably lower extent than F3, having no effect on the transduction of *Ac*-luc (I, Fig. 7C). The nonrelevant cationic peptide, RKK (Ivaska et al., 1999), had no declining impact on the viral binding, internalization, or transduction (I, not shown; III, Figs. 2 and 3).

To elucidate the possible role of nucleolin during baculovirus transduction, the nucleolin antibody, NCL3, was tested for neutralization of gene delivery of baculovirus. Preincubation of HepG2 cells with the NCL3 inhibited the transduction of *Ac*-EGFP approximately by 50% compared to the antibody-free control, whereas the streptavidin control antibody had no statistically significant effect (III, Fig. 6C). The control antibody raised against transferrin receptor, a marker of CME, improved viral transduction up to 2-fold (III, Fig. 6C).

5.4 Viral targeting and entry into human cancer cells

5.4.1 Kinetics of viral entry

To compare the kinetics of viral internalization, it was first verified that the viruses displaying the tumor-homing peptides use the common, low pH-dependent entry route of wild-type baculovirus in HepG2 cells (Kukkonen et al., 2003; Matilainen et al., 2005; van Loo et al., 2001). With increasing concentrations of ammonium chloride, viral transduction was gradually decreased, being completely abolished at 6 mM with each virus (I, Fig. 5). Next, the kinetics of viral uptake between the modified and control viruses was more carefully evaluated by determining the ratio of membrane bound and internalized virus by quantitative confocal imaging at different time intervals following synchronized viral binding to HepG2 cells. The *Ac*LyP-1-luc, *Ac*F3-luc, and *Ac*CGKRK-luc were progressively endocytosed, and their internalization followed similar kinetics with the control virus *Ac*-luc up to 30 min p.t. (II, Fig. 3A-B). By 60 min p.t., the percentage of internalized virus was approximately 40% with the *Ac*LyP-1-luc, and 29% with the *Ac*-luc (II, Fig. 3B), *Ac*F3-luc, as well as *Ac*CGKRK-luc (II, not shown), indicating nearly 1.4-fold increase in the uptake of *Ac*LyP-1-luc. Moreover, the initial course of endosomal release was similar for the EGFP expressing viruses, *Ac*LyP-1-EGFP and the control *Ac*-EGFP (II, Fig. 3C). By 4 h p.t., a maximal quantity of the internalized *Ac*LyP-1-EGFP capsids had escaped from the endosomes and the virus had reached the highest transduction efficiency as opposed to 6–8 h p.t. with the *Ac*-EGFP (II, Fig 3C). At 8 h p.t., the intensity of nucleus-associated fluoresence of *Ac*LyP-1-luc capsids was approximately 50% lower (II, Fig. 3D), while the luciferase activity was 2-fold higher than those of *Ac*-luc (II, Fig. 3E). These results showed faster internalization, an earlier nuclear entry, subsequent capsid dissociation, and genomic release by the LyP-1 displaying baculovirus.

The kinetics of viral internalization and endosomal escape were similarly investigated with the capsid-modified *Ac*F3-VP39, and *Ac*F3-VSVG/F3-VP39 to identify the entry step, during which the capsid-fused F3 peptide executed its inhibitory effect on baculovirus transduction. At 4 h p.t., the percentage of internalized *Ac*F3-VP39 or *Ac*F3-VSVG/F3-VP39 was equal to that of the control virus, *Ac*VP39, detected as early as 1 h p.t. (III, Fig. 4B), suggesting a delay

during the later stages of the uptake process with the capsid-modified viruses. In support, the half-time of the endosomal release of the *Ac*VP39, *Ac*F3-VP39, and *Ac*F3-VSVG/F3-VP39, was estimated to be roughly 85±5, 95±10, and 60±5 min (III, Fig. 4C), and the maximal expression levels 28, 40, and 32 h p.t. (III, Fig. 5), respectively, reflecting a considerable delay in the nuclear accumulation of the capsid-modified viruses.

The internalization of *Ac*WT ODV occurred to a detectable extent by 30–45 min p.t. at 4ºC and at 37ºC in both HepG2 (IV, Figs. 1C and D) and A549 (IV, not shown) cells, suggesting the usage of direct membrane fusion as the mode of entry. By 4 h p.t., the proportion of internalized ODV from the bound virions was approximately 30%, and no apparent increase in viral uptake was observed over time (4–24 h p.t.), at alkaline pH, or with lowered transduction temperatures (20–28 ºC; IV, not shown). The intracellular transport of ODV was confined to vesicular structures peripheral to the plasma membrane, impeding subsequent nuclear entry and transgene expression (IV, Fig. 1D). Similar to the *Ac*WT ODV, the *Ac*ZZP74 BV became incapable of entering and transducing HepG2 cells (IV, Fig. 6) due to the unanticipated inclusion of the ZZP74 fusion protein into the viral nucleocapsid (IV, Figs. 4 and 5). The *Ac*ZZP74 BV-positive vesicles were negative for the early endosomal antigen (EEA-1; IV, Fig. 6D), as well as for the late endosomal and lysosomal marker (LAMP-2; IV, Fig. 6E), used to visualize vesicular compartmentalization.

5.4.2 Tumor targeting *in vivo*

After demonstrating that the LyP-1, F3, and CGKRK tumor-homing peptides promote baculovirus binding, internalization, and transduction *in vitro*, tumor homing of the viruses was evaluated in BALB/c *nu/nu* mice bearing tumor xenografts derived from the MDA-MB-435 (II, Fig. 4A) or MDA-MB-231 (II, Fig. 4B) cells. As the baculovirus transduction of MDA-MB-435 cells was impeded during the early steps of viral entry inhibiting subsequent gene delivery *in vitro* (II, Fig. 6), the tumor accumulation and the systemic biodistribution of the injected viruses were assessed by immunolabeling of the virus particles to reliably compare the distributed virus dose. The *Ac*LyP-1-luc accumulated within these xenografts with a significantly higher specificity and efficiency than the control virus *Ac*-luc (II, Fig. 4). The targeting of *Ac*LyP-1-luc was more specific in the MDA-MB-435 (II, Fig. 4A) than in the MDA-MB-231 (II, Fig. 4B) xenografts as demonstrated by higher accumulation in the tumors (II, Fig. 5) and lower distribution in nontarget organs (II, not shown). No difference in the tumor targeting between the F3 or CGKRK peptide displaying viruses and the control virus was observed (II, not shown). No apparent colocalization of the LyP-1 displaying virus was observed with the markers expressed in the endothelium of the lymphatic system, LYVE-1 or VEGFR-3, or with the blood vessel marker CD31 (II, not shown). Analyses of different organs from the tumor-bearing mice showed accumulation of both the *Ac*LyP-1-luc and *Ac*-luc in liver (II, Fig. 5) and spleen (II, not shown), while weaker labeling was

distinguished in kidneys (II, Fig. 5) and lungs (II, not shown), and the lowest quantities in the heart or brain (II, not shown).

