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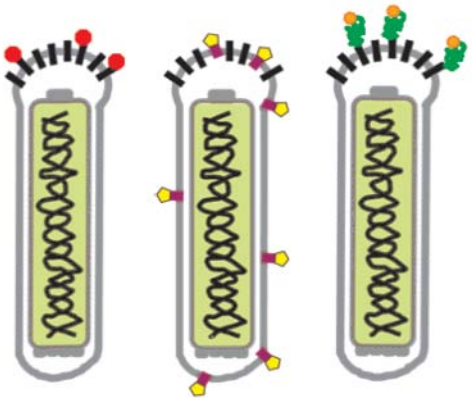
168

Heli Matilainen

Development of Baculovirus Display Strategies Towards Targeting to Tumor Vasculature



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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2006

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Johan sitä masentuu, kun vain ajatteleekin kaikkia niitä, jotka tekevät työtä ja raatavat, ja mitä hyötyä siitä muka on. Eräs sukulaiseni luki trigonometriaa tuntokarvansa lerpalleen, ja kun hän oli oppinut kaiken, tuli Mörkö ja söi hänet suuhunsa. Joopa joo, Mörön vatsassa hän sitten lojui niin erinomaisen viisaana!

Juksu

ABSTRACT

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Diss

Targeting of viral vectors to specific cells has received an increasing interest in the area of therapeutic gene transfer. However, specific targeting to tumors, especially to tumor vasculature has been impeded by the limitations of present vector systems and lack of selective markers. Therefore, peptides that home to specific sites in the tumor vasculature or tumors, are attractive as targeting moieties for gene therapy purposes. *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), a prototype of the *Baculoviridae* family, is a promising new vector candidate for gene therapy. Moreover, baculovirus display -technology enables the presentation of targeting moieties on the viral surface. The goal of this study was to develop baculovirus vectors for targeted and enhanced gene transfer to cancer cells. To this end, specific tumor and tumor vasculature targeting peptides, such as RGD, RKK, LyP-1, F3 and CGKRK, were successfully displayed on the baculoviral surface. Most importantly, the recombinant viruses showed enhanced binding and gene delivery to their target cells *in vitro*. In order to develop a functional targeted virus vector, the entry mechanism of baculovirus was elucidated. Baculovirus was shown to enter human hepatocarcinoma cells using the endosomal entry route, possibly via clathrin coated vesicles. In addition, the use of macropinocytosis was suggested. Additionally, to study the receptor of RKK targeting motif, α2β1 integrin, echovirus 1 (EV1) entry was studied. The α2β1 integrin was shown to internalize in concert with EV1 using caveolae endocytosis. Together, the results of this thesis demonstrate the feasibility of baculovirus display of targeting peptides to enhance gene delivery to target cells and, thus, suggests baculovirus to possess potential for targeted tumor therapy.

Keywords: baculovirus; viral entry; display; echovirus 1; gene transfer; integrin; tumor homing peptides; targeting.

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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 Matilainen, H.*, Rinne, J.*, Gilbert, L., Marjomäki, V., Reunanen, H. & Oker-Blom, C. 2005. Baculovirus entry into human hepatoma cells. J. Virol. 79: 15452-15459.

II Marjomäki, V., Pietiäinen, V., Matilainen, H., Upla, P., Ivaska, J., Nissinen, L., Reunanen, H., Huttunen, P., Hyypiä, T. & Heino, J. 2002. Internalization of echovirus 1 in caveolae. J. Virol. 76: 1856-1865.

III Matilainen, H., Mäkelä, A.R., Riikonen, R., Saloniemi, T., Korhonen, E., Hyypiä, T., Heino, J., Grabherr, R. & Oker-Blom, C. 2006. RGD motifs on the surface of baculovirus enhance transduction of human lung carcinoma cells. J. Biotechol. 125: 114-126.

IV Riikonen, R., Matilainen, H., Rajala, N., Pentikäinen, O., Johnson, M., Heino, J. & Oker-Blom, C. 2005. Functional display of an alpha2 integrin-specific motif (RKK) on the surface of baculovirus particles. Technol. Cancer. Res. Treat. 4: 437-445.

V 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. J. Virol. 80: 6603-6611.

* Equal contribution

RESPONSIBILITIES OF HELI MATILAINEN IN THE ARTICLES OF THIS THESIS

Article I: I am responsible for planning the experiments for this paper. I and Leona Gilbert performed the expression studies. Johanna Rinne is responsible for most of the confocal microscopy experiments with participation of myself. Hilkka Reunanen primarily conducted the electron microscopy studies in collaboration with Johanna Rinne and myself. Rinne wrote the article and I took part in finalizing it.

Article II: I am responsible for many of the double labeling studies for confocal microscopy, the EV1 protein production studies and some inhibition experiments. In addition, I have participated in the electron microscopy studies, the sucrose gradient centrifugations and the infectivity titration experiments.

Article III: I am mainly responsible for the design and implementation of this study and I also wrote the article. Anna Mäkelä constructed the luciferase-expressing viruses, conducted the transduction experiments, wrote the corresponding part in the paper, and participated in finalizing the manuscript.

Article IV: I participated in designing the genetic constructs and some cloning procedures. In addition, I helped in writing the article.

Article V: I am responsible for the design of the basic concept of this article. Anna Mäkelä constructed and characterized the recombinant viruses as well as planned and performed the binding and transduction experiments. I am responsible for the binding cross inhibition studies together with Mäkelä and Daniel White. Anna Mäkelä wrote the article and I participated in finalizing it.

Studies I, III-V were carried out under the supervision of Prof. Christian Oker-Blom and study II under the supervision of Dr. Varpu Marjomäki.

ABBREVIATIONS

5'UTR	5' untranslated region
A549	human lung carcinoma cell line
aa	amino acid
AAV	adeno-associated virus
*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
APN	aminopeptidase N
BHK	baby hamster kidney cell line
BV	budded virus
CAR	coxsackievirus-adenovirus receptor
CAV9	coxsackie A virus 9
CD-34	sialomucin, tumor lymphatic endothelial marker
CD63	lysosomal membrane glycoprotein
CEA	carcinoembronic antigen
CGKRK	cysteine-glycine-lysine-arginine-lysine
CHO	Chinese hamster ovary cell line
CI-MPR	cation-independent mannose 6-phosphate receptor
CMV	cytomegalovirus
CT	cytoplasmic
DAF	decay accelerating factor
DGEA	aspartic acid-glycine-glutamic acid-alanine
DMEM	Dulbecco's modified eagle medium
EAHY	human endothelial aortic hybridoma cell line
ECM	extracellular matrix
EEA1	early endosomal antigen 1
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EV	echovirus
F	major envelope protein on group II BV
F3	tumor homing peptide
FACS	fluorescent activated cell sorter
FBS	fetal bovine serum
FCS	fluorescent correlation spetroscopy
FISH	fluorescence i*n situ* hybridisation
FMDV	foot-and–mouth-disease virus
GFP	green fluorescent protein
GluR-D	glutamine receptor signal sequence
GMK	green monkey kidney cell line
gp120	HIV major surface glycoprotein
gp41	HIV envelope glycoprotein
gp64	baculovirus group 1 major envelope glycoprotein 64
gp75	orthomyxoviridae envelope glycoprotein 75

GPI	glycosyl-phosphatidylinocitol
GS-linker	glycine-serine -linker
GST	glutathione-S-transferase
GTPase	guanosine triphosphate hydrolyzing enzyme
GV	granulovirus
HepG2	human hepatoma cell line
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMGN2	human high mobility group protein 2
HPEV1	human parechovirus 1
HPV14	human rhinovirus 14
HRP	horseradish peroxidase
Huh7	human hepatoma cell line
IE	immediate early
Ig	immunoglobulin
IRKK	isoleucine-arginine-lysine-lysine
LDL	low–density lipoprotein
*Ld*MNPV	*Lymantia dispar* MNPV
LLC-MK2	monkey rhesus kidney cell line
luc	firefly luciferase
LyP-1	tumor homing peptide
LYVE-1	lymphatic endothelial hyaluronan protein
mAb	monoclonal antibody
MDA-MB-435	human breast carcinoma cell line
MEM	minimal essential medium
MG63	human osteosarcoma cell line
MHC	major histocompability complex
MIDAS	metal ion-dependent adhesion site
MMP	matrix metalloproteinase
MNPV	multiple nucleopolyhedrovirus
MOI	multiplicity of infection
mRNA	messenger RNA
NHO	normal human osteoblast cell line
NG2	melanoma-associated high molecular weight antigen
NGR	asparagine-glycine-arginine
NPV	nucleopolyhedrovirus
ODV	occluded nucleopolyhedrovirus
*Op*MNPV	*Orgyia pseudotsugata* MNPV
p.i.	post infection
p.t.	post transduction
p53	tumor suppressor protein
p67	antigen of *Theileria parva*
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PDGF	platelet-derived growth factor
PDI	protein disulfide isomerase
PFA	paraformaldehyde
pfu	plaque forming unit
PI	phosphoinositol
Pk1	porcine kidney cell line
PKCα	protein kinase Cα
PLC	phospholipase C
polyAla	poly-alanine
PV	poliovirus
RGD	arginine-glysine-aspartic acid
RKK	arginine-lysine-lysine
RKKH	arginine-lysine-lysine-histidine
RT	room temperature
SAOS	human osteosarcoma cell line
SARS	severe acute respiratory synrome
scFV	single–chain antibody
sCR1	soluble complement receptor type 1
SDS	sodium docedyl sulphate
*Se*MNPV	*Spodoptera exigua* MNPV
sf	*Spodoptera frugiperda*
SNPV	single nucleopolyhedroviruses
ss	signal sequence
SV40	simian virus 40
TGN	trans golgi network
TM	transmembrane
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VLP	virus-like particle
VP	viral protein
vp39	baculovirus capsid viral protein 39
VSV	vesicular stomatitis virus
VSV-G	vesicular stomatitis virus G-protein
wt	wild type
Z domain	synthetic IgG-binding domain of protein A

CONTENTS

1 INTRODUCTION

Viruses are currently regarded as the most efficient gene delivery vehicles. The proof of viral therapeutic gene transfer concept has been demonstrated in a large number of animal studies and viruses are being utilized in numerous gene transfer applications. However, there is no perfect vector and several factors have hampered the progress of viral gene delivery. As the use of pathogenic viral vectors has been drawn into question during the latest years, the urge of non-pathogenic safer gene therapy vectors is apparent. To this end, the need for targeting has become evident. Specific targeting to cells or tissues would diminish side effects of possibly toxic gene products to healthy tissues and improve the efficacy of the treatment.

For treatment of cancer, gene delivery through vasculature is an attractive method, but has been largely restricted by the lack of vector specificity. The discovery of specific vascular signatures from tumor blood vessels by *in vivo* phage display has increased the opportunities for specific targeting to tumor vasculature.

In this thesis work, baculovirus *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), was utilized to develop display strategies for targeting to tumor vasculature. Baculovirus has a long history for production of recombinant proteins in insect cells and it is regarded as a safe vector candidate due to the lack of replication in mammalian cells. More recently, the gene delivery to numerous mammalian cell lines of different origin has also been demonstrated. The baculovirus display -technology has been successfully used for several different applications and has lately been diversified into presentation of targeting moieties on the surface of baculovirus. Therefore baculovirus can be regarded as an attractive candidate for targeted gene delivery.

Thus, the main goal for this study was to create recombinant baculovirus vectors displaying various tumor cell and/or tumor vasculature homing peptides on the surface of the virions. The peptides were expressed as fusions to either the second copy of viral membrane protein gp64 or the truncated vesicular stomatitis virus G membrane protein (VSV-G), thus, enabling

presentation of the targeting peptides on the surface of recombinant viruses. The recombinant targeting peptide displaying viruses were subjected to binding and transduction studies in the corresponding human cancer cell lines.

In order to develop efficient viral gene delivery vehicles, the entry route of the virus and receptor targeted should be known in detail. For this purpose, entry mechanisms of baculovirus and echovirus 1 (EV1), together with its receptor α2β1, were studied. More detailed knowledge of viral entry mechanisms will further be of use both in gene therapy applications and in the remedy of pathogenic virions.

2 REVIEW OF THE LITERATURE

2.1 Baculoviruses

The *Baculoviridae* family is characterized by "a large double-stranded DNA genome, which is packed in a rod-shaped capsid and enveloped by a unit membrane". The name baculovirus derives from the Latin word *baculum*, meaning "rod" or "stick" (Miller 1996). Baculoviruses infect predominantly insects of the order of Lepidoptera (Blissard & Rohrmann 1990). Nucleopolyhedroviruses (NPVs) have many virions within intranuclear crystal called polyhedra, granuloviruses (GVs) have only one virion inside each crystal or granule (Volkman 1997). NPVs can be further divided into multiple nucleopolyhedroviruses (MNPVs) containing many nucleocapsids per envelope and single nucleopolyhedroviruses (SNPVs) containg one nucleocapsid per envelope (Blissard & Rohrmann 1990).

Occluded form (ODV) of the NPV, surrounded by the crystalline protein matrix of polyhedrin is responsible for the primary infection of the larvae (Blissard 1996). Although extremely stable in the environment, the matrix is solubilized in the alkaline midgut of the larva, releasing the viruses, which then enter the midgut epithelial cells by fusion with microvilli (Blissard 1996, Blissard & Rohrmann 1990, Volkman 1997). During infection, two forms of the virion are produced. Occluded virions are produced and packed into the polyhedras inside the nucleus and are eventually released back to the environment as a consequence of cell lysis (Blissard 1996). In addition, some capsids are transported to cytoplasm and bud from the basal side of the midgut epithelial cells to the hemocoel. These budded viruses (BVs) are responsible for the secondary infection in the host and also infection of cell cultures (Blissard 1996, Blissard & Rohrmann 1990). The infection in cell cultures can be divided into three phases: early phase preceding viral DNA replication (0-6 hours p.i.), late (6-24 h p.i.) and very late phase (18-24 to 72 h p.i.) (Miller 1996). The budded virus is produced during the late phase and the occluded form during the very late phase of the infection (Blissard 1996).

2.1.1 Biology and structure of budded virus

Autographa californica multiple nucleopolyhedrovirus, *Ac*MNPV, isolated from the alfalfa looper *Autographa californica*, is a prototype virus within the family *Baculoviridae* (Blissard & Rohrmann 1990, Miller 1996). The budded form of the virus (BV) has a single rod-shaped nucleocapsid surrounded by a loose-fitting envelope (Figure 1) (Blissard 1996). The viral DNA (134 kbp) is associated with the DNA binding protein p6.9 in the rodshaped nucleocapsid composed of the vp39 major capsid protein together with several other minor proteins (Ayres et al. 1994, Blissard 1996, Thiem & Miller 1989). The structure of capsid and viral DNA of BV is similar to ODV, but the lipid and protein composition of the membrane differs, reflecting the distinct roles of the viral forms in the infection process (Blissard 1996). The membrane of BV, composed largely of phosphatidylserine, is derived from the plasma membrane of the host cell as the virus buds (Blissard 1996, Braunagel & Summers 1994).

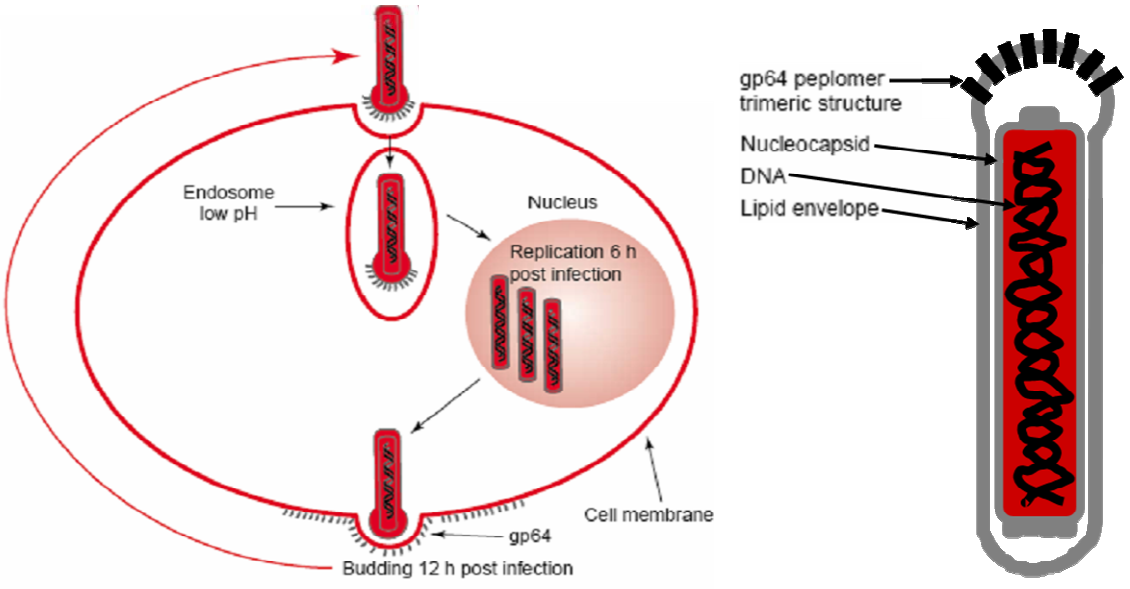

FIGURE 1 Life cycle and structure of budded baculovirus (BV). The budded virion contains a dsDNA genome surrounded by nucleocapsid and lipid membrane. Major membrane glycoprotein gp64 forms trimers that are concentrated on one end of the virion. BV enters insect cells by endocytosis resulting in the release of viral nucleocapsids to the cytoplasm. Nucleocapsids are subsequently transported to the nucleus for replication. Budding of assembled virus capsids out of the cell is mediated by gp64 molecules. Reprinted from Trends Biotechnology, 19, Grabherr, R., Ernst, W., Oker-Blom, C., Jones, I. 231-236, Developments in the use of baculoviruses for the surface display of complex eucaryotic proteins, Copyright (2001), with permission from Elsevier.

2.1.2 Major membrane glycoprotein gp64

A major envelope glycoprotein, gp64, is found in BVs of lepidopteran NPV group I viruses (Herniou et al. 2001, Okano et al. 2006, Zanotto et al. 1993), like *Autographa californica Ac*MNPV (Whitford et al. 1989) and *Orgyia pseudotsugata* (*Op*MNPV) (Blissard & Rohrmann 1989). This highly conserved glycoprotein forms spike-like peplomers that are mainly located at the end of the virion and become incorporated into the viral particles during the budding process (Volkman et al. 1984, Volkman 1986). Gp64 is a type I integral membrane protein having an N-terminal signal sequence and a transmembrane (TM) domain near the C-terminus (Blissard & Rohrmann 1989) (Figure 2). Gp64 is synthezised throughout the infection cycle, and the maximal rate of synthesis is observed in the period of 24-26 h p.i. (Blissard & Rohrmann 1989, Jarvis & Garcia 1994, Oomens et al. 1995, Volkman 1997). The glycoprotein is present in trimeric form on the surface of the cell and virion (Oomens et al. 1995). Trimerization, mediated by the oligomerization domain (Monsma & Blissard 1995), is a rapid but inefficient process involving intermolecular disulfide bond formation. Monomeric proteins are subsequently being degraded in infected cells (Oomens et al. 1995, Volkman & Goldsmith 1984). Gp64 is phosphorylated (Volkman & Goldsmith 1984), N-glycosylated (Volkman et al. 1984) and acylated by palmitoylation (Roberts & Faulkner 1989, Zhang et al. 2003). N-linked glycosylations are present in four out of five potential sites in *Ac*MNPV gp64 (Jarvis et al. 1998). Some of the glycosylation sites may contain complex sugars. N-glycosylation is necessary for the optimal gp64 function, however, the structure of the glycoforms is relatively irrelevant (Jarvis & Garcia 1994). Gp64 is responsible for the host cell receptor binding activity (Hefferon et al. 1999) and is both essential and adequate for mediating pH-dependent membrane fusion during viral entry (Blissard & Wenz 1992) with the help of the membrane fusion domain (Monsma & Blissard 1995). In addition, the gp64 protein is necessary for cell-to-cell transmission of the BV (Monsma et al. 1996) (See 2.1.3) and is important for efficient virion maturation and budding (Oomens & Blissard 1999).

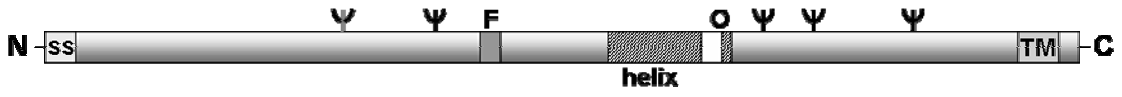

FIGURE 2 Schematic representation of the *Ac*MNPV gp64 major membrane glycoprotein. ss, signal sequence; helix, predicted alpha-helical region; TM, transmembrane domain; ψ predicted N-glycosylation site; F, fusion domain; O, oligomerzation domain. Based on Monsma & Blissard 1995.

The group I lepidopteran NPVs, including *Ac*MNPV, encode an additional envelope fusion protein F that does not seem to mediate membrane fusion. However, the F fusion protein is functional in baculoviruses lacking gp64 e.g. in group II lepidopteran NPVs and GVs (Okano et al. 2006).

2.1.3 Baculovirus entry

The infection of BV (*Ac*MNPV) in *Spodoptera frugiperda* 9 (*sf*9) cell culture is thought to represent the secondary infection in the insect host (Miller 1996). In addition to insect cells, BV has been found to enter mammalian cells without viral gene expression (Volkman & Goldsmith 1983). The precise steps in the binding and entry of budded virions to insect and mammalian cells are however not yet characterized.

Entry to insect cells. The receptor of BV is unknown. Scatchard analysis of virus-insect cell -interactions indicate a strong and specific receptor mediated binding (Wang et al. 1997, Wickham et al. 1992). However, the rate of BV attachment appears to be the limiting step in the infection and viral binding reaches equilibrium only after 3-5 hours at 4°C (Wickham et al. 1992). The involvement of insect cell surface proteins in the binding is controversial. According to two studies based on experiments in the presence of metabolic inhibitors, enzymes and competitive substances, cellular glycoprotein component(s) might be involved in the binding process (Wang et al. 1997, Wickham et al. 1992). In contrast to this, the study of Tani and colleagues (2001) showed no involvement of cell surface proteins, but they suggested the involvement of phospholipids in the binding or entry process of recombinant BV displaying extra amount of gp64 protein on the membrane (Tani et al. 2001). Additionally, specific bivalent cations, Ca^{2+} and Mg^{2+}, not just simple electrostatic charge, are likely to be involved in the binding by a yet uncharacterized mechanism (Wang et al. 1997).

To date, no specific antibody blocking of the viral binding to the insect cell surface has been reported. Although neutralizing antibodies, like AcV1, recognise gp64, the antibodies neutralize the infection without blocking the viral binding (Roberts & Manning 1993, Volkman et al. 1984, Volkman & Goldsmith 1985). Despite this, evidence supports the use of gp64 for the receptor binding molecule of BV. The elimination of one or more N-glycosylation sites in gp64 have been shown to impair the viral binding in insect cells (Jarvis et al. 1998). The active role of the gp64 in binding of the virion to the insect cell surface was confirmed by soluble gp64 competition (Hefferon et al. 1999).

Early studies of BV entry to insect cells, based on inhibition of the infection by ammonium chloride or chloroquine treatments that prevent the endosomal acidification, proposed the use of adsorptive endocytosis as the productive entry pathway (Volkman 1986, Volkman & Goldsmith 1985). These results were later confirmed with fluorescence microscopy analysis of the membrane protein gp64 and capsid protein vp39. As a consequence of the chloroquine treatment the vp39 capsid protein remained in the cytoplasm with gp64 and was not able to enter the nucleus (Charlton & Volkman 1993). Additionally, Wang and coworkers successfully inhibited endocytosis of the virus with chloroquine and sodium azide (Wang et al. 1997). The release of

nucleocapsids from endosomes, measured with ammonium chloride inhibition assays, took place between 15 and 30 min p.i., the halftime being 25 min (Hefferon et al. 1999).

The importance of gp64 in the endocytosis was demonstrated by blocking the endocytosis, and destroying the sensitivity to weak bases with the mAb AcV1 (Volkman & Goldsmith 1985). The same antibody was also found to prevent the capsid from entering nucleus, suggesting gp64 to have a role in the capsid release (Charlton & Volkman 1993). Later, the role of gp64 was confirmed in studies demonstrating the direct membrane fusion activity of gp64. This was accomplished by detection of syncytia formation by gp64 expressed on the insect cell surface. pH values of 5.5-5.7 were needed to trigger the fusion activity (Blissard & Wenz 1992, Leikina et al. 1992). Membrane fusion mediated by gp64 was further established by Wang and colleagues with fluorescently labeled viruses and *in vivo* membrane fusion kinetic studies (Wang et al. 1997). Together these studies confirmed that gp64 functions as the viral fusion protein mediating the release of nucleocapsid from endocytic vesicles into the cytoplasm of insect cells. This acid-induced membrane fusion activity is triggered by acidification of the endosome and presumably leads to the fusion of the BV envelope and endosomal membrane. By mutational analysis, a short fusion domain of gp64 was located. It shares features with the fusion domains of Semliki Forest virus and vesicular stomatitis virus. The fusion activity was abolished by a polyclonal antibody against this region and also with AcV1 gp64 antibody (Monsma & Blissard 1995). The membrane fusion of BV has been characterized in more detail (Chernomordik et al. 1995, Kingsley et al. 1999, Markovic et al. 1998, Oomens & Blissard 1999, Plonsky et al. 1999, Plonsky & Zimmerberg 1996). A model of this phenomenon suggests trimeric gp64 to form a multimeric complex on the membrane due to the conformational change triggered by low pH (Markovic et al. 1998).

Interestingly, a minor alternative pathway characterized as direct fusion at the plasma membrane, not inhibited by gp64 antibody AcV1 or weak bases, has been reported to exist (Volkman et al. 1986, Volkman & Goldsmith 1985). However, the mechanism and importance of this phenomenon have remained unknown. Later, this phenomenon was studied by Wang and colleagues, who, nevertheless, detected no visible fusion of the virus to the plasma membrane even in the presence of inhibitory drugs of endocytosis (Wang et al. 1997).

After uncoating, the nucleocapsids have been shown to induce the polymerization of actin cables in the cytoplasm and are transported towards the nucleus with the help of myosin-like motor (Charlton & Volkman 1993, Lanier et al. 1996, Lanier & Volkman 1998). Instead, depolymerization of microtubules seem to be a necessary event in the infection (Volkman & Zaal 1990). The major capsid protein vp39 has been detected in the nucleus from 1 to 6 h p.i. by immunofluorescent staining (Charlton & Volkman 1993). The mechanism of the genome release is not known, but the removal of stabilizing zinc ions is involved in the process (van Loo et al. 2001).