5.4.3 Mechanism and regulation of viral entry

The nature and regulation of baculovirus uptake were characterized in HepG2 and 293 cells. The notion that approximately 40% of HepG2 and 80% of 293 cells were positive for EGFP expression after 24 h transduction with 200 PFU/cell of *Ac*-EGFP clearly indicates that both of these cell types are highly permissive to baculovirus transduction. While merely 100% of the 293 cell nuclei were positive for the viral capsid antibody, the transgene expression levels were somewhat higher in HepG2 cells (unpublished results).

To characterize the involvement of CME in baculovirus entry, various approaches; e.g. marker proteins, siRNA, chemical inhibitors, and transfection of DN and CA mutant plasmids were used. Clathrin-coated vesicles were not required for baculovirus internalization as no colocalization between baculovirus and clathrin heavy chain (Fig. 1A-C) or transferrin (V, Suppl. 1A) was detected. In addition, no alteration in the distribution of the expressed light chain clathrin was recognizable upon baculovirus internalization (V, Suppl. 1B-C). In contrast, nearly 70% decrease in viral internalization (V, Fig. 1D) and transduction (V, not shown) was observed in cells treated with the dynamin-inhibitor dynasore, while the use of DN dynamin-2 and the interference of dynamin-2 expression with siRNA failed to efficiently inhibit viral entry due to incomplete knock-down of dynamin (V, not shown).

Extensive ruffle formation at the cell surface of both cell lines was evident early following viral administration (V, Fig. 2A, Suppl. 2). The virus appeared to exploit the extended cellular protrusions for its attachment and further traveling towards the plasma membrane followed by engulfment from cellular ruffles (V, Video S1). Nearly 3-fold increase in ruffle formation was observed in the cells transduced with baculovirus for 30 min compared to the virus-free control cells (V, Fig. 2B). In addition, baculovirus entered these cells along the fluid phase markers horse-radish peroxidase (HRP; V, Fig. 2D-E) and dextran (V, not shown). The longer the viral internalization was allowed to proceed, the higher was the percentage of vesicles positive for both HRP and baculovirus (V, Fig. 2E).

As several fluid-phase pathways originate from the plasma membrane raft areas, the effect of drugs, namely methyl-ß-cyclodextrin, filipin and nystatin, that interfere with cholesterol content or function at the plasma membrane was tested. The results obtained with these drugs reflected the role of raft-derived vesicles in baculovirus uptake as viral internalization and/or transduction was clearly inhbited (V, Fig. 3A-B). The dynamin-independent, raft-derived GPI-anchored protein-enriched early endosomal compartment (GEEC) (Kalia et al., 2006) and flotillin (Glebov et al., 2006) pathways were not involved in baculovirus transduction due to the absence of colocalization between the virus and flotillin-1 or glycosylphosphatidylinositol (GPI)-anchored protein GPI-

EGFP (V, Fig. 3C). In addition, the expression of WT, CA, or DN mutant forms of Cdc42 showed no effect (V, not shown).

Next, the involvement of macropinocytosis in baculovirus entry was examined. The chemical inhibitors of Na+/H+ exchanger (EIPA), PI3K, as well as PLC, all involved in macropinocytosis and in additional pathways, inhibited baculovirus transduction roughly by 50% (V, Fig. 3D, Suppl. 3A-B). Milder concentrations of EIPA, however, caused no decrease in the virus-mediated expression (V, Fig. 3E). Additionally, the regulators of macropinocytosis, Pak1 and Rab34, were not involved (V, Fig. 3F), showing no direct association of baculovirus entry with macropinocytosis.

In cells transfected with the DN and CA forms of Arf6, a regulator of clathrin-independent entry and recycling (Brown et al., 2001; Donaldson, 2003), the internalization and transduction of baculovirus was reduced up to 80% in comparison to the cells transfected with the WT form of Arf6 (V, Fig. 4A-B). In addition, the nuclear entry of baculovirus was decreased following the knock-down of the expression of endogenous Arf6 (V, Fig. 4C). Additionally, reorganization of actin was detected with Arf6 CA and DN mutant constructs (V, not shown). In support, knock-down of Arf6 expression by siRNA notably reduced the nuclear internalization of baculovirus (Fig. 4C).

Similar to the mutant forms of Arf6, lower cytoplasmic quantities of baculovirus were detected in cells transfected with the DN and CA mutant forms of RhoA (V, Fig. 5A, D). Also the nuclear localization was reduced by a minimum of 50% with all RhoA constructs including the WT RhoA compared to the EGFP-expressing control (V, Fig. 5B). In support, lower nuclear entry of baculovirus was evident after knock-down of endogenous RhoA by siRNA (V, Fig. 5C). The dynamin- and RhoA-dependent IL2-receptor pathway (Lamaze et al., 2001) was not involved in baculovirus entry as evidenced by the lack of colocalization between the virus and the expressed, internalized NTB domain of IL2-receptor (V, Suppl. 4C). The knock-down of Rac1, another RhoGTPase family protein, by siRNA, or the expression of DN and CA mutant forms of Rac1 had no statistically significant effect on baculovirus internalization (V, Suppl. 4A-B).

Due to the efficient ruffle formation and involvement of actin, RhoA, PLC, PI3K as well as rafts (V, Fig. 2, Suppl. 2), the utilization of phagocytosis-like entry by baculovirus was evaluated using heat-inactivated, green fluorescent *E. coli* particles as phagocytic tracers. Clear colocalization of baculovirus and *E. coli* was observed at 5 and 10 min p.t. (V, Fig. 6A-B). Moreover, baculovirus was able to stimulate the uptake of *E. coli* up to 49-fold in the non-phagocytic HepG2 and 293 cells following coadministration, while untransduced control cells contained no apparent *E. coli*-specific fluorescence (V, Fig. 6C). When baculovirus was fed to the cells before the administration of *E. coli*, the bacteria could no longer efficiently enter the cells (V, Fig. 6C). Treatment of HepG2 cells with the dynasore and filipin reduced the baculovirus-triggered internalization of *E. coli* by approximately 50% (V, Fig. 6D). These results propose the ability of baculovirus to induce transient phagocytosis, allowing bacterial entry after simultaneous administration.

Taken together, unmodified baculovirus enters human cells via a clathrin-independent pathway, which originates from the raft areas, is regulated by dynamin, Arf6 and RhoA, and is able to induce ruffle formation and coordinated phagocytic uptake of *E. coli*. The virus enters the cells progressively (II, Fig. 3B; III, Fig. 4B), and roughly 40% of the bound virions are internalized by 4 h p.t. (III, Fig. 4B). Nuclear localization of the virus capsids is detectable starting at 4 h p.t. (Laakkonen et al., 2007) in tandem with the transgene expression reaching maximal expression levels at 28-34 h p.t. (I, Fig. 4B; III, Fig. 5) depending on the expression cassette.