Entry to mammalian cells. Based on the first reports showing transduction of mammalian cells to be liver-specific (Boyce & Bucher 1996, Hofmann et al. 1995), the use of asiaglycoprotein receptor by BV in mammalian cells was suggested. This possibility was however excluded later with two different experimental settings (Hofmann et al. 1995, van Loo et al. 2001). Despite this, Hofmann and colleagues indicated the presence of a receptor in hepatocytes, based on competition experiments and clear dose-response curves of baculovirus-mediated gene transfer (Hofmann et al. 1995). However, the wide range of mammalian cell types that *Ac*MNPV BV enters (Kost et al. 2005, Kost & Condreay 2002), suggests BV to interact with the molecule that is conserved and present on many cell types or that the interaction is relatively non-specific (Hefferon et al. 1999, Shoji et al. 1997). Results obtained by Duisit and colleagues with binding inhibition and competition studies strongly suggest the binding of BV to involve a large number of membrane motifs and low affinity docking. Additionally, negatively charged motifs, especially heparin sulphate seem to be required for the viral binding, although also the presence of other negatively charged binding motifs is likely. The involvement of gp64 in the binding to mammalian cells is unclear (Duisit et al. 1999). As the charge of gp64 is negative under physiological conditions (Volkman & Goldsmith 1984), it is possible that other proteins or phospholipids in the viral membrane take part in the binding process (Duisit et al. 1999). Also Tani and coworkers suggested the use of phospholipids in the binding or entry process. More specifically, phosphoinositol (PI) and phosphatidic acid (PA) might be involved in the entry to HepG2 cell line (Tani et al. 2001).

The first evidence for the use of endosomal pathway in baculoviral entry into mammalian cells was provided by inhibition of transduction in HepG2 and Huh7 cells in the presence of chloroquine (Boyce & Bucher 1996, Hofmann et al. 1995). Further, van Loo and colleagues (2001) demonstrated strong inhibition of marker gene expression in Pk1 cells by chloroquine, bafilomycin A1 and ammonium chloride, all known to inhibit endosomal acidification. The halftime of endosomal escape was estimated to be about 50 min, detected by ammonium chloride inhibition assay (van Loo et al. 2001). In line with biochemical studies, virions were detected inside cytoplasmic vesicles and in the cytoplasm by electron microscopy (Condreay et al. 1999, Hofmann et al. 1995). Lately, Kukkonen and coworkers observed GFP fused viral capsids inside structures positive for early endosomal antigen 1 (EEA1) 30 min p.t. in HepG2 and EAHY cells, but not at 4 h p.t. by confocal imaging (Kukkonen et al. 2003). Additionally, they reported monensin, the inhibitor of endosomal acidification, to block the virus capsid entry into the cytoplasm, thus causing the virus to be present in the early endosomes still at 4 h p.t. The dynamitin overexpression did not affect the nuclear import of capsids, although it caused the accumulation of nucleocapsids in the vesicles at the cell periphery. Thus, it seems that dynamitin is not needed for BV cytoplasmic transport (Salminen et al. 2005).

Similarly to insect cells, gp64 has an important role in the entry process also in mammalian cells; the gp64 mAb AcV1, seems to block the transgene expression in hepatocytes, but not the binding in Huh7 cells studied (Hofmann et al. 1998). Additionally, recombinant virus expressing extra gp64 on the viral surface seems to possess higher expression of the marker gene in broader range of mammalian cell types than the control virus (Tani et al. 2001).

Uncoated BV nucleocapsids were seen in the cytoplasm at 4 h p.t. in HepG2 and Pk1 cells by electron microscopy (Kukkonen et al. 2003, van Loo et al. 2001). Endocytic modifications did not seem to be necessary for the nuclear transport of the BV, as microinjected nucleocapsids entered the nucleus normally (Salminen et al. 2005). Similarly to insect cells, the importance of the actin filaments in the virus entry was confirmed using several different actin disrupting agents that inhibited both the transgene expression and the entry of the capsid into nucleus (Salminen et al. 2005). Based on two studies, microtubulus network is not needed in viral entry as demostrated by toxin treatments causing depolymerization of microtubules. Microtubules seemed to be even a structural constraint in the nuclear import of BV as increased transgene expression in the presence of microtubule disrupting agents was detected (Salminen et al. 2005, van Loo et al. 2001).

Van Loo and colleagues (2001) reported the non-dividing mammalian cells to be susceptible to BV transduction. The marker gene expression detected in nondividing Pk1 cells arrested in S phase with aphidicolin indicates that the viral genome is transported into the nucleus. The presence of the viral genome and capsid proteins inside the nucleus was further demonstrated by FISH and confocal immunofluorescence microscopy. By electron microscopy, the uncoated intact nucleocapsids were suggested to be transported through the nuclear pore into the nucleus in Pk1 cells, a transport mechanism different from other DNA viruses (van Loo et al. 2001). In agreement with this, Kukkonen and coworkers (2003) have found viral capsids in the nucleus of HepG2 cells at 4 h p.t.

2.1.4 Baculovirus applications

The interest in baculoviruses was raised in 1949, when the development of insect pathogens into biological pesticides was established. Registration of the first insect pathogen as a pesticide took place in 1975 and since then baculoviruses has become commonly used as pesticides. As a result of resistance to chemical pesticides, new genetically engineered baculovirus pesticides have been examined in field tests (Miller 1996).

The development of *Ac*MNPV into a eukaryotic protein expression system during the early 1980s marked the start of a wide utilization of the virus to express heterologous proteins in insect cells (Oker-Blom et al. 2003). Starting from the production of human β-interferon (Smith et al. 1983), thousands of proteins, including complex eukaryotic ones, have succesfuly been produced (Kost et al. 2005). One of the latest proteins produced has been the avian

influenza H5N1 hemagglutinin molecule (Nwe et al. 2006). Major advantages in baculovirus expression system include large insertion capacity, relatively easy construction, strong promoters and post-translational processing abilities (O'Reilly et al. 1994a). In contrast to prokaryotic expression, insect cells are able to drive post-translational modifications e.g. proteolytic processing, phosphorylation, N- and O-glycosylation and acylation required for the biological activity for many eukaryotic proteins (O'Reilly et al. 1994a). However, some limitations in modification of complex N-linked oligosaccharides, e.g. terminal sialic acid have been reported (Jarvis & Finn 1996, Marchal et al. 2001, Tomiya et al. 2004). This is a clear hindrance especially in clinical applications, since N-glycans, terminal sialic acid in particular, have an important impact on the glycoprotein function (Kost et al. 2005). There have been several attempts to improve and humanize the N-glycan processing pathway by expressing mammalian genes encoding N-glycan processing activities in transgenic insect cells (Aumiller et al. 2003, Hollister et al. 1998, 2002, Hollister & Jarvis 2001, Jarvis 2003) as well as creating recombinant baculoviruses encoding N-glycan processing activities (Hill et al. 2006, Jarvis et al. 2001, Jarvis 2003, Jarvis & Finn 1996, Seo et al. 2001). Additionally, difficulties in phosphorylation have been reported. These problems can be a result of very high expression levels obtained in late phase of infection when the post-translational capacity of the cells declines (O'Reilly et al. 1994a).

The baculovirus expression system has been used for a variety of applications. One of them is the production of virus-like particles (VLPs) that have been used to study viral assembly processes, produce novel vaccines and proteins for diagnostic assays and gene transfer (Kost et al. 2005). The widely used baculovirus display system is reviewed in 2.7.3.

2.2 Picornaviruses

Echoviruses (EV), belong to enteroviruses in the family *Picornaviridae*. Their virions are nonenveloped and the genome consists of positive single stranded RNA. Picornaviruses constitute a vast selection of important human and animal pathogens, like hepatitis A virus, rhinovirus and foot-and–mouth-disease virus, (FMDV) (Racaniello 2001, Stanway et al. 2002). Enteroviruses include several human pathogens, such as poliovirus (PV), coxsackieviruses (CAV) and EVs, but also include other animal viruses. Human enteroviruses can be further divided into polioviruses and human enteroviruses A-D (Pallansch & Roos 2001, Stanway et al. 2002).

2.2.1 Biology and structure of Echovirus 1

Echoviruses (EV) belong to the human enterovirus B species (Stanway et al. 2002). Echovirus 1 (EV1, Farouk strain) is known to cause respiratory infections

and symptoms in the faecal-oral route most commonly among neonates and infants (Grist et al. 1978).

The structure of EV1 has been determined by cryo-crystallography (Filman et al. 1998). Among enteroviruses, the structure of polioviruses is best characterized and represents the common structural properties of the capsids (Filman et al. 1989, Hogle et al. 1985). The capsid of EV1, representing common structure of enterovirus capsid, is approximately 30 nm in diameter and consists of an icosahedral arrangement of 60 identical protomers (Figure 3). Each protomer is composed of four capsid proteins (VP1-4) (Filman et al. 1998, Hogle et al. 1985, Racaniello 2001). The major capsid proteins, VP1, 2 and 3, form the surface of the protomer, whereas VP4 is internal (Hogle et al. 1985). Myristic acid is covalently bound to the N-terminal residue of VP4 and may be involved in the capsid assembly or in the entry of the virus into cells (Chow et al. 1987). The major capsid proteins share a similar structure. Connecting loops and C-terminus of the protomer core are responsible for the creation of the antigenic sites of the virion particle (Hogle et al. 1985). Enteroviruses, including echoviruses, contain a large canyon at each fivefold vertex. At the base of the canyon there is a hydrophobic pocket that is occupied by a "pocket factor", a fatty-acid-like ligand that may stabilize the capsid (Hogle 2002).

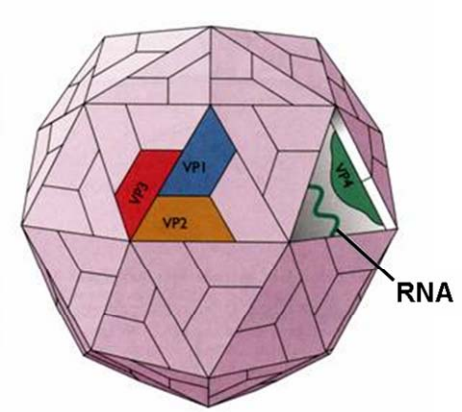

FIGURE 3 Structure of enterovirus capsid. One protomer is highlighted and the opening reveals viral genome. Reproduced with permission, from Flint et al. 2000.

The positive ssRNA genome of picornaviruses (7-8.5 kb) contains a single open reading frame (Bedard & Semler 2004), poly(A) at the 3' end (Bedard & Semler 2004, Spector & Baltimore 1974) and a covalently attached viral protein, VPg at the 5' end (Nomoto et al. 1977).

2.2.2 Echovirus 1 entry

An enterovirus infection begins with the binding of the virus to the cell surface receptor or receptors. Receptor binding may result in conformational changes essential for viral entry and RNA release (Rossmann et al. 2002). Uncoating involves loss of the structural protein VP4 and release of the RNA from the

capsid. Viral replication takes place in the cytoplasm in replication complexes that are enclosed in a rosette-like shell of virus-induced vesicles (Bienz et al. 1992). The viral genome acts directly as mRNA and is translated to a single polyprotein that is subsequently cleaved to mature polypeptides by viral proteases. In capsid assembly, immature protomers self-assemble into a pentamer that combine and form icosahedral virus particles. After packaging the genome into the capsid, maturation cleavage leads to the formation of infective viral particles. Progeny virus particles are being released by lysis of the host (Hogle 2002).

The replication cycle of EV1 takes approximately eight hours (Huttunen et al. 1997) and involves and the partial shut down of host cell protein sythesis (Pietiäinen et al. 2000). EV1 infection activates specific signal pathways that in turn up-regulate expression of certain cellular genes and may prolong the survival of the host cell (Huttunen et al. 1997, 1998, Pietiäinen et al. 2000).

EV1 is known to use α2β1 integrin and β2-microglobulin as receptors on the cell surface (Bergelson et al. 1992, Ward et al. 1998). The virus binds solely to the I domain of α2β1 integrin. In contrast to the binding of collagen, echovirus binding is not dependent on functional form of integrin, and does not involve metal ion-dependent adhesion site (MIDAS) (Bergelson et al. 1993, 1994, Emsley et al. 2000, King et al. 1995, 1997). Later, the residues important for EV1 binding to the I domain have been identified (Dickeson et al. 1999, King et al. 1997). The structural model of EV1 interaction with I domain created by cryo-electron microscopy has been recently presented (Xing et al. 2004). In the model, the I domain binds to the canyon of the viral capsid with extensive contacts to the outer surface wall. Electrostatic interactions are involved in the binding. According to the model, the simultaneous binding of collagen and EV1 is impossible and, thus, EV1 competes for the binding with natural ligand of integrin α2β1. The avidity of EV1 binding to the I domain was found to be 10 times that of collagen. Moreover, binding of EV1 to I domain was found to induce integrin clustering demostrated by *in vivo* experiments and molecular modeling. In the model, binding sites around the 5-fold axis of the capsid pentamer are occupied by five I domains without steric hindrance (Xing et al. 2004). In contrast to some other picornaviruses, receptor binding *in vitro* does not induce uncoating of the EV1 (Hoover-Litty & Greve 1993, Kaplan et al. 1990, Racaniello 1996, Xing et al. 2004).

Infection of the majority of echoviruses, including EV1, have been shown to be blocked with mAbs against β2-microglobulin in certain, but not all cell lines studied. β2-microglobulin is a small protein that is in complex with the class I HLA heavy chains and presents antigenic peptides. The mode of the inhibition of EV infection was speculated to be block in the formation of virus-receptor complex (Ward et al. 1998).

2.3 Endocytosis routes utilized by viruses

Endocytosis is characterized as internalization of macromolecules and particles into plasma membrane invaginations that pinch off to form vesicles thereby enabling entry of these substances into the cell. Endocytosis can be divided into two broad categories, phagocytosis, "cell eating" e.g. uptake of large particles, and pinocytosis, "cell drinking", e.g. uptake of solutes. Phagocytosis is restricted to certain specialised cell types, such as macrophages, whereas pinocytosis is utilized by all cells. There are at least four mechanisms in pinocytosis: clathrin-mediated endocytosis, macropinocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis (Conner & Schmid 2003). The effciency of pinocytosis depends on the putative interaction of the internalized substances with the cell surface. Uptake following interaction with high affinity receptor (receptor-mediated endocytosis) is most efficient, whereas adsorptive endocytosis, relying on non-specific cell surface interactions, or simple uptake of fluid phase markers are more inefficient (Conner & Schmid 2003).

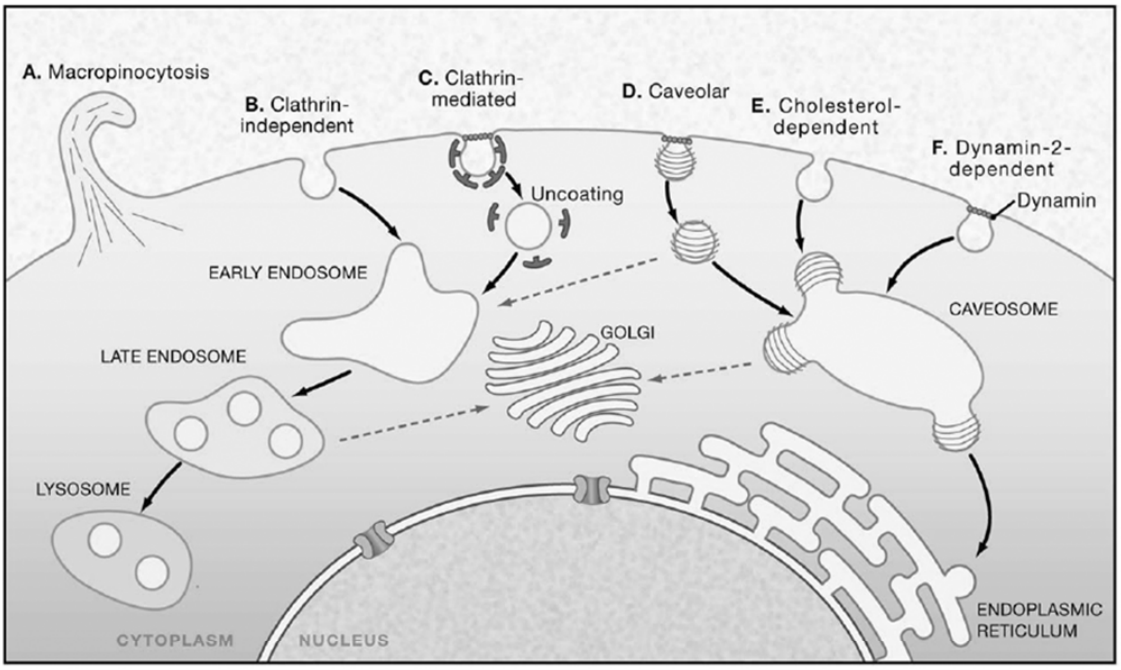

FIGURE 4 Endocytic routes utilized by viruses. See text for details. Reprinted from Cell, 124, Marsh, M., Helenius, A., Virus entry: open sesame, 729-740, Copyright (2006), with permission from Elsevier.

Viruses are intracellular parasites, whose replication depends on the ability to deliver their genome from infected to noninfected cells. Most viruses with DNA genomes enter the nucleus, whereas most RNA viruses replicate in the cytosol (Smith & Helenius 2004). Entry and uncoating of viruses is firmly controlled and includes several consecutive steps involving conformational alterations and leading to genome release (Marsh & Helenius 2006). Viruses utilize a wide

variety of cellular membrane proteins for binding. Viral receptors actively promote the entry and determine the entry mechanism utilized. This can be accomplished by inducing conformational change of the virus, by initiating cellular signals and by mediating specific endocytotic internalization (Marsh & Helenius 2006).

To deliver the genome into the cell, the genome, usually at that time inside the viral capsid, must somehow penetrate the cellular membrane (Pelkmans & Helenius 2003). This membrane can be either the plasma membrane or the membrane of an intracellular organelle (Marsh & Helenius 2006). For enveloped viruses, the mechanism of penetration is membrane fusion, which is mediated by fusion proteins on the viral envelope. This fusion event is triggered by receptor binding or low pH and leads to irreversible conformational changes in the fusion protein. Nonenveloped viruses either induce the lysis of the membrane or form a pore through it. Similarly to enveloped viruses, penetration of nonenveloped virions is induced by receptor binding or low pH (Smith & Helenius 2004). Although some enveloped and nonenveloped viruses may penetrate the cell directly through plasma membrane, a majority of viruses have adapted to exploit endocytic mechanisms to gain access to host cells. Inside the endocytic vesicles, viruses are conveniently transported deep into the cytoplasm without a trace of viral proteins on the plasma membrane (Marsh & Helenius 2006). In addition to penetration, capsid destabilization and viral genome release from the capsid, must be achieved. Similarly to penetration, these changes are a result of viral structural alteration during the entry, triggered by viral interactions with the cell e.g. receptor binding and exposure to low pH (Marsh & Helenius 2006). After penetration of the cellular membrane, the viral genome must usually be transported either to the nucleus or to specific cytosolic membranes. This can be performed by exploiting the cytoskeleton and cellular motor proteins (Smith & Helenius 2004).

Altogether, virus uptake seems to be a complex phenomenon, not mediated by only one entry mechanism. Identification of various new entry mechanisms is ongoing. Results will hopefully further enlighten the genious but still partly mysterious mechanisms of virus internalization.

2.3.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (Figure 4C) is a continuous process that transports e.g. low-density lipoprotein and transferrin bound to its receptor (Schmid 1997). Clathrin coated pits are composed of lattice-like assembly of clathrin molecules. Pits form in response to an internalization signal, present in the cytoplasmic tail of the receptor, by clathrin assembly to the cytoplasmic face of plasma membrane. Several other molecules interact with clathrin. Dynamin GTPase is responsible for the release of the vesicle from the plasma membrane, pinching off, to form clathrin coated vesicle (Marsh & McMahon 1999). Internalized clathrin coated vesicles rapidly loose their coat and fuse with early endosomes (Mellman 1996), 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 (Hopkins et al. 1990, Mellman 1996, Tooze & Hollinshead 1991). Late endosomes are perinuclear more spherical multivesicular structures. They differ from early endosomes by their lower luminal pH and different protein composition (Piper & Luzio 2001). Additionally, they associate with different small GTPases of the rab family than early endosomes. Rab proteins are responsible for the coordination of vesicular transport in the cell, including vesicle formation and motility as well as targeting of vesicles to appropriate compartment (Zerial & McBride 2001). Late endosomes transport some cargo to lysosomes that contain degradative acid hydrolases. Late endosomes can be distinguished from lysosomes by the presence of mannose-6-phosphate receptors and recycling plasma membrane receptors (Luzio et al. 2003).

The clathrin route is the most commonly utilized entry pathway of viruses. It is used by viruses such as adenoviruses, orthomyxoviruses (e.g. influenzavirus), alphaviruses (e.g. Semliki Forest virus), rhabdoviruses (e.g. vesicular stomatitis virus) and certain picornaviruses (DeTulleo & Kirchhausen 1998, Marsh & Pelchen-Matthews 2000). Clathrin-mediated endocytosis transports viruses associated with their receptors efficiently and rapidly to early and late endosomes. The acidic surroundings of endosomes facilitate the penetration of many viruses by inducing conformational changes in the virus particle. The place of this acid-induced penetration depends on the pH threshold of the virus (Marsh & Helenius 2006). Some viruses, such as Ebola virus or SARS coronaviruses, need proteolytic processing of viral proteins mediated by acid dependent endosomal proteases to facilitate the penetration process (Chandran et al. 2005, Simmons et al. 2005).

Semliki Forest virus was the first virus reported to use clathrin mediated endocytosis (Helenius et al. 1980), and nowadays its entry steps are well characterized. The virus is first bound to the receptor(s) on the cell membrane followed by subsequent lateral diffusion on the surface that leads the virus into clathrin coated pits. Internalization then takes place by receptor-mediated endocytosis i.e. clathrin coated vesicles leading to early endosomes. Low pH induces viral spike glycoprotein-mediated membrane fusion that leads to the penetration of the capsid to the cytoplasm. Uncoating of the genome, translation and replication all take place in the cytoplasm (Pelkmans & Helenius 2003). According to the latest reports, viruses can also induce directly the formation of the clathrin coat. This was observed with influenza virus that was shown to establish clathrin coated pit at the binding sites, detected with single –particle tracking (Rust et al. 2004).

2.3.2 Lipid raft-mediated endocytosis

Lipid rafts are structurally and functionally distinct plasma membrane microdomains that are enriched with cholesterol, glycosphingolipids and certain proteins (Pietiäinen et al. 2005, Simons & Ikonen 1997). Later studies have stated rafts as small shells formed by the interaction of certain proteins

with cholesterol-rich regions that are able to form larger rafts upon activation (Anderson & Jacobson 2002, Pietiäinen et al. 2005). Despite intensive research, the composition and function of lipid rafts are still controversial (Mayor & Rao 2004). Raft-mediated internalization can be dynamin-mediated or not, depending on the case (Marsh & Helenius 2006) (Figure 4E and F).

SV40 may use lipid raft-mediated dynamin-independent entry as an alternative pathway for caveolin-mediated entry (Damm et al. 2005). Lipid-raft-mediated endocytosis is also likely employed by some picornaviruses, papillomaviruses, filoviruses and retroviruses (Pelkmans & Helenius 2003). This caveolae-independent entry mechanism seems to be dependent of raft clustering. Avian sarcoma and leukosis virus belonging to retroviruses, has been shown to internalize this route when bound to GPI-anchored form of receptor, ending up in the endosomes (Narayan et al. 2003). The echovirus 11 (EV11) receptor, DAF, is also coupled to GPI anchor and mediates virus entry via this route (Stuart et al. 2002). Coxsackie A9 (CAV9) virus may also utilize lipid rafts in the entry. Viral receptors including αvβ3 intergin, were found to accumulate in lipid rafts followed by virus infection. Furthermore, viral infection was shown to be inhibited with raft-distrupting drugs (Triantafilou & Triantafilou 2003).

2.3.3 Caveolae-mediated endocytosis

Caveolae are small flask-shaped plasma membrane invaginations in rafts that possess caveolin proteins on the cytoplasmic side of the plasma membrane (Rothberg et al. 1992, Schlegel & Lisanti 2001). Caveolae are static structures and caveolae internalization seems to be triggered upon signaling events caused by clustering of the ligand bound receptors (Figure 4D). Dynamin and actin are needed for this slow caveolae-mediated endocytosis leading to intracellular structures called caveosomes. Caveolin coat does not dissociate during this process. Caveosomes are stable structures with neutral pH and multiple flask-shaped caveolar domains enriched with caveolin-1. They differ from classical endocytic and biosynthetic organelles by the lack of appropriate markers, such as rab GTPases (Pelkmans et al. 2001, Pelkmans & Helenius 2002, 2003). Caveolae interact with the ER and the golgi complex as well as endosomes (Pelkmans 2005). Also caveolin-independent transport mechanisms from rafts to caveosomes have been suggested to exist (Damm et al. 2005, Kirkham et al. 2005).

SV40 was the first virus reported to use caveolae –mediated endocytosis (Anderson et al. 1996). Several other viruses, such as coronaviruses (Nomura et al. 2004), filoviruses (Empig & Goldsmith 2002), influenza viruses (Nunes-Correia et al. 2004) and polyomaviruses (Richterova et al. 2001) utilize caveolae in their entry.

SV40 is known to be redistributed to the cell surface caveolae due to binding and subcequent clustering of MHC class I molecule used as viral receptors (Anderson et al. 1998, Stang et al. 1997). The virus may also induce

formation of new caveolae. The virus, but not the receptor, is internalized via caveolae, in a slow but efficient process dependent on dynamin, actin and signaling events (Pelkmans & Helenius 2002, Stang et al. 1997). SV40 is transported inside caveolae to caveosomes (Pelkmans et al. 2001), however also non-caveolin mediated transport of SV40 from the plasma membrane has been reported (Damm et al. 2005). After several hours in caveosomes, the virus uses a novel caveolin-independent transport mechanism to travel to the smooth ER. Finally SV40 enters the nucleus for replication (Pelkmans et al. 2001, Pietiäinen et al. 2005).

2.3.4 Macropinocytosis

Macropinocytosis is an induced process that mediates internalization of large amounts of fluid or membrane (Figure 4A). It takes place upon formation of large vacuoles by the closure of plasma membrane ruffles and is dependent on polymerization of actin, but does not need dynamin. Not many viruses are known to utilize this rather non-specific pathway (Pelkmans & Helenius 2003, Swanson & Watts 1995). The large vaccinia virus likely uses this pathway in its entry, inducing actin-mediated membrane rearrangements and cell-surface protrusions resembling initial stage of phagocytosis (Locker et al. 2000) that is mechanistically similar to macropinocytosis utilized by viruses (Meier & Greber 2004). HIV-1, normally entering by direct fusion of plasma membrane, also utilizes macropinocytosis in macrophages (Marechal et al. 2001). Adenovirus type 2 has been shown to utilize macropinocytosis in its entry process in concert with clathrin mediated endocytosis. Binding to αv integrin coreceptor was shown to trigger signaling leading to internalization of virus by actin-dependent and dynamin-independent process using macropinocytic vesicles. Subsequent penetration of viruses to cytosol occurred simultaneously from endosomes and macropinosomes, however, the mechanism of macropinocytic release is unknown (Meier et al. 2002, Meier & Greber 2004).