6 DISCUSSION

Baculovirus represents an attractive alternative to the traditional pathogenic viral vectors for gene therapy. Despite the absence of pre-existing anti-baculovirus humoral immunity in humans, the exploitation of baculovirus for systemic gene delivery is limited by vector immunogenicity, activation of innate immune responses, and transduction of non-target tissues. To address these challenges, two parallel and complementary lines of research, coupled to this thesis work, were recently established in our research group: one focusing on the development of targeted cancer-specific baculovirus vectors for the delivery of pro-apoptotic genes and proteins, while the other aims at attenuating complement- and innate immunity-mediated inactivation of these vectors. Thus, the long-term goal of these studies is to develop a comprehensive baculovirus-based therapeutic strategy that will target pre-selected cancer cells to promote tumor clearance and inhibition of tumor growth. The present study was performed to pursue a preliminary step towards these ambitious goals by 1) engineering cancer-selective baculovirus gene delivery vectors displaying a selection of tumor-homing peptides on the viral envelope (I-III), 2) developing novel, complementing baculovirus-based tools for a parallel use (IV), and 3) characterizing the nature and regulation of baculovirus entry to human cancer cells for further refinement of transductional targeting (V).

6.1 Baculovirus display: a multifunctional tool for viral vector targeting and studies on virus-cell interactions

6.1.1 Display on budded virus

Surface glycoproteins of enveloped viruses have served as versatile platforms for the functional display of heterologous peptides and proteins despite the generally limited structural knowledge regarding their three dimensional crystal structures. Display methods in general are limited in terms of the length and the amino acid sequence of the translated polypeptide, rendering these

issues particularly relevant for virus display, in which virion functionality is of utmost relevance. The major envelope glycoprotein GP64 is the most frequently applied endogenous scaffold in baculovirus surface display. The importance of GP64 in viral infection and high-titer vector production, however, limits both the level of manipulation and the applicability of the platform to baculovirus targeting. This has consequently led to the expansion of alternative display platforms, comprising both type I and II heterologous viral and cellular transmembrane glycoproteins with distinct benefits (Makela and Oker-Blom, 2006; Makela and Oker-Blom, 2008). For example, the application of either a full-length or truncated VSVG as a fusion partner enables display on the lateral virion surfaces (Chapple and Jones, 2002; Guibinga et al., 2004; Makela and Oker-Blom, 2006; Ojala et al., 2004), while GP64 restricts the presentation of the foreign (poly)peptides solely at the apical pole of the virion (Boublik et al., 1995).

In the present study, cancer-selective tropism of baculovirus was pursued by introducing three previously identified tumor targeting peptides, LyP-1, F3, and CGKRK, on the baculovirus envelope using the transmembrane anchor of VSVG as the display platform (I-II). Earlier, these peptides have effectively been attached to different payloads varying in size, structure and chemical composition, such as T7 phage via a genetic fusion to the capsid protein 10b, or quantum dots and fluorescein by chemical coupling, and subsequently delivered to the relevant vascular target sites in xenografted tumors (Akerman et al., 2002; Christian et al., 2003; Hoffman et al., 2003; Laakkonen et al., 2002; Laakkonen et al., 2004; Porkka et al., 2002). Here, the adaptation potential of these peptides to the baculovirus envelope (I-II) and capsid display systems (III) was demonstrated. Baculovirus represents by far the largest and the most complex cargo of these peptides. In earlier studies, a truncated VSVG composed of a 21-amino-acid ectodomain together with the TM and CT domains was shown to enhance display and enable uniform distribution of the fusion proteins on the baculoviral surface (Chapple and Jones, 2002; Kaikkonen et al., 2006; Ojala et al., 2004). To improve specificity, the platform was further revised herein by substituting the truncated VSVG ectodomain, shown to mediate unselective and enhanced viral binding and transduction (Kaikkonen et al., 2006; Makela and Oker-Blom, 2006; Ojala et al., 2004), with an inert linker to provide distance and flexibility for the uninhibited display and functioning of the peptides (I). This strategy proved to be sufficient for promoting high-level incorporation of these fusion proteins to the plasma membrane of the infected cells and subsequently to budded virions (I). In parallel with the full-length VSVG as well as truncated VSVG fusion proteins, these peptide-VSVG chimeras were expectedly expressed as SDS-resistant trimers (Chapple and Jones, 2002; Kaikkonen et al., 2006; Kreis and Lodish, 1986; Ojala et al., 2004; Robison and Whitt, 2000).

The present study emphasized the possibility to alter both the efficiency and the specificity of baculovirus-mediated gene delivery to human cancer cells via envelope display of the tumor targeting peptides (I-II). The differences in the structures of the displayed peptides had also a detectable influence on the

mode and kinetics of binding and internalization, as well as tumor targeting of these vectors (see Chapter 6.2). The corresponding soluble peptides inhibited viral attachment and transduction to a variable extent using relatively high peptide concentrations (I). The evidence of sequence specific as opposed to charge-mediated enhancement was strongest for the LyP-1 peptide with an apparent dissociation constant in the range of hundreds of micromolar, CGKRK and F3 peptides being less specific. In contrast to the redirected binding by the LyP-1, the F3 and CGKRK peptides appeared to augment the natural entry process of the virus through both specific and non-specific interactions.

Unexpectedly, the F3 peptide exerted a dramatic reduction in the nuclear accumulation and subsequent transgene delivery after coincubation with both the virus displaying the peptide and the unmodified control virus, suggesting direct interaction of the soluble peptide with the virus particles. The preliminary investigations showed that the F3 had no apparent effect on the cellular attachment of baculovirus (I, III), but instead inhibited viral entry at a step following viral attachment and early uptake (III). These consequences were also translated into the recombinant viruses, which displayed the peptide on the viral nucleocapsid by an N-terminal fusion to the major capsid protein VP39 (III) (Kukkonen et al., 2003). These results together suggest that the F3 acts during cytoplasmic entry or trafficking of the virus by directly interacting with the virus particles, rendering the peptide a functional molecular tool for studies on baculovirus entry and trafficking.

The mechanism underlying the effects of the F3 remain speculative thus far, but may involve neutralization of conformational epitopes of possible negative charge at the viral envelope or capsid that are critical for molecular interactions during baculovirus entry. Likewise, the highly negatively charged heparin has been shown to strongly interfere with the viral gene delivery but not the binding by directly interacting with the virus particle itself (Duisit et al., 1999). The cytoplasmic trafficking of baculovirus in insect cells is mediated by capsid-induced actin polymerization (Charlton and Volkman, 1993; Lanier and Volkman, 1998). Similarly in mammalian cells, viral nucleocapsids may be able to stimulate the formation of actin filaments, as cytochalasin D strongly reduces the transduction efficiency, but not the delivery of the nucleocapsids into the cytoplasm (van Loo et al., 2001). It is therefore possible that F3 hinders the interaction of the VP39 and/or P78/83 capsid protein(s) with actin (Lanier and Volkman, 1998), and thereby reduces the efficacy of intracellular trafficking of the virus. Interestingly, the synthetic α-helical domain of HMGN2 (amino acids 18-48), corresponding to the full-length F3, has been demonstrated to possess antimicrobial properties as a consequence of potential membrane activity (Feng et al., 2005a; Feng et al., 2005b). Thus, in addition to the activity against certain microbes, F3 appears to act as an antiviral peptide. The F3 also shares sequence homology with a hypothetical protein of *Cf*MNPV, a close relative to *Ac*MNPV (Blissard and Rohrmann, 1990; Slack and Arif, 2007), but not with any other protein from the virus taxon. The biological significance of this structural relation regarding F3-mediated inhibition of *Ac*MNPV transduction is, however, unclear.