2.3.5 Clathrin- and caveolin-independent endocytosis

Some viruses have been reported to use clathrin- and caveolin-independent endocytosis pathways. These pathways are not yet understood, but at least dynamin-dependent and -independent internalization mechanism are known to exist (Conner & Schmid 2003, Marsh & Helenius 2006, Pelkmans & Helenius 2003). Influenzavirus has been reported to utilize clathrin- and caveolin-independent pathway in parallel with clathrin mediated endocytosis. The details of this route are not known, but internalization is likely dynamin-dependent and lead to early endosomes (Rust et al. 2004, Sieczkarski & Whittaker 2002b) (Figure 4B). In addition, polyomavirus seems to enter cells in uncoated vesicles by dynamin-independent manner (Gilbert & Benjamin 2000).

2.4 Integrins

Integrins are cell curface adhesion receptors that mediate cell-extracellular matrix and cell-cell –interactions (Hynes 1992, Ruoslahti 1991). They mediate anchorage and migration of cells through extracellular matrix proteins and generate intracellular signals that regulate the cell survival, differentiation and proliferation. Inflammation and tumor progression are examples of pathological conditions that integrins participate in (Koistinen & Heino 2002).

Integrins are heterodimers that are composed of noncovalently associated α and β subunit (Figure 5). In mammals 18 α and 8 β subunits associate to form a total of 24 different heterodimers. Both subunits are membrane glycoproteins, with single transmembrane segment, large extracellular domain and short cytoplasmic tail (Hynes 1992). The structure of the extracellular domains of αvβ3 integrin was determined using x-ray crystallography by Xiong and colleagues (2001). The head of the integrins consist of seven N-terminal repeats known as β-propeller fold in α subunit and a βA domain looping out from immunoglobulin (Ig)-like hybrid domain of β integrin (Xiong et al. 2001)

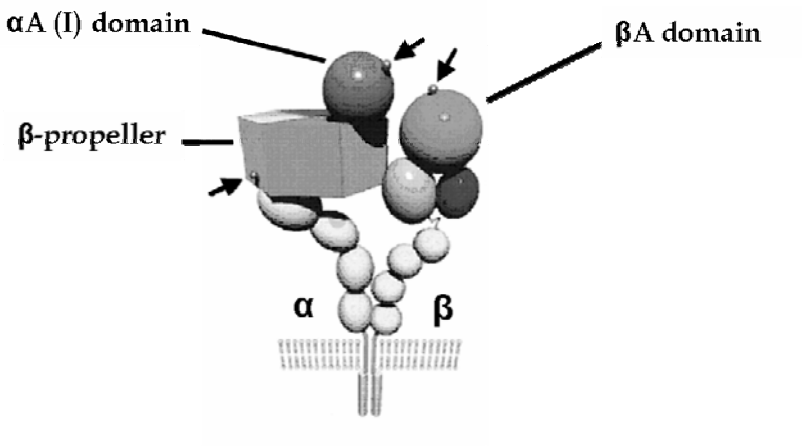

FIGURE 5 Schematic representation of the domain structure of an integrin dimer with an αA (I) domain. α subunit (left) contains β-propeller that αA (I) domain attaches. βA domain of β subunit (right) is indicated. Bivalent cation-binding sites are shown as small spheres (arrows). Reproduced with permission, from Humphries, 2000, *Biochemical Society Transactions*, 28, 311-339. © the Biochemical Society.

The natural ligands of integrins include extracellular matrix proteins e.g. collagens, fibronectin, laminin, vitronectin and immunoglobulin superfamily receptors (Hynes 1992). Moreover, numerous viruses, including picornaviruses (Bergelson et al. 1992, Berinstein et al. 1995, Roivainen et al. 1994), cytomegaloviruses (Wang et al. 2005) and adenoviruses (Wickham et al. 1993) use integrins as receptor molecules. Integrins can often bind several ligands and individual ligands are recognized by more than one integrin (Hynes 1992).

Many integrins, including α2 integrin (Takada & Hemler 1989), contain I (or A) domain inserted into α subunit that comprises the ligand binding site of

these integrins (Dickeson et al. 1999). αA-like domain, βA, present in β subunit is involved in the ligand binding of integrins lacking I domain. Both the αI and βA domains contain a metal ion-dependent adhesion site (MIDAS) that is important for the integrin ligand binding (Lee et al. 1995a, 1995b, Xiong et al. 2001). Intracellular signals activate integrins into a high affinity ligand binding state. Ligand bound active integrin causes further conformational changes that result in integrin clustering and subsequent cell signaling leading to various cellular responses (Ginsberg et al. 2005, Hynes 2002a, Qin et al. 2004).

2.4.1 RGD as integrin ligand

Many integrin ligands, such as fibronectin, vitronectin, collagens, fibrinogen and von Willenbrand factor utilize an arginine-glycine-aspartic acid (RGD) motif in the binding (Ruoslahti 1996, Ruoslahti & Pierschbacher 1987). Integrins interacting with RGD include all αv integrins (αvβ1, αvβ3, αvβ5, αvβ6, αvβ8), α5β1, α8β1 and αIIbβ3 integrin (Hynes 2002a).

The integrin specificity of RGD is dependent on the conformation and environment of the RGD site (Pierschbacher & Ruoslahti 1987) and the nature of the surrounding amino acids. RGD is an absolute requirement for binding, but amino acid following it may vary (Pierschbacher & Ruoslahti 1984a, 1984b). In linear RGD peptides, fourth residue may specify the receptor preference by altering the overall shape of the RGD motif. Cyclization of the specific RGD motif may increase the affinity for the receptor, but may also change the selectivity of the ligand. The specific conformational requirements of different integrins in the RGD binding enable the design of more selective probes for individual receptors (Piersbacher & Ruoslati 1987).

In the structure of αvβ3 integrin in complex with RGD, determined by Xiong and colleagues (2002) (Figure 6), RGD was shown to bind at the major interface between integrin subunits contacting directly the MIDAS site in βA. Binding was associated with structural changes in integrin and a divalent cation, Mn^{2+}, participated in the formation of the bonds. In contrast to the structure obtained with the short RGD peptide, in physiological integrin-ligand interactions residues outside RGD participate in the binding process (Takagi 2004).

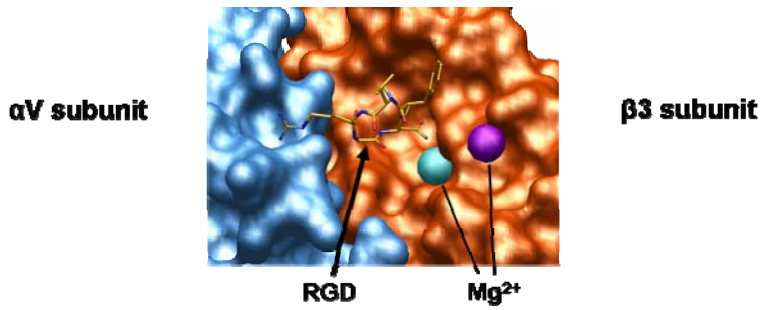

FIGURE 6 Integrin αvβ3 in complex with RGD. Alpha V subunit in blue, beta 3 subunit in red and RGD as a ribbon structure. Magnesium atoms are indicated. RGD binds to the interface between subunits and makes extensive contact with both. Model by Olli Pentikäinen based on Xiong et al. 2002.

RGD is employed by several viruses that use it to bind integrin and gain entry into cells (Ruoslahti 1996). Below, picornaviruses utilizing RGD are introduced. Moreover, for example, adenoviruses employ this motif in the binding to their integrin receptors (Mathias et al. 1994, 1998, Wickham et al. 1993).

Coxsackie A virus 9 (CAV9) possesses an RGD sequence at the C-terminus of its capsid protein VP1 (Chang et al. 1989). According to the crystal structure of CAV9, RGD-containing C-terminus is flexible and is exposed on the outer surface of the virus (Hendry et al. 1999). In contrast to surrounding residues, comparisons of CAV9 isolates have revealed the highly conserved nature of RGD highlighting its importance (Chang et al. 1992, Santti et al. 2000). The virus is known to use RGD (Roivainen et al. 1991) in binding to αVβ3 integrin on the cell surface (Roivainen et al. 1994). This interaction is not, however, sufficient for cell entry and several other coreceptors have been identified (Triantafilou et al., 1999, 2000b, 2002). The virus has later been shown to bind also αVβ6 integrin in an RGD dependent manner (Williams et al. 2004). However, some mutants lacking the RGD motif are known (Hughes et al. 1995) and a RGD-independent entry route is known to exist (Roivainen et al. 1996). The C-terminal region of VP1 containing RGD is highly antigenic, and viral infection has been neutralised by antisera against this region (Pulli et al. 1998a, 1998b).

Human parechovirus 1 (HPEV1), previously known as echovirus 22, has been reported to compete for receptor binding with CAV9 (Roivainen et al. 1994). Similarly to CAV9, HPEV1 possesses an RGD motif in the C-terminus of VP1 (Hyypiä et al. 1992). The importance of RGD in the receptor binding of HPEV was demonstrated by using synthetic peptides (Pulli et al. 1997, Stanway et al. 1994). The use of αvβ1 integrin as a receptor molecule was demonstrated by antibody blocking (Pulli et al. 1997), but later the virus was demostrated to preferentially use αvβ3 integrin (Triantafilou et al. 2000a). Joki-Korpela and colleagues (2001) confirmed receptor preferency for αvβ3 and concluded the virus to use this receptor mainly in the early interactions on the cell surface. Thus, other receptors might be needed for the internalization of the virus (Joki-Korpela et al. 2001). RGD containing peptide has been shown to be antigenic and able to induce production of neutralizing antibodies in a rabbit (Joki-Korpela et al. 2000).

Foot-and–mouth-disease virus (FMDV) has RGD motif located in the middle of VP1 protein seen in crystal structure as a disordered, flexible loop structure exposed on the surface of the virus (Acharya et al. 1989). This site has been identified as a major antigenic area of the virus (Bittle et al. 1982, Pfaff et al. 1982). The RGD mediates binding of the virus to αvβ3 integrin (Baxt & Becker 1990, Berinstein et al. 1995, Fox et al. 1989, Jackson et al. 1997, Mason et al. 1994). FMDV has been also shown to bind to heparan sulphate (Jackson et al. 1996), αvβ6 integrin (Jackson et al. 2000a) and α5β1 integrin (Jackson et al. 2000b).

2.4.2 RKK as integrin ligand

The arginine-lysine-lysine (RKK) motif was originally found in the snake (*Bothrops jararaca*) venom disintegrin/metalloproteinase jararhagin by Ivaska and colleagues (1999a). This circular peptide, CTRKKHDNAQC, present in the primary sequence of jararhagin metalloproteinase domain (aa241–248), was shown to inhibit collagen binding to α2I domain. Three amino acids, RKK, and the cyclic conformation were determined to be essential for the function of the peptide (Ivaska et al. 1999a). According to models and docking simulations by Pentikäinen and colleagues (1999) RKK sequence interacts with α2I domain using ionic as well as hydrophobic interactions (Figure 7). The binding site for RKK was identified to the vicinity surrounding the MIDAS by binding studies with mutated α2I domain. The binding of RKK containing metalloproteinase was suggested to block collagen binding at the MIDAS site leaving metalloproteinase active to degrade other proteins, likely β1 integrin itself (Pentikäinen et al. 1999). Later, two RKK containing peptides derived from jararhagin have competitively inhibited collagen I binding to α1I domain (Nymalm et al. 2004).

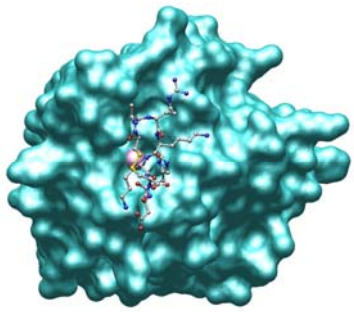

FIGURE 7 Model of RKK binding to α2 I domain of α2β1 integrin. RKK binds to the vicinity of a metal ion dependent adhesion site (MIDAS). Model by O. Pentikäinen based on Pentikäinen et al. 1999.

2.5 Tumor vasculature

Tumor growth is dependent on the supply of nutrients and oxygen, namely blood. If vasculature is not in reach, tumor growth is impaired, tumor cells become necrotic and/or apoptotic, and the size of the tumor nodule is restricted to a diameter of 0.4 mm. Thus, in order for tumors to grow, new blood vessels have to be formed. This process, angiogenesis, the formation of new blood vessels developing from existing vessels, occurs in response to appropriate stimulus from the tumor (Hanahan & Folkman 1996). Angiogenesis is triggered by hypoxia in tumor mass, which in turn activates the expression of specific factors, such as vascular endothelial growth factors (VEGFs), platelet-derived growth factors (PDGFs) and angiopoietins. During the process, supporting

cells, pericytes, and/or smooth muscle cells, as well as ECM surround the endothelial tube (Ruoslahti 2002b). The tumor vasculature has many distinct features (Hanahan & Folkman 1996, Hida & Klagsbrun, M., 2005, Ruoslahti 2002b). They are permeable, convoluted, irregular in diameter, thin-walled and surrounded by too few or abnormal pericytes. Due to the "leakiness" of the tumor vasculature, tumor cells can be found in the luminal side of the vessel. Most importantly tumor blood vessels are in a constant state of angiogenesis.