To conclude this part, the unselective nature of baculovirus binding motivates further refinement of the surface display system and the discovery of alternative display platforms. The most obvious solution to conquer the tropism mediated by the WT GP64 would be the exclusion of the protein from the BVs. However, results obtained with GP64-null viruses have shown that these vectors suffer from low-titer vector production due to inefficient virus budding and propagation in insect cells (Kitagawa et al., 2005; Mangor et al., 2001; Monsma et al., 1996; Oomens and Blissard, 1999; Zhou and Blissard, 2008b). Application of the recently identified VSVG stem region (91 C-terminal amino acids) to rescue viral budding and the minimal GP64 cell surface targeting domains (38 N-terminal amino acids of mature ectodomain and 52 C-terminal amino acids) (Zhou and Blissard, 2008b) in combination with multimeric display of high-affinity targeting ligands may facilitate more efficient and specific targeting of GP64-null virions. Yet, the presence of VSVG stem, shown to broaden baculoviral tropism (Barsoum et al., 1997; Kaikkonen et al., 2006; Kitagawa et al., 2005; Makela and Oker-Blom, 2006), and the N-terminus of GP64, involved in host cell receptor binding (Zhou and Blissard, 2008a), in the virions render this display platform susceptible to unspecific interactions. Therefore, identification of alternative proteins capable of compensating the budding and propagation functions of GP64 in the context of GP64-null virus is of immense importance. Alternatively, molecules capable of neutralizing the GP64-mediated background binding to target cells in the presence of a targeting moiety, but retaining the low-pH-triggered membrane fusion activity could be able to serve in a similar context.

6.1.2 Display on occlusion-derived virus

Baculoviruses are unique compared to other virus families by having two distinct viral phenotypes, ODV and BV, of a shared genotype. ODV is a specialist as it only infects the highly differentiated columnar epithelial cells within the alkaline conditions of the larval midgut. BV, on the other hand, is a generalist being highly infectious to the tissues of the host as well as cultured insect and mammalian cells. Most of the established data on baculovirus–mammalian cell interactions and baculovirus display technology relates to BV (Braunagel and Summers, 2007; Hu, 2006; Makela and Oker-Blom, 2008). ODV envelope proteins, have provided unique tools to comprehend the molecular events that sort and traffic integral membrane proteins from the ER to nuclear membranes and to ODV envelope, as well as regulate the formation of virus-induced intranuclear membrane microvesicles (Braunagel and Summers, 2007). Although BV has proven multifunctional in biotechnology and research, diversification of baculovirus-based tools would facilitate a more comprehensive exploitation of each viral phenotype, BV, ODV as well as polyhedra, in applications, for which they are best suited.

The present study was the first to examine the interaction of ODV with human cells, and the functionality of the P74 ODV envelope protein for display of heterologous peptides (IV). The preliminary details of the mode and kinetics

of ODV binding, internalization, and gene transduction were studied in baculovirus-permissive HepG2 cells (Hofmann et al., 1995; Matilainen et al., 2005), and in A549 lung carcinoma cells investigated already in the early 1980s for the propensity of ODV uptake (Volkman and Goldsmith, 1983). The results obtained herein showed the binding of ODV to these cells to be concentration-dependent and saturable at 4ºC, which suggests specific binding and occupation of receptor attachment sites similar to the natural target cells (Haas-Stapleton et al., 2004; Ohkawa et al., 2005). Accordingly, based on the number of physical virus particles per cell, our rough evaluations suggested that a 2,000-3,000-fold higher quantity of ODV was required to achieve binding efficiencies comparable to BV in HepG2 cells. Likewise, based on PFU per pictogram viral protein, BV has been shown to be 1700-fold more infectious compared to ODV in cultured insect cells (Volkman et al., 1976). While the low temperature prevents internalization of BV by low pH-dependent endocytosis (Volkman and Goldsmith, 1985), ODV enters its natural host cells by direct membrane fusion both at 27ºC and 4ºC (Horton and Burand, 1993). As the internalization of ODV in HepG2 and A549 cells was observed to occur to a detectable extent not only at 37ºC but also at 4ºC, direct fusion of the virus envelope with the plasma membrane of the target cell is the probable mode of entry for ODV also in human cells. The intracellular transport of ODV was confined peripheral to the plasma membrane, impeding subsequent nuclear entry and transgene expression. Furthermore, viral transduction could not be rescued by mimicking the preferred alkaline environment and lowered temperature of the ODV infective entry, or following treatment of the cells with drugs interfering with microtubule polymerization or histone deacetylation (Condreay et al., 1999; Salminen et al., 2005; van Loo et al., 2001). These results are in an agreement with the early electron microscopy studies conducted with A549 cells and additional human and nonhuman vertebrate cells, in which ODV nucleocapsids were detected in cytoplasmic vacuoles, in cytoplasmic projections at the cell surface as enveloped virions, or in the cytoplasm as nonenveloped nucleocapsids (Volkman and Goldsmith, 1983). To conclude, these results left no doubt that, although capable of cellular binding and limited internalization, phenotypically unmodified ODV is incapable of mediating successful transduction in human cells.

In contrast to other enveloped viruses, ODV lacks apparent spikes and a characteristic fusion protein, and the key protein mediating the fusion event is yet to be identified. These details render ODV exceptional among enveloped viruses with respect to its mechanism of facilitating functional entry (Haas-Stapleton et al., 2004; Ohkawa et al., 2005). Therefore, a modified ODV, displaying a tandem repeat of IgG-binding Z domain via an N-terminal fusion to an extra copy of the ODV-specific P74 envelope protein (ZZP74) (Faulkner et al., 1997; Slack et al., 2001), was engineered. In contrast to direct membrane fusion, it was anticipated to improve the efficiency and selectivity of ODV uptake and consequently rescue the transductional block of ODV in human cells. The ZZP74 chimera was observed to localize in the intranuclear ring zone, and was enriched in virus-induced microvesicles similar to WT P74 (Faulkner et

al., 1997; Slack et al., 2001). However, subsequent analysis of the viral envelope and nucleocapsid fractions revealed an unexpected incorporation of the ZZP74 fusion protein into viral nucleocapsids of both ODV and BV, obstructing the ODV envelope display. The ZZP74 BV preserved normal infectivity, polypeptide profile, and morphology, but became incapable of entering and transducing human cells. This unexpected incorporation did not compromise cellular binding, but led to the aggregation of the virus peripheral to the plasma membrane. This is an extreme demonstration of how the infectious titer obtained in insect cells does not directly translate into the transducing ability of baculovirus (Chan et al., 2006). A similar phenomenon was observed with the baculoviruses displaying the F3 peptide on the viral nucleocapsid that possessed normal infectivity in insect cells, but were transductionally-challenged in HepG2 cells (III). This effect, however, was likely peptide-specific rather than a general consequence of modifying VP39 or the viral capsid (Kukkonen et al., 2003).