The presence of lymphatic vessels within certain tumors has only been established recently along with the availability of molecular markers for lymphatics (Ruoslahti 2002b). However, these intratumoral vessels may not be completely fuctional and lymphatics nearby tumor might have greater influence to tumorigenesis (Alitalo & Carmeliet 2002). Tumor cells and tumor-associated macrophages secrete lymphangiogenic growth factors (VEGF-C or VEGF-D) leading to lymphangiogenesis and lymphatic endothelial cells in turn attract tumor cells by secreting chemokines. This facilitates metastasis that tumor lymphatics are known to conduct (Alitalo & Carmeliet 2002, Schoppmann et al. 2002). The abundance of lymphatic vessels in and around the tumor has been shown to correlate with the metastatic activity of the tumor (Ruoslahti 2002b).

Cancer cells themselves might be able to form vessel-like structures without endothelial cell participation. This controversial phenomenon, vasculogenic mimicry, is predictive of a poor prognosis. These channels might connect blood and lymphatic vessels inside the tumor mass and contribute to blood and nutrient supply of the tumor (McDonald et al. 2000, Ruoslahti 2002b).

2.6 Targeting tumor vasculature

Tumor vasculature is an attractive target for cancer therapy for several reasons. Their accessibility is good and treatment is efficient; elimination of blood supply can profoundly suppress tumor growth. Tumor endothelial cells have been considered genetically stable and unlikely to develop drug resistance (Ruoslahti 2002a). Recent findings of the possible aneuploidy and instability of tumor endothelial cells however signifies the potential for acquiring drug resistance (Hida & Klagsbrun 2005).

The exploitment of *in vivo* phage display ten years ago demonstrated the heterogeneity of blood vessels showing that each tissue expresses unique vascular markers that can be exploited in targeting (Pasqualini & Ruoslahti 1996, Rajotte et al. 1998). By using the same approach, it was further shown that vessels of normal tissues can be distinguished from, for example, vessels present in tumors mostly by identification of changes related to angiogenesis (Arap et al. 1998, Pasqualini et al. 2000, Porkka et al. 2002). Thus, tumor vasculature is morphologically distinct from normal vasculature, and the presence of several specific cell-surface and extracellular matrix proteins can be

employed to distinguish tumor vasculature from normal vasculature. These specific marker proteins can be used to target and destroy the vessels without significantly affecting normal vessels. These features make tumor blood vessels major targets in inhibiting tumor growth (Ruoslahti 2002b).

As angiogenesis is an important phenomenon of tumor vasculature, markers of tumor vasculature are mostly angiogenesis-related (Ruoslahti 2002b) (Figure 8). They include αvβ3 integrin (Brooks et al. 1994a), matrix metalloproteinases (MMPs) (Nelson et al. 2000), aminopeptidase N (APN) (Pasqualini et al. 2000) and receptors for angiogenetic growth factors, such as VEGFRs (Plate et al. 1993) and endoglin (Fonsatti & Maio 2004, Li et al. 1999). Tumor endothelial cells also express certain normally intracellular proteins, like nucleolin (Christian et al. 2003) and annexin (Oh et al. 2004), on the cell surface. Pericytes surrounding the angiogenic vessels express membrane proteolgycan, melanoma-associated high molecular weight antigen (NG2) (Schlingemann et al. 1990).

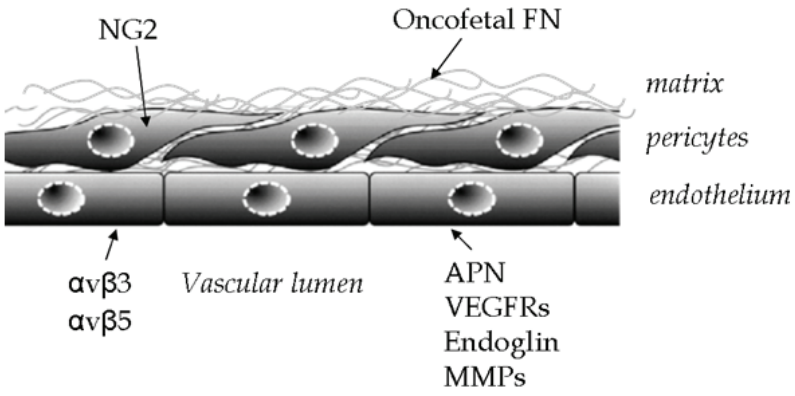

FIGURE 8 Angiogenesis marker proteins. See text for details. Reprinted from Seminars in Cancer Biology, 10, Ruoslahti, E., Targeting tumor vasculature with homing peptides from phage display, 435-442, Copyright (2000), with permission from Elsevier.

Targeting to angiogenesis-related markers has been the major object in tumor vasculature targeting. Several strategies targeting to VEGF-A or its receptors, VEGFR-1 and VEGFR-2, overexpressed in angiogenic blood vessels, have been developed (D'Andrea et al. 2006, Ferrara et al. 2004, Ferrara 2004). The anti-VEGF therapy has been shown to eliminate angiogenic blood vessels in certain tumors. Kim and colleagues were first to report that antibodies against VEGF suppress angiogenesis and tumor growth *in vivo* (Kim et al. 1993). Later, Avastin, humanised antibody against VEGF, has been approved as therapeutic agent for treatment of metastatic colorectal cancer (Ferrara et al. 2004). Also VEGF itself has been utilized in targeting to its receptor; diphteria toxin conjugated growth factor was reported to selectively target to tumor neovasculature and possess significant anti-tumor activity (Olson et al. 1997). Retrovirus encoding dominant negative mutant of VEGF has been utilized to infect tumor endothelial cells resulting suppression of tumor growth (Millauer et al. 1994). These studies, among others, have demostrated the feasibility of

anti-VEGF therapy in inhibiting angiogenesis (D'Andrea et al. 2006, Ferrara et al. 2004).

Certain integrins, such as αvβ3, αvβ5 and α5β1, play a role in angiogenesis, and are upregulated in angiogenic vessels (Eliceiri & Cheresh 1999, Kim et al. 2000). Especially targeting to αvβ3 integrin in the angiogenic blood vessels has been extensively studied (See 2.6.2). MMPs, especially, MMP-2 and MMP-9 have been shown to be associated with αvβ3 integrin on the surface of endothelial cells (Brooks et al. 1996). Phage selected to bind these two MMPs has been reported to specifically target to tumor vasculature (Koivunen et al. 1999). In a recent study, MMP-9 activated prodrug targeted to multiple myeloma-diseased bone marrow was exploited (Van Valckenborgh et al. 2005). Homing peptides targeting to NG2, specifically expressed on the surface of tumor cells and pericytes of angiogenic tumor vasculature, have been obtained by phage screening and these peptides have specifically homed to tumor vasculature *in vivo* (Burg et al. 1999). Aminopeptidase N, was identified as an angiogenesis marker by identification of the receptor (Pasqualini et al. 2000) for tumor-homing peptide NGR, obtained by phage display (Arap et al. 1998). NGR has been successively utilized in selective targeting of anticancer drug doxorubicin or proapoptotic peptide to tumors showing efficient anti-cancer activity (Arap et al. 1998, Ellerby et al. 1999). Antibodies specific for the extra domain B of fibronectin have been exploited in tumor targeting (Ebbinghaus et al. 2004) and they have been reported to block angiogenesis (Kim et al. 2000). Moreover, angiostatin, a fragment of plasminogen, and endostatin, a cleavage product of collagen XVIII, are factors produced by the primary tumor. They have been shown to specifically target angiogenic blood vessels, inhibiting angiogenesis and thereby growth of metastasis (Cao & Xue 2004, O'Reilly et al. 1994b, 1997). Both angiostatin and endostatin are being evaluated to be used as therapeutics (Cao & Xue 2004).

Different tumor types express unique molecular signatures as well; *in vivo* phage screening of two transgenic tumors, pancreatic islet cell tumor and skin cancer, has yielded identification of distinct homing peptides. Furthermore, distinct molecular signatures can be found in different stages of tumorigenesis (Hoffman et al. 2003, Joyce et al. 2003). A combination of *in vivo* and *ex vivo* phage display has improved the sorting power even further and resulted in peptides with mutual specificity among tumor cells, tumor blood vessels and bone marrow cells (Porkka et al. 2002) or tumor cells and tumor lymphatic endothelium cells (Laakkonen et al. 2002) (See 2.6.1).

The identification of lymphatic endothelial cell markers, such as VEGFR-3 (Jussila et al. 1998), podoplanin (Breiteneder-Geleff et al. 1999) and LYVE-1 (Prevo et al. 2001) has enabled the study of tumor lymphatics in detail (Jackson et al. 2001, Ruoslahti 2002b). According to the latest studies, lymphatic vessels in tumors are specialised and carry specific markers showing molecular heterogeneity in the lymphatics as well (Alitalo et al. 2005, Fiedler et al. 2006, Laakkonen et al. 2002, 2004, Zhang et al. 2006). It seems that markers defining lymphatics of a particular tumor are distinct from blood vessel markers of the

same tumor (Ruoslahti 2002b). Recently, Fiedler and colleagues (2006) reported the identification of the first tumor lymphatic endothelial marker, sialomycin CD34. This marker, earlier identified as a marker for blood endothelial cells (Hirakawa et al. 2003), was present in lymphatics of human colon, breast, skin and lung tumors, but not in lymphatics of normal tissues. CD34 seems to be specific for intratumoral lymphatics of human origin as mouse tumor-associated lymphatics were devoid of this marker (Fiedler et al. 2006). Specific targeting to tumor lymphatics *in vivo* has been accomplished with LyP-1 peptide identified by *in vivo* phage display by Laakkonen and colleagues (Laakkonen et al. 2002, 2004) (See 2.6.1). Zhang and coworkers (2006) recently shed light on this issue by identifying several distinct lymphatic zip codes by *in vivo* phage display. This study confirmed the finding of Laakkonen and coworkers (2002, 2004) indicating that tumor development is affiliated to organ- and state specific changes in lymphatics (Zhang et al. 2006). Further characterization of tumor endothelial cells for specific markers will provide new important targets for therapy. Thus, specific targeting to tumor lymphatics might lead to the reduction of metastasis (Ruoslahti 2002b).

Lymphangiogenic growth factors VEGF-C and D and their receptor VEGFR-3, has been employed to target tumor lymphatics. For this purpose, specific antibodies or soluble growth factor receptors has been utilized to suppress tumor growth, lymphangiogenesis and tumor metastasis induced by these lymphangiogenic growth factors. One approach has utilized adenovirus gene therapy to express soluble VEGFR-3, which was shown to inhibit tumor associated lymphangiogenesis. However, a better understanding of the role of tumor lymphatics in this matter is crucial for these applications (Alitalo et al. 2005, Karpanen, T. & Alitalo 2001).

2.6.1 Tumor homing peptides

F3, a linear peptide of 31-aa (KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK), was identified from *ex vivo* and *in vivo* phage display of screening a cDNA library by Porkka and coworkers (2002). It binds to both tumor vasculature and bone marrow endothelial progenitor cells. The F3 peptide, identified as a N-terminal fragment of human high mobility group protein 2 (HMGN2), was reported to home to certain tumors, such as human breast carcinoma xenograft MDA-MB-435 by intravenous injection accumulating in the nuclei of tumor and tumor endothelial cells. The uptake of the peptide was demostrated to be energy dependent (Porkka et al. 2002). The ability of F3 to carry a payload was demostrated by F3 coated fluorescent inorganic nanoparticles that were able to home to blood vessels of MDA-MB-435 tumors (Akerman et al. 2002). Later, the receptor of F3 was identified as nucleolin by affinity chromatography assay (Christian et al. 2003). Nucleolin, a nucleolar protein, is preferentially found in the nucleus and cytoplasm, but recently it has been reported to exist also on the cell surface (Said et al. 2002, Sinclair & O'Brien 2002). In the study of Christian and coworkers (2003), nucleolin was detected on the surface of actively growing cells that were also able to bind and internalize F3. In contrast, F3 was not able

to bind to serum starved cells possessing solely nuclear nucleolin. As both F3 and intravenously injected antinucleolin 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).

LyP-1, a cyclic nonapeptide (CGNKRTRGC), with a targeting specificity to lymphatic vessels in certain tumors, was isolated by combining *ex vivo* and *in vivo* phage display screening by Laakkonen and colleagues (2002). Intravenously injected LyP-1-displaying phage was demostrated to home to human breast carcinoma, MDA-MB-435, and human osteosarcoma, KRIB, tumors *in vivo*. Both LyP-1 phage and fluorescein-conjugated LyP-1 peptide colocalized in the MDA-MB-435 tumors with markers of lymphatic endothelia, but not with blood vessel markers, strongly suggesting homing to lymphatic vessels. LyP-1 –displaying peptide was found to accumulate in the nuclei of both lymphatic endothelial and tumor cells. *In vitro* studies further demonstrated the internalization of fluorescein-conjugated LyP-1 peptide (Laakkonen et al. 2002). In further studies by Laakkonen and colleagues (2004), LyP-1 was shown to remarkably accumulate in primary MDA-MB-435 breast cancer xenografts and their metastases as visualised in a whole body imaging of tumor-bearing mice. Interestingly, LyP-1 was reported to induce cell death both *in vitro* and *in vivo*. LyP-1 peptide treatment reduced the number of lymphatic vessels and inhibited growth of the xenografted tumors induced with MDA-MB-435 breast cancer cells. Peptide localised primarily to hypoxic areas in tumors. As the primary target of LyP-1 is lymphatics, the antitumor effect mediated by the peptide concentrates to tumor cells in the vicinity of lymphatics. This could be very beneficial because these cells are most likely to spread by metastasis (Laakkonen et al. 2004). LyP-1 has additionally been demostrated to target inorganic nanoparticles to tumor lymphatics in the MDA-MB-435 tumor model in mice *in vivo* (Akerman et al. 2002).

CGRKR, a five residue linear peptide, identified in a combination of *ex vivo* and *in vivo* phage display screen, has been reported to home to neovasculature in tumors and at lower levels to dysplastic skin vasculature (Hoffman et al. 2003). Fluorescein-CGKRK peptide was shown to home to endothelial cells in various transplant tumors localizing in the cytoplasm and nuclei of the cells and was also reported to bind a range of tumor cells *in vitro*. The homing was reported to be specific for a distinct state of the dysplacia, indicating that vessels of different stages in tumorigenesis have distinctive qualities. By the virtue of the overall charge of the peptide (+3), the recognition of heparan sulphate or phosphatidylserine on the cell surface by CGKRK was suggested (Hoffman et al. 2003).

2.6.2 Targeting to αvβ3 and α2β1 integrins

Integrin αvβ3 is a receptor for several extracellular matrix proteins, including vitronectin, fibronectin, osteopontin, thrombospondin, proteolyzed collagen and von Willenbrand factor, all using RGD in the binding. αVβ3 integrin is known to be expressed on the surface of angiogenic blood vessels and has been identified as a marker of angiogenic vascular tissue (Brooks et al. 1994a, Eliceiri & Cheresh 1999). The overexpression of αvβ3 integrin on the tumor-associated vessels in human carcinomas and practical absence in normal tissues of adult animals make it an attractive candidate for cancer targeting (Max et al. 1997, Ruoslahti 2002a). As many tumors express αVβ3 integrin (Max et al. 1997), and tumor vasculature is "leaky", targeting to vasculature enables targeting to tumor as well (Pasqualini et al. 1997). αVβ3 integrin has an important role in the angiogenesis induced both by cytokines or tumors (Brooks et al. 1994a, Eliceiri & Cheresh 1999). It also promotes tumor progression and metastasis in e.g. human melanoma (Felding-Habermann et al. 1992, Seftor et al. 1999) and breast carcinoma (Sung et al. 1998, Wong et al. 1998).

Abolishing the ligand binding of this integrin is known to inhibit angiogesis by causing apoptosis in endothelial cells of newly formed blood vessels (Brooks et al. 1994b, Friedlander et al. 1995). Peptide-mimicking RGD-ligands or anti-integrin antibodies have the same antitumor effect in tumor models (Brooks et al. 1994b, 1995). A monoclonal antibody against αvβ3 integrin (Brooks et al. 1994a), has been shown to suppress the growth and angiogenesis of MCF-7PB-derived human breast cancer in the microenvironment of human skin in chimeric SCID mouse model (Brooks et al. 1995). The humanised version of this antibody, vitaxin, and its second generation derivative, vitaxin-2, have been clinically tested for targeted antiangiogenic cancer therapy (Tucker 2003).

Later, the role and mode of action of αvβ3 in angiogenesis has been under debate. αvβ3 integrin might act as a negative regulator for angiogenesis, rather than being proangiogenic. For therapeutical use, the mode of action of αvβ3 integrin should be further clarified (Hynes 2002b).

RGD motif has a natural role in the αvβ3-mediated migration process. This has been demonstrated in vitro, for example, by inhibition of invasion with RGD peptides, RGD analogs and integrin blocking antibodies (Gehlsen et al. 1988, Sung et al. 1998, Wong et al. 1998). The RGD containing ligand-mimetics selective for αvβ3 integrin have been extensively employed in the αvβ3 integrin targeting to achieve antiangiogenic response. The cyclization of the RGD peptides and selection of flanking sequences for best affinity and selectivity for αvβ3 integrin have enabled the design of more specific peptides (D'Andrea et al. 2006, Ruoslahti 1996, Temming et al. 2005).

These selective RGD motifs can be inserted or coupled into a payload in order to target to αvβ3 integrin expressing cells. This system was demonstrated by Hart and colleagues (1994), who displayed the RGD on the surface of filamentous phage and demosntrated RGD dependent entry in vitro (Hart et al.

1994). The cyclic RGD motif, the RGD-4C peptide (CDCRGDCFC), discovered by phage display, binds selectively to human αvβ3 and αvβ5 integrins. Phage displaying RGD-4C peptide has been reported to selectively and specifically home to αvβ3 integrin expressing tumor vasculature and from there to tumor parencyma (Koivunen et al. 1995, Pasqualini et al. 1997). Later, phage displaying adenoviral penton base protein, carrying RGD motif, has been shown to transduct mammalian cells RGD-dependently in vitro (Di Giovine et al. 2001, Piersanti et al. 2004).

Several studies have employed the display of RGD on the surface of adenovirus in order to target them to tumor cells or tumor endothelial cells (Temming et al. 2005). Wickham and colleagues (1997) displayed RGD-4C on the C-terminus of the fiber protein of adenovirus, and showed increased gene delivery of this vector into cells expressing integrins in vitro. Later, the display of RGD in HI loop of fiber knob has enhanced the targeting and transduction efficiency of several cancer cell lines in vitro (Dmitriev et al. 1998, Haviv et al. 2002, Nagel et al. 2003, Wesseling et al. 2001, Yotnda et al. 2004) and *in vivo* (Nakamura et al. 2002, Okada et al. 2002, 2004, Rein et al. 2004, Staba et al. 2000) and lead in effective anti-tumor activity (Okada et al 2002, 2004, Nakamura et al. 2002). In the study of Okada and coworkers (2004) a combination treatment of two RGD displaying adenoviruses encoding either tumor necrosis factor alpha or interleukin-12 gene in a murine melanoma B16BL6 tumor was demostrated. Numerous studies have utilized RGD coupled to nonviral vectors. For example, cationic nanoparticles have been successfully used in αvβ3 integrin targeting *in vivo* (Temming et al. 2005).

RGD has been coupled to drugs to obtain specifically targetable chemotherapeutics. Coupling of RGD-4C to doxorubicin or a cytotoxic anti-cancer drug has enhanced the efficacy and reduced the toxic effects in nude mice bearing MDA-MB-435-derived human breast cancer xenografts (Arap et al. 1998, Ellerby et al. 1999). Recently, Chen and colleagues (2005) reported αvβ3 specific accumulation of the paclitaxel prodrug coupled to bicyclic RGD motif to MDA-MB-435 human breast tumors.

Integrin α2β1 expression is known to be associated in tumor progression and invasion in several carcinomas. The essentiality of this integrin in the cancer cell migration, invasion and metastasis has been demonstrated by many experimental settings (Koistinen & Heino 2002). α2β1 integrin expression has been shown to be upregulated by VEGF in cultured endothelial cells. Combination of antibodies blocking signaling of α2β1 and α1β1 integrins inhibit VEGF-dependent angiogenesis and tumor growth *in vivo.* Hence, α2β1 (and α1β1) antagonists may be used in inhibition of VEGF induced angiogenesis in cancer (Senger et al. 1997, 2002). Circular RKK peptide motif found by Ivaska and colleagues (1999a), has been shown to block collagen binding to α2I domain, thus showing potential for therapeutic studies. However, further optimization of the cyclic peptide conformation would improve the therapeutic value, and targeting or blocking experiments remain to be reported (Ivaska et al. 1999a, Ivaska 1999).

2.7 Towards baculovirus-mediated gene therapy

Gene therapy can be defined as a transfer of genetic material into a cell, tissue or whole organ, aiming at curing a disease or introducing a new trait. Vectors based on different virus systems, including retroviruses, lentiviruses, adenoviruses and adeno-associated viruses, are being utlized, and are currently the best choice for the efficient gene delivery. However, no optimal vector system exists and further development is necessary (Verma & Weitzman 2005).

Baculoviruses have recently raised interest as a potential gene therapy vector. They have long been used as safe and efficient gene delivery tools in insect cells (O'Reilly et al. 1994a), and later have been found to transduce mammalian cells (Hofmann et al. 1995). Baculoviruses have many beneficial features for gene transfer into mammalian cells. Viruses can be easily manipulated, can be grown to high titers (10^8-10^9 pfu/ml) and have a large insertion capacity (up to 47 kb tested). AcMNPV is unable to replicate in mammalian cells, but with the choice of appropriate mammalian promoters, many mammalian cells can be transduced resulting in either transient or stable expression of transgenes. Additionally, baculovirus transduction induces only little or no cytotoxicity in host cells (Cheshenko et al. 2001, Ghosh et al. 2002, Kost & Condreay 2002). However, integration of viral DNA to the host cell genome (Merrihew et al. 2001) raises the possibility of immediate early (IE) gene product (IE-1) expression, detected in BHK-21 cells by yet unknown mechanisms. Thus more effort should be taken to determine to what extent BV genes are transcribed in mammalian cells (Murges et al. 1997).

2.7.1 *In vitro* gene delivery

The use of BVs as pesticides raised an interest to study baculovirus interactions with non-target e.g. mammalian cells. In the first studies replication of BV seemed to occur also in nonpermissive mammalian cells. However, as later attempts to detect replication have failed, BVs are considered nonreplicative in mammalian cells (Kost & Condreay 2002). Volkman and Goldsmith (1983) tested a number of mammalian cell lines for *Ac*MNPV entry and reported that especially occlusion-derived form of the virus was able to enter certain mammalian cells. However, the internalization of BV was observed at lower levels (Volkman & Goldsmith 1983). The first attempt to express transgenes in mammalian cells was made by Carbonell and Miller (1987), who detected a reporter enzyme activity in a human lung carcinoma A549 cell line. However, the detected enzyme activity was not *de novo* i.e. due to expression of the viral genome, but associated with the virion itself (Carbonell & Miller 1987). The first successful transgene delivery to mammalian cells was performed by Hoffman and colleagues (1995), who reported that the recombinant baculoviruses were able to efficiently deliver and transiently express luciferase under mammalian cytomegalovirus (CMV) IE promoter in human and rabbit hepatocytes *in vitro*.

Consistent with this, Boyce and colleagues reported expression of a marker gene, lacZ, under the control of the Rous sarcoma virus promoter in HepG2 cells and rat hepatocytes (Boyce & Bucher 1996). Gene delivery seemed to be restricted to cells of hepatic origin, as most other mammalian cell lines tested failed to efficiently express the transgene. The block in expression seemed to lie in uncoating or cellular transport thereafter (Boyce & Bucher 1996, Hofmann et al. 1995). However, later several other tumor cell lines of human, rat and porcine origin were succesfuly transduced. In these studies, a composite promoter consisting of CMV IE enhancer, chicken b-actin promoter or CMV promoter was employed (Shoji et al. 1997, Yap et al. 1997). Gene expression was transient (Boyce & Bucher 1996, Hofmann et al. 1995, Shoji et al. 1997, Yap et al. 1997), until Condreay and colleagues developed a system for obtaining stable gene expression based on antibiotic selection and isolated CHO cell lines stably expressing transgene. In these studies, the possibility of random integration of baculoviral DNA was suggested (Condreay et al. 1999). Another approach employed baculovirus-AAV hybrid utilizing the site-specific integration of AAV, leading to prolonged expression (Palombo et al. 1998). Later, BV has been reported to transduce numerous cell lines (Kost et al. 2005, Kost & Condreay 2002), including several primary cell lines like human and mouse pancreatic islet cells (Ma et al. 2000), primary rat chondrocytes (Ho et al. 2004) and mouse primary kidney cells (Liang et al. 2004). Additionally, human neural cells (Sarkis et al. 2000) and human mesenchymal stem cells (Ho et al. 2005) can be efficiently transduced. Human osteosarcoma cells proved to be excellent target cells for BV, with even 140-times greater transduction efficiency than that of HepG2 cells, which are considered to be the most susceptible to BV (Clay et al. 2003, Song et al. 2003, Song & Boyce 2001).

The capability to express a transgene varies greatly between different cell types. According to Kukkonen and colleagues, the block in nonpermissive cell lines presumably lies in the cytoplasmic transport or entry to the nucleus (Kukkonen et al. 2003), not in the viral escape from the endosomes as suggested in the earlier studies (Barsoum et al. 1997, Boyce & Bucher 1996). Several attempts have been utilized to obtain better transduction efficiency of BV. This can be achieved by optimization of transduction conditions e.g. incubation time and temperature, buffer used or cell growth phase (Ho et al. 2004, Hsu et al. 2004, Hu et al. 2003). Additionally, butyrate or trichostatin A have been found to enhance expression of transgene in a wide variety of cell lines. Both substances act as histone deacetylase inhibitors thereby alleviating the susceptibility of used expression cassettes to silencing by histones (Airenne et al. 2000, Condreay et al. 1999, Hu et al. 2003, Leisy et al. 2003, Sarkis et al. 2000). The choice of the promoter seemes to be of importance in obtaining efficient expression (Shoji et al. 1997, Spenger et al. 2004). Baculovirus surface modifications, especially peudotyping with VSV-G protein have increased the transduction efficiency of several cell lines (Barsoum et al. 1997, Facciabene et al. 2004, Park et al. 2001, Pieroni et al. 2001, Tani et al. 2001, 2003).