The results from these studies (III-IV) revealed an apparent correlation between the modification of the viral nucleocapsid and compromised viral transduction, rendering the surface architecture of the nucleocapsid relevant in mediating receptor interactions and/or signaling events during baculovirus entry in human cells. Baculovirus has generally been considered to use a similar endocytic route to enter both insect and mammalian cells, whereas the behavior of the ZZP74 BV clearly reflects fundamental species-specific differences in the entry processes, and/or involvement of specialized receptor molecules. P74, a common denominator of both ODV and ZZP74 BV, may be one of the key factors impeding the internalization of these virion phenotypes in human cells. Furthermore, P74 may require a specific binding partner absent from mammalian cells. Functional engineering of ODV vectors is therefore required to facilitate ODV-mediated gene transduction in human cells. In addition to viral targeting to a preferred receptor and endocytic entry pathway, ODV should be equipped with a viral fusogenic machinery to facilitate endosomal escape at an appropriate pH, and subsequent gene transduction.

6.2 Tumor targeting of baculovirus: potential for cancer therapy

Lately, tumor vasculature has received increased attention as a target for potential anti-cancer therapies. Several peptides and antibodies that recognize tumor specific vascular signatures have been discovered by novel methods such as *in vivo* screening of phage display libraries (Laakkonen et al., 2008; Ruoslahti, 2002; Ruoslahti et al., 2005). The LyP-1, F3, and CGKRK represent such peptides possessing tumor homing ability and binding specificity towards the cells lining the tumor blood vessels or lymphatics. Notably, these peptides are able to concentrate in the target tissue and penetrate the target cells in a cell type-specific manner, being particularly efficient delivery vectors for the targeting of

therapeutic moieties and imaging agents (Akerman et al., 2002; Hoffman et al., 2003; Laakkonen et al., 2002; Laakkonen et al., 2004; Porkka et al., 2002).

The present study was the first to scrutinize the applicability of the LyP-1, F3, and CGKRK peptides for tumor targeting of a bulky and complex payload, the enveloped baculovirus. As anticipated, each of these peptides intensified viral attachment and transduction in both MDA-MB-435 and HepG2 cells (I-II). Apparent specificity of peptide-mediated viral binding, however, was observed only in the case of the LyP-1 peptide as the attachment was inhibited with the corresponding soluble peptide in a concentration-dependent manner with a high magnitude (I). Furthermore, the LyP-1 peptide contributed to saturable viral binding in both cell types leading to earlier nuclear accumulation and enhanced transgene expression in HepG2 cells. The virus displaying LyP-1 also showed stronger competitiveness against transduction with WT baculovirus (II). These data suggest the involvement of a specific receptor in cellular attachment and entry (Fogal et al., 2008). Despite the higher efficiency of viral attachment and transduction, the binding pattern and internalization kinetics of baculoviruses displaying the F3 and CGKRK tumor-homing peptides were similar to that of the control virus (I-II). This indicates that the improvement in viral binding and gene delivery is largely mediated by the positive charge of the F3 and CGKRK peptides as opposed to a sequence-specific enhancement with the LyP-1 peptide. These results confirmed that a positive charge of the displayed peptide or the VSVG membrane anchor were not attributable to the improved behavior of the LyP-1 displaying virus.

After demonstrating that the LyP-1 promotes the performance of baculovirus *in vitro*, tumor targeting of the virus in mice was evaluated following intravenous administrations (II). The LyP-1 peptide was originally identified using tumors derived from MDA-MB-435 cells (Laakkonen et al., 2002). The same model, in addition to the MDA-MB-231 breast carcinoma xenograft model, was applied to preserve the molecular characteristics of the original tumor. The virus displaying the LyP-1 peptide accumulated within these tumors with higher specificity and efficiency than the control virus, and the viral targeting was more specific in the MDA-MB-435 than in the MDA-MB-231 xenografts. The tumor accumulation of the modified virus likely was at least 10- to 20-fold higher than that of the control vector, which exhibited low fluorescence barely distinguishable from the background. The LyP-1 virus-specific labeling was scattered over large regions of the tumor in a heterogeneous pattern, showing stronger labeling at the tumor periphery. Minor labeling was also detected in single cells within the tumor mass. Thus, the specificity of the LyP-1 peptide was able to prevail over the dominant tropism of the wild-type GP64. The F3 and CGKRK peptide displaying viruses did not accumulate into the MDA-MB-435 or MDA-MB-231 tumors, indicating that the VSVG membrane anchor, a positive charge, or a general tumor-homing ability of the displayed peptide, are not sufficient to target baculovirus to these tumors. Consistent with previous studies (Kim et al., 2006; Kircheis et al., 2001), both the modified and the control virus were distributed in the non-target

organs especially in liver and spleen with diminished amounts in kidneys and lungs, and negligible amounts in brain and heart.

As expected, the characteristics of the soluble LyP-1 peptide were not completely translated into the modified virus. For example, while the binding of fluorescein-conjugated LyP-1 peptide was increased by 2.5-fold to MDA-MB-435 cells grown in low serum (Laakkonen et al., 2004), no obvious increase was observed in viral binding or gene delivery to serum-starved MDA-MB-435 or HepG2 cells (II; not shown). This reflects the dominancy of GP64 over the LyP-1 peptide in viral tropism after serum-depletion and the involvement of more than a single receptor in the cellular binding of the virus. Also the biodistribution of the modified virus, which differs greatly in size and complexity from fluorescein, was dissimilar to that of the conjugated LyP-1 peptide. The fluorescent peptide, allowed to circulate for 5-15 min, colocalized with the lymphatic markers LYVE-1, podoplanin, and VEGFR-3 in vessel-like structures within MDA-MB-435 tumor tissue (Laakkonen et al., 2002). Although the virus displaying LyP-1 was clearly concentrated to vessel-like structures resembling lymphatic vessels, no apparent colocalization of the virus with the LYVE-1 or VEGFR-3 was detected (II; not shown). In parallel, virus-specific fluorescence was absent from the vessels positive for the blood vessel marker, CD31 (II; not shown). To mimic a therapeutic situation enabling virus-mediated transgene expression with sufficient quantities for a functional effect, the viruses were allowed to circulate for 24 h in contrast to <20 min with the LyP-1 peptide. By 24 h post injection, the viruses had partly infiltrated through the leaky tumor vessels since virus-specific labeling was detectable in single cells within the tumor. Also the LyP-1 peptide has been shown to accumulate in the tumors outside the structures positive for the lymphatic markers, including the tumor cells (Laakkonen et al., 2002). While the LyP-1 peptide homes to tumors within 5-15 min post injection with increasing accumulation over time (Laakkonen et al., 2002; Laakkonen et al., 2004), baculovirus clears more slowly from the blood. Its slow clearance may be attributable to the complexity of its surface architecture and large size, impeding its extravasation.

In conclusion, the virus displaying the lymphatic homing peptide, LyP-1, on the viral envelope showed the greatest potential for targeted therapies of all the modified viruses by selectively accumulating to xenografted human MDA-MB-435 and MDA-MB-231 tumors in mice after systemic administration (II). This type of tropism modification enables elevation of the targeting efficacy and/or selectivity and thereby broadens the scope of baculovirus vectors for disease therapies. The LyP-1 peptide may enable the specific targeting of tumor lymphatics and their adjacent tumor tissues for destruction by baculovirus vectors carrying proapoptotic genes or proteins. These vectors could be specifically suitable for transient, acute expression of factors (e.g. proapoptotic secreted cytokines) that jeopardize the survival of proliferating target cells without the need of transducing each cell within the tumor mass. Together with a multivalent display of targeting moieties and introduction of innate immunity-resistance into these vectors, such modified viruses may enable more effective use *in vivo* for potential therapeutic applications in cancer treatment.

The benefits of these baculoviral gene delivery vectors compared to unmodified and currently used pathogenic vectors would be several including: (I) reduction of "back-ground" transduction and thereby greater specificity, (II) specific cancer killing, (III) escape from the inactivation by the complement system and/or generation of neutralizing antibodies, and (IV) scalable and easy production and purification procedures. Thus, refinement of the targeting strategies and attenuation of complement- and innate immunity-mediated inactivation of baculovirus will be essential issues of future studies.

6.3 Baculovirus entry: importance for therapeutic gene delivery

Until now, the detailed mechanisms contributing to functional entry of baculovirus to human cells have been inadequately characterized. The mode and kinetics of viral binding and uptake have been studied using enzymatic modification of the target cells, charged compounds and different chemical inhibitors to obstruct virus–cell interactions or distinct endocytic processes. Structurally modified baculoviruses or modern molecular inhibitors in the form of dominant negative cellular proteins or siRNA, for example, have been applied to a lesser extent. To further develop transductional targeting and gene delivery of baculovirus vectors, the study presented herein attempted to shed light on many important but yet unclear aspects of the recently suggested endocytic route of baculovirus entry. The overall strategy was to test inhibitors known to affect diverse endocytic processes to evaluate the involvement of particular pathways. Although often used, this strategy is complicated and difficult even in the clearest of cases requiring many controls and multiple parallel perturbants for confirmation.

Baculovirus has previously been proposed to use CME together with macropinocytosis for cellular uptake (Long et al., 2006; Matilainen et al., 2005). The role of CME in baculovirus entry was suggested based on the effects of chlorpromazine and a DN mutant of Eps15 in non-human BHK21 cells (Long et al., 2006), as well as occasional viral attachment with plasma membrane-bound coated pits in HepG2 cells (Matilainen et al., 2005). However, the virus has not been detected within budded clathrin-coated vesicles in human cells despite early endosomal targeting (Kukkonen et al., 2003; Matilainen et al., 2005). Instead, enveloped virus particles were internalized into numerous large plasma membrane invaginations and non-coated vesicles associated with plasma membrane ruffling (Matilainen et al., 2005), reflecting the involvement of a more efficient pathway. The results obtained herein showed that baculovirus is able to induce ruffle formation and exploits filopodial extensions for its attachment, transport towards the plasma membrane, and final engulfment into the cell (Figure 5A-B). The mechanism involves a raft-dependent pathway leading to viral uptake into smooth-surfaced vesicles devoid of clathrin (Figure 5C). This requires actin rearrangement and dynamin assembly, and is regulated by signaling pathways involving the actin mediators

Arf6 and RhoA (Figure 5). Additionally, the virus is able to induce a coordinated phagocytic uptake of *E. coli* and is internalized into the same vesicular compartments (V). A role for nucleolin during baculovirus trafficking was also proposed (III).

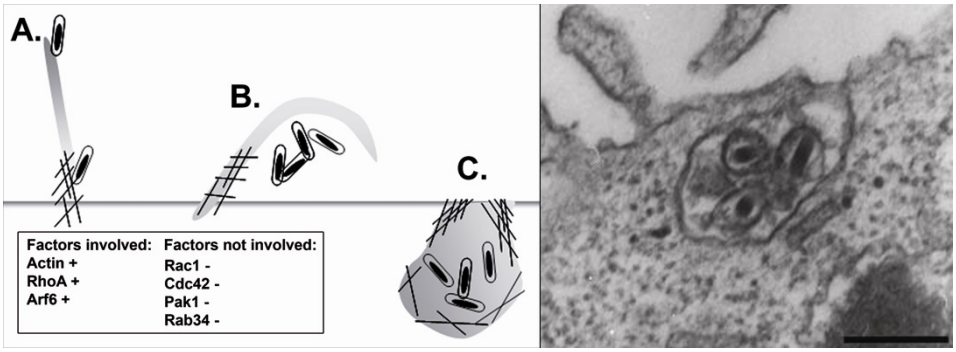

FIGURE 5 Mechanism and regulation of baculovirus entry to human cancer cells. See text for details. The figure was kindly provided by Johanna P. Laakkonen.

Macropinocytosis is a form of clathrin and caveolin-independent uptake often associated with frequent actin-driven ruffles on the plasma membrane. The route is often elicited by growth factors, and phorbol esters and relies on molecules such as Rac1, Pak1, PI3K, PLC, Rab34, and actin for entry (Jones, 2007). Few bacteria and viruses, such as *Shigella flexneri*, *Salmonella typhimurium*, *Haemophilus influenzae*, HIV-1, vaccinia and Ad have been shown to utilize macropinocytosis for their entry (Liu et al., 2002; Meier et al., 2002; Meier and Greber, 2004; Mercer and Helenius, 2008; Nhieu and Sansonetti, 1999). This relatively random uptake of particles by macropinocytosis-like movements has also been called triggered phagocytosis (Swanson, 2008). Phagocytosis is a solid particle uptake pathway, but resembles macropinocytosis in many aspects by engulfing large amounts of membrane into the cytoplasm. In addition to professional phagocytes, a number of additional cell types are able to engulf material by a phagocytic mechanism (Niedergang and Chavrier, 2005). There are rather well–accepted diagnostic criteria for phagocytosis such as, low pH-dependence, activation of Rho GTPases (RhoA, Rac1, and/or Cdc42), a requirement for actin dynamics, and dependence on dynamin- 2 (Caron and Hall, 1998; Clement et al., 2006; Niedergang and Chavrier, 2005). The pathway does not result in increased fluid-phase uptake (Niedergang and Chavrier, 2005), and does not depend on clathrin (Conner and Schmid, 2003; Conner and Schmid, 2003), caveolin (Bishop, 1997; Pelkmans, 2005), flotillin (Glebov et al., 2006), or so-called GEEC (Kalia et al., 2006) pathways.