2.7.2 *In vivo* gene delivery

For gene therapy purposes, efficient *in vivo* transduction by baculovirus vectors need to be achieved. Initial *in vivo* gene transfer trials using baculovirus were directed to the mouse and rat liver. However, gene transfer was not successful, presumably because of the complement-mediated inhibition (Sandig et al. 1996). This was verified by Hoffman and colleagues, who showed the inactivation of the virus to be due to the classical complement system. This effect was circumvented by preincubation with antibody against the C component 5 or cobra venom factor (Hofmann et al. 1998, Hofmann & Strauss 1998). The i*n situ* perfusion method allowed the avoidance of the complement as demonstrated by Sandig and colleagues who obtained successful transgene expression *ex vivo* in human liver tissue by this method (Sandig et al. 1996). Direct injection of viruses into the livers of complement resistant (C5-deficient) mice by Hofmann and colleagues (1998) demonstrated, for the first time, the success of baculovirus-mediated gene transfer *in vivo.* Similar gene transfer was successful even into induced human liver tumor in nude mice even though intact complement system was present (Hofmann et al. 1998). Hofmann and collegues further demonstrated recombinant soluble complement receptor type 1 (sCR1) to protect the virus against complement lysis *in vitro* (Hofmann et al. 1999). Further, display of DAF on the viral surface was shown to generate complement resistant vector that was able to mediate enhanced gene transfer into neonatal rats *in vivo* by injection to liver (Huser et al. 2001). Additionally, a synthetic inhibitor of complement activation pathway, FUT-175, was shown to protect virus *in vitro* (Tani et al. 2003). Recently, the use of soluble complement inhibitor 1 (sCR1) injected together with AcMNPV into the tail vein of mice led to successful transgene expression in hepatic cells *in vivo.* However, significant, possibly complement-mediated toxicity was reported. In contrast to suggestions by Hofmann and Strauss (1998), the use of both classical and alternative complement pathway in the inactivation, was suggested, as IgM antibody binding, known to mediate the alternative pathway, was reported (Hoare et al. 2005).

Several other ways to avoid complement has been utilized. One is to choose the site of the delivery not exposed to the complement system. With this strategy, efficient gene transfer was accomplished by injecting viruses directly to mouse brain (Sarkis et al. 2000). Also Lehtolainen and colleques (2002) showed successful transgene delivery in rat brain. The virus exhibited cell type specificity into choroid plexus cells. Additionally, gene transfer in mouse retinal pigment epithelial cells has been reported (Haeseleer et al. 2001). Transient delivery was also achieved to rabbit carotid arteries by collar-mediated delivery with an efficiency comparable to adenovirus (Airenne et al. 2000).

Another choice to avoid complement has been the expression of the VSV-G protein, reported to display complement resistance (Ory et al. 1996), on the viral membrane (Barsoum et al. 1997, Tani et al. 2001). This strategy has lead to the greater resistance to serum and delivery of transgenes *in vivo* in skeletal muscle in mice (Pieroni et al. 2001) and in the celebrum and testis of mice (Tani

et al. 2003). However, Tani and colleagues (2003) suggested another mechanism of inhibition to exist as gene transfer by VSV-G displaying virus was not successful by intravenous, intraperitoneal or intrahepatic route.

Promising for cancer gene therapy applications, recombinant baculovirus encoding p53 gene under CMV promoter has been reported to induce p53-mediated apoptosis of SAOS-2 cells *in vitro*. Experiments were performed to induce programmed cell death of cancer cells in combination with the anticancer drug adriamycin (Song & Boyce 2001). These results suggest that baculovirus can be exploited in the cancer gene therapy.

2.7.3 Baculovirus display

The expression of foreign proteins on the baculovirus membrane, baculovirus display system, was developed in the mid 1990s (Boublik et al. 1995), and has since been used in several applications including screening of expression libraries (Ernst et al. 1998) and antigen presentation (Abe et al. 2003, Kaba et al. 2003, Lindley et al. 2000, Tami et al. 2004). The finding that baculoviruses efficiently entered certain mammalian cells has provided unique opportunities in gene delivery. Thus, the baculovirus display system, can be exploited to specifically target the viruses for gene therapy purposes (Oker-Blom et al. 2003).

While phage display is a powerful technique for selecting peptides from huge libraries (Clackson & Wells 1994, Paschke 2006), baculovirus display allows the expression of large complex eukaryotic proteins requiring correct post-translational modifications and proper folding (Oker-Blom et al. 2003). There are several strategies of displaying fusion partners on the BV surface, e.g. fusion to the second copy of gp64, to the native gp64 or to the vp39 capsid protein. Moreover, foreign membrane anchor can be used as a platform (Oker-Blom et al. 2003) (Figure 9).

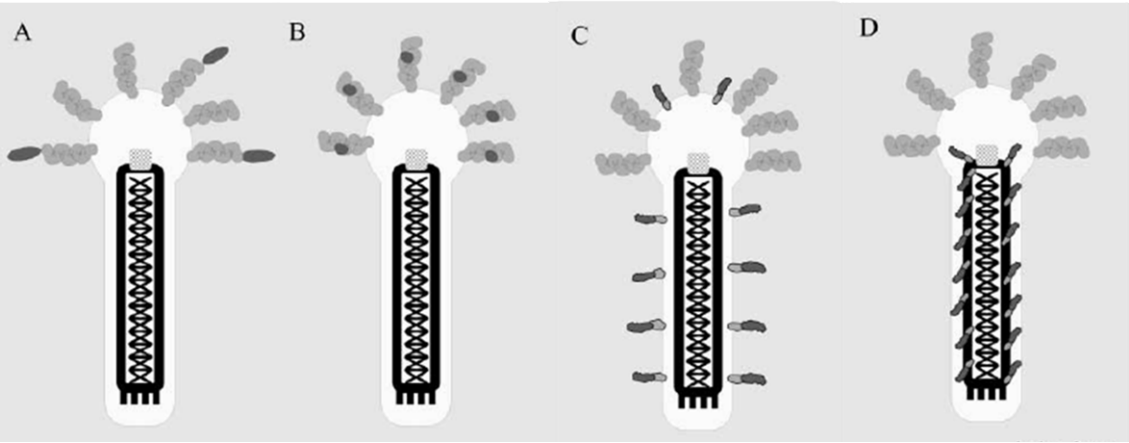

FIGURE 9 Baculovirus display strategies. A) fusion to the N-terminus of the second copy of gp64 B) peptide insertion to the native gp64 C) fusion to the VSV-G protein or its anchor D) fusion to the N- or C-terminus of major capsid protein vp39. Modified from Oker-Blom et al. 2003 by permission of Oxford University Press.

Gp64 as a fusion partner. As a basis of novel eukarytorstaotic display system, Boublik and coworkers showed that foreign proteins can be displayed on the surface of *Ac*MNPV. They displayed glutathione-S-transferase (GST) and HIV major surface glycoprotein gp120 as a fusion to the gp64 protein N-terminus. Fusion was implemented to the second copy of gp64 protein so that both native and modified protein was present on the viral surface presumably as co-oligomers. Gp120 was reported to be functional in binding to its ligand (Boublik et al. 1995). After this, GFP and rubella virus envelope proteins (Mottershead et al. 1997) and the ectodomain of HIV-1 envelope protein gp41 (Grabherr et al. 1997) were displayed on the viral surface on a similar manner.

Gp64 surface display has been utilized to specifically target mammalian cells by displaying ligands for specific cellular receptor proteins. Mottershead and colleagues reported successful display of single–chain antibodies (scFV) and synthetic IgG binding domain (Z /ZZ) from protein A as a fusion to gp64 N-terminus. The biological activity of the displayed peptides highlighted the possibilities of this approach (Mottershead et al. 2000). Ojala and co-workers then showed enhanced binding ability of single chain anti-CEA antibody fragments to mammalian target cells expressing the carcinoembronic antigen (CEA) (Ojala et al. 2001). *Plasmodium berghei* circumsporozoite protein displayed on the BV surface was found to bind HepG2 hepatocarcinoma cells 12-fold better than the wild-type virus (Yoshida et al. 2003). Räty and colleagues displayed avidin on the virus surface as a gp64 fusion and obtained enhanced transduction of mammalian cells due to the positive charge of avidin. Cell biotinylation was shown to further enhance the transduction. Specific targeting to biotinylated epidermal growth factor was demonstrated. Additionally, magnetic targeting with biotinylated paramagnetic particles was accomplished (Räty et al. 2004).

Gp64 based display strategy has also been utilized in several other applications. The successful presentation of antigenic sites of FMDV (Tami et al. 2000) and p67 antigen of *Theileria parva* (Kaba et al. 2003) suggested this system to have applications in the biosynthetic vaccines. Lindley and collegues generated monoclonal antibodies against human nuclear receptors expressed on the viral surface as gp64 fusions (Lindley et al. 2000). By the same method, antibody responses to a variety of other proteins, including human peroxisome proliferator-activated receptor (Tanaka et al. 2002), rodent malaria *Plasmodium berghei* circumsporozoite protein (Yoshida et al. 2003), hemagglutin protein of Rinderpest virus (Rahman et al. 2003) and FMDV proteins (Tami et al. 2004) have been obtained. The ability of raising a strong immunogenic reaction by inoculation of the antigen-presenting baculovirus was further demostrated by displaying influenza virus hemagglutinin on the surface of BV. A strong innate immune response protecting mice from lethal challenge of influenza virus was indicated (Abe et al. 2003). Additionally, this display system has been utilized to protect the BV from complement lysis *in vivo* by displaying DAF on the viral surface (Huser et al. 2001).

The use of the second copy of gp64 as a fusion partner allows the native gp64 to mediate the viral infection process (Grabherr et al. 2001). However, the rather low incorporation of gp64 fusions to the viral surface has occasionally been of concern (Grabherr et al. 1997, Ojala et al. 2001, 2004). This can be avoided by utilizing the native gp64 for display. The oligonucleotide sequence encoding HIV-1-gp41 epitope tag has been fused at a naturally occurring *NotI* site in gp64 genome (Ernst et al. 2000) and several other locations of the native gp64 (Spenger et al. 2002). This system has been demonstrated to work with peptides up to 23 aa (Spenger et al. 2002), while insertion of large molecules, such as green fluorescent protein (GFP), have been unsuccessful, highlighting the importance of gp64 in the infection process. Later, display system based on the streptavidin binding was introduced, by inserting streptavidin binding peptide into the native gp64 Not-1 site. This system was suggested to be suitable for the display of cDNA libraries for the direct selection of protein function (Ernst et al. 2000).

Alternative display strategies. The expression of foreign viral membrane proteins on the BV surface, pseudotyping, has been accomplished mostly by using the VSV-G protein. The protein was shown to enhance the transduction and also broaden the range of susceptible mammalian cell types (Barsoum et al. 1997, Facciabene et al. 2004, Park et al. 2001, Pieroni et al. 2001, Tani et al. 2001). In these studies, the gp64 membrane protein was present, however, later it was confirmed that VSV-G alone on the surface of a gp64-deficient virus was capable of complementing the role of gp64. The VSV-G pseudotyped virus was also able to efficiently transduce mammalian cells (Kitagawa et al. 2005, Mangor et al. 2001). AcMNPV has been successfully pseudotyped with the F protein of *Lymantia dispar* MNPV (*Ld*MNPV), *Spodoptera exigua* MNPV (*Se*MNPV) by transposition of corresponding gene into gp64-deficient bacmid DNA (Lung et al. 2002). Additionally, "inverse" pseudotyping has been carried out by displaying measles virus receptors in gp64-deficient viruses. Recombinant viruses exhibited ligand directed transduction of the cells expressing measles virus envelope glycoprotein (Kitagawa et al. 2005). Tani and colleagues pseudotyped *Ac*MNPV with rhabdovirus envelope proteins and obtained gene delivery into neural cells (Tani et al. 2003). Additionally, extra copy of gp64 gene introduced to the baculovirus genome has lead to the expression of higher amount of gp64 and resulted in enhanced transduction of mammalian cells (Tani et al. 2001).

The use of the membrane anchor of the VSV-G has been reported to improve the display of fusion proteins on the viral surface. Chapple and Jones displayed GFP fused to the anchor consisting of 21-amino acid ectodomain together with the transmembrane (TM) and cytoplasmic (CT) domains of the VSV-G protein. They directed the fusion protein to the membrane by using the gp64 signal sequence (ss) and reported this fusion protein to have a nonpolar distribution in comparison to the polar display of gp64 (Chapple & Jones 2002). Ojala and colleagues used the same approach with IgG binding ZZ domains

and obtained enhanced display in comparison to corresponding gp64 fusion proteins. The transduction efficiency of the target cells was, however, not improved when compared to the control virus (Ojala et al. 2004). Further, the 21-amino acid ectodomain of the VSV-G together with the TM and CT domains was demonstrated to improve transduction of several mammalian cell lines by recombinant baculovirus (Kaikkonen et al. 2006).

The TM region of neuraminidase has also been utilized to display fusion partners as N-terminal fusion on the viral surface obtaining nonpolar distribution (Borg et al. 2004). Borg and colleagues displayed EFGP and introduced a cDNA library approach that should allow for full-length expression of cDNA libraries (Borg et al. 2004). Ernst and colleagues created a library expressing HIV-1-gp41 antigenic site inserted into the influenza virus hemagglutinin on the BV surface and isolated a single baculovirus clone with increased binding capacity out of a pool of 8000 variants (Ernst et al. 1998). Also C-terminal membrane anchor of gp64 has been utilized to display the ectodomain of HIV-1 envelope protein gp41 successfully (Grabherr et al. 1997).

vp39 as a fusion partner. Another interesting approach to baculovirus display has been obtained by utilizing vp39 capsid protein as a fusion partner (Kukkonen et al. 2003). EGFP, used as a proof of principle for the system, was fused to the N- and C-terminus of the protein and was shown to be displayed efficiently without compromising the viral infectivity. As also other peptides and even oligomeric proteins can be successfully displayed according to unpublished results by Kukkonen et al. capsid display may provide another system of eukaryotic display. Additionally, capsid display system could be employed together with surface display to "double target" the virus (cell surface and nuclear targeting) for gene therapy purposes (Kukkonen et al. 2003, Oker-Blom et al. 2003).

3 AIMS OF THE STUDY

Specific targeting and gene delivery to tumor cells and tumor vasculature has lately been of specific interest. Baculovirus possesses many beneficial features for gene delivery