Exploitation of multiple cellular entry mechanisms appears to be a common phenomenon among viruses, for example in the cases of HIV-1 (Liu et al., 2002), Ad serotype 2 (Meier et al., 2002; Meier and Greber, 2004) and influenza virus (Nunes-Correia et al., 1999; Nunes-Correia et al., 2004), thus complicating the transductional targeting of viral gene therapy vectors. The

mechanism of baculovirus entry appears to share many features of phagocytosis, hence the name "phagocytosis-like", and macropinocytosis. The present results demonstrated that baculovirus is able to cointernalize with the fluid-phase markers HRP and dextran (V), and the entry was stimulated by the treatment of the cells with human epidermal growth factor up to 10-fold (unpublished results). However, viral entry was neither inhibited by the application of the amiloride analog, EIPA, which has commonly been regarded as an inhibitor of macropinocytosis despite its effects also in other pathways (Jones, 2007), nor was it regulated by the Rac1, Pak1 or Rab34 GTPases (V). Clear inhibition of viral transduction was observed following treatment with the inhibitors of PI3K and PLC required for both macropinocytosis and phagocytosis (Araki et al., 1996; Greenberg, 1999; Jones, 2007) (V). Consistent with the notion that baculovirus entry could occur by a phagocytosis-like uptake, the pH-dependent mechanism was dependent on dynamin, but independent on the clathrin, caveolin, flotillin, and GEEC-pathways, and the virus was able to induce phagocytosis of *E. coli* following cointernalization of the virions with these phagocytic tracers (V). However, in the light of fluid-phase uptake, baculovirus entry does not directly resemble a phagocytosis-like mechanism. In addition, the involvement of the actin mediators Arf6 and RhoA (Ridley, 2006), regulators of baculovirus uptake, have been implicated in both macropinocytosis and phagocytosis (Jones, 2007; Niedergang and Chavrier, 2005), making them indefinite markers for the conclusive identification of phagocytic endocytosis. Also, the involvement of Rab34 in macropinocytosis is thought to be cell type specific (Goldenberg et al., 2007). Thus, the neutral effect of the DN Rab34 (V) does not directly exclude macropinocytosis as an internalization mechanism. Colocalization of baculovirus with rabankyrin-5, a Rab5 effector that localizes in large vacuolar structures corresponding to macropinosomes (Schnatwinkel et al., 2004), and knockdown of its expression could define the involvement of macropinocytosis in baculovirus entry. Moreover, the relatively high quantity of baculovirus used may trigger cellular responses that are different from single viruses.

Phagocytosis is a dynamin-dependent and raft-derived process. Likewise, the early uptake of baculovirus as well as the virus-triggered internalization of *E. coli* was sensitive to dynasore and to drugs that affected raft domains. These results suggest a similar regulation for both of these processes. The induction of phagocytosis by baculovirus was unexpected since it has been generally supposed that phagocytosis could not be induced by particles smaller than 500 nm in diameter (Rabinovitch, 1995; Sansonetti, 2001). The criterion of particle size, however, is not sufficient to generally predict the mechanism of internalization, since, for example, clathrin and caveolin are also involved in the internalization of particles larger than 1 μm (Li et al., 2005; Veiga and Cossart, 2005). Furthermore, recent studies have demonstrated that phagocytosis could be triggered by tracers corresponding to the size of baculovirus including latex beads as small as 130 nm (Desjardins and Griffiths, 2003), or 170-200 nm enveloped HSV-1 virions (Clement et al., 2006). The phagocytosis-like internalization of HSV-1 was regulated by RhoA but not Cdc42 or Rac1

(Clement et al., 2006), while Arf6 was shown to facilitate the phagocytic uptake of red blood cells in macrophages and entry of a small bacterium *Chlamydia* by reorganizing actin (Balana et al., 2005). Interestingly, baculovirus-mediated activation of innate immune responses, specifically the viral inactivation by the complement system, has been demonstrated (Hofmann and Strauss, 1998; Hofmann et al., 1999; Huser et al., 2001; Huser and Hofmann, 2003), and complement-mediated phagocytic uptake has been reported for viruses such as HSV (Van Strijp et al., 1989). Furthermore, phagocytosis or a phagocytosis-like mechanism has been implicated in the entry of viruses including many paramyxoviruses smaller in size than baculovirus (Silverstein and Marcus, 1964), the giant mimivirus (Ghigo et al., 2008), as well as vaccinia (Locker et al., 2000). Also trapping of baculovirus by the phagocytic pathway and subsequent transportation to intracellular compartments positive for the Toll-like receptor-9 has been demonstrated in the non-permissive macrophages (Abe et al., 2005).

To conclude, the functional entry of baculovirus occurs in highly-permissive human cancer cells through a low pH-dependent process that commences from cellular raft areas leading to viral engulfment into large, smooth-surfaced vesicles and does not involve raft-derived IL2-receptor, flotillin or GEEC pathways. The mechanism reminds of phagocytosis as it is regulated by dynamin, Arf6 and RhoA and induces the uptake of *E. coli* in non-phagocytic cells. As baculovirus holds potential for gene therapy strategies, an understanding of its entry mechanism may prove valuable in designing effective baculovirus-mediated delivery routes and aid in defining potential cell-type-specific targets for therapy. Moreover, as this study describes the strategy used by baculovirus to enter non-host human cells, it also has the potential to provide novel insights into the field of host-pathogen interactions.

7 CONCLUSIONS

The main conclusions of this thesis are:

1. Differences in the structures of the surface displayed tumor-targeting peptides have a detectable influence on the efficiency, mode and kinetics of binding, internalization and gene delivery of baculovirus vectors. Tumor targeting of baculovirus *in vivo* is feasible by display of a tumor-homing peptide, such as LyP-1, with high specificity and affinity.

2. Of the baculovirus phenotypes, the BV is the most efficient in mediating gene delivery to human cells and facilitating modification of vector tropism using display techniques. Albeit capable of cellular binding and limited internalization, phenotypically unmodified ODV is incapable of mediating successful transduction in human cancer cells.

3. BV enters highly permissive human cancer cells along fluid-phase markers from the raft areas into smooth-surfaced vesicles devoid of clathrin. The internalization is regulated by dynamin, and the actin mediators Arf6 and RhoA. In addition, the virus is able to induce ruffle formation and the uptake of fluorescent *E. coli* bioparticles in non-phagocytic cells. The mechanism thus shares some features of professional phagocytosis and macropinocytosis.

The potential involved in the transductional targeting of baculovirus in the light of the present understanding of the molecular mechanisms of viral entry and display technologies encourage further studies in this field. It seems realistic to expect that if and when the limitations associated with the modification of viral tropism and activation of innate immune responses can be better addressed, baculovirus could become an efficient gene therapeutic tool in the treatment of diverse diseases including cancer. Despite the appealing potential of baculovirus, only the future will tell when the first clinical application of this versatile insect virus becomes a reality.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Kohti terapeuttista geeninsiirtoa: bakuloviruksen kohdennus ja sisäänmeno ihmisen syöpäsoluihin

Geeniterapiaa pidetään yhtenä tulevaisuuden hoitomuodoista useiden perinnöllisten ja ympäristön aiheuttamien sairauksien kuten syövän sekä sydän- ja verisuonisairauksien hoidossa. Hoidon edellytyksenä on onnistunut geeninsiirto kohdekudokseen kuljettimen eli vektorin avulla. Virukset ovat evoluution myötä kehittyneet luonnostaan tehokkaiksi geeninsiirtäjiksi ja ovatkin nykyään käytetyimpiä geeninsiirtovektoreita. Yksi geenihoidon yleistymisen esteistä on kuitenkin ollut virusvektoreiden puutteellinen kohdennus haluttuun kudokseen. Geeninsiirtoa on pyritty tehostamaan lisäämällä viruksen määrää, mikä voi aiheuttaa vakavia sivuvaikutuksia. Geeninsiirron kohdentamista tarvitaan erityisesti syövän hoidossa, jotta hoito vaikuttaisi pääasiallisesti pahanlaatuisiin soluihin. Molekyylejä, jotka sitoutuvat syöpäkasvaimen veri- ja imusuonien endoteelisolujen tai kasvainsolujen pinnalla ilmentyviin kudoksen tunnusmerkillisiin reseptoreihin, on eristetty viime vuosina erilaisista peptidi- ja proteiinikirjastoista. Näiden avulla geenihoito voidaan kohdentaa vain haluttuun elimeen tai kasvaimeen liittämällä peptidiosoitelappu geeninsiirtovektoriin.

Bakulovirukset ovat hyönteisiä infektoivia viruksia ja luontaisesti vaarattomia ihmisille. Mallibakulovirus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), tuottaa elinkiertonsa aikana kahta erilaista virustyyppiä: silmikoituvaa virusta (*engl.* budded virus; BV) sekä okluusioperäistä virusta (*engl.* occlusion-derived virus; ODV). Nämä ilmiasut ovat perimältään samanlaisia, mutta poikkeavat rakenteeltaan ja toiminnaltaan toisistaan. BV on sauvamainen ja sen proteiinikuorta eli kapsidia ympäröi isäntäsolun solukalvosta peräisin oleva vaippa, jonka toisessa päässä on GP64-glykoproteiineja. GP64 välittää BV:n tunkeutumista isäntäsoluunsa sekä kuroutumista infektoidusta solusta ulos ja on siten merkittävässä roolissa viruksen elinkierrossa. ODV sisältää puolestaan useita kapsideja, joita ympäröi tuman kalvorakenteista peräisin oleva vaippa. ODV vastaa infektion aloituksesta, kun taas BV on erikoistunut solusta-soluun infektioon, jonka tarkoituksena on levittää infektiota järjestelmällisesti hyönteisen koko elimistöön. Bakulovirus säilyy luonnossa polyhedriini-proteiinista koostuvassa matriisissa, polyhedrassa, joka sisältää useita ODV-virioneja. *Ac*MNPV:n perintöaines on 133 894 emäsparin mittainen ja muodostuu kaksisäikeisestä, rengasmaisesta DNA:sta, joka koodaa teoreettisesti 150:tä proteiinia.

Bakuloviruksella on useita ominaisuuksia, joiden ansiosta se on turvallinen ja tehokas vektoriehdokas geeniterapiaan. Näihin ominaisuuksiin sisältyvät mm. kyky ilmentää siirtogeenejä sekä jakautuvissa että jakautumattomissa nisäkässoluissa ja kyky siirtää suuria määriä hoitavaa DNA:ta kohdesoluun. Koska bakulovirus ei kykene lisääntymään nisäkässoluissa, se on turvallinen vaihtoehto patogeenisille virusvektoreille.

Lisäksi sen ohjautumista tiettyihin kohdesoluihin voidaan manipuloida useilla vaihtoehtoisilla tekniikoilla kuten muuntelemalla geneettisesti viruksen pintaproteiineja, liittämällä kohdennus- tai tarttumispeptidejä viruksen pinnalle, käyttämällä kohdennusvasta-aineita tai korvaamalla viruksen omia pintaproteiineja vierasta alkuperää olevien virusten vastaavilla proteiineilla. Kohdennuspeptidejä tai -proteiineja voidaan ilmentää bakuloviruksen pinnalla osana viruksen omia tai vierasperäisiä vaipan glykoproteiineja.

Bakulovirus kykenee siirtämään ja ilmentämään vieraita geenejä monissa nisäkässolutyypeissä. Suurin osa bakuloviruksen ja nisäkäsolujen välisiin vuorovaikutuksiin sekä geeninsiirtoon liittyvä tutkimus on tehty BV:lla, kun taas ODV:n hyödyntämistä vastaavissa sovelluksissa on tutkittu vähän tai ei lainkaan. Sekä BV:n että ODV:n sitoutumis- ja sisäänmenomekanismit tunnetaan melko hyvin hyönteissoluissa, mutta näiden tapahtumien yksityiskohdat nisäkässoluissa ovat vielä laajalti epäselviä. Tämän väitöskirjatyön tavoitteena oli kehittää syöpäsoluihin kohdentuvia bakulovirusperäisiä geeninsiirtovektoreita ja tutkia viruksen sisäänmenomekanismeja ihmisen syöpäsoluihin sekä viljelmissä että koe-eläimissä.

Tutkimuksessa kehitettiin useita vaipaltaan muokattuja BV-vektoreita, jotka rakennettiin geeniteknisesti liittämällä lyhyitä, syöpäkasvaimiin ohjautuvia peptidejä (LyP-1, F3 ja CGKRK) vesicular stomatitis-viruksen G-proteiinin kalvoankkurin välityksellä viruksen pinnalle. Nämä räätälöidyt vektorit sitoutuivat ja tunkeutuivat viljeltyihin rinta- ja maksasyöpäsoluihin selvästi tehokkaammin ja osittain myös valikoidummin verrattuna kontrollivirukseen. Peptidien onnistunut ilmentäminen johti myös huomattavasti parempaan geeninsiirtoon. Lisäksi hiiren verisuonistoon ruiskutettu, LyP-1-peptidiä ilmentävä vektori kohdentui valikoidusti syöpäkasvaimiin, kun taas F3- ja CGKRK-virusten parantunut tehokkuus soluviljelmissä ei toistunut eläinkokeissa. Sekä liukoinen että bakuloviruksen kapsidissa ilmennetty F3-peptidi esti merkittävästi viruksen geeninsiirtoa maksasyöpäsoluissa ja tarjoaa näin käyttökelpoisen peptidityökalun bakuloviruksen sisäänmenon ja kulkeutumisen tarkasteluun nisäkässoluissa. Tutkimuksessa osoitettiin myös, että pinnaltaan muokkaamaton ODV sitoutuu BV:n tavoin melko tehokkaasti ihmisen maksa- ja keuhkosyöpäsoluihin, mutta vaillinaisen sisäänmenon ja tehottoman solulimaan vapautumisen seurauksena se ei kykene välittämään geeninsiirtoa ja -ilmentymistä kohdesoluissa. Jotta ODV:ta voitaisiin hyödyntää BV:n tavoin geeninsiirrossa, ODV:n pintaproteiinien toiminnallinen muokkaus tehokkaan sisäänmenon mahdollistamiseksi on avainasemassa.

Lopuksi BV:n sisäänmenon ihmisen syöpäsoluihin osoitettiin olevan aikaisemmista ehdotuksista poiketen riippumatonta klatriinivälitteisestä endosytoosista. Sen sijaan sisäänmeno muistutti mekanismiltaan fagosytoosin kaltaista prosessia, jonka säätelyyn vaikuttivat aktiinin polymerisaatio, dynamiini sekä Arf6- ja RhoA-GTPaasit.

Tämän tutkimuksen tulokset edesauttavat syöpäsoluihin sekä syöpäkasvaimien veri- ja imusuonistoon kohdentuvien bakulovirusvektoreiden kehitystyötä mahdollisiin geeniterapiasovelluksiin.

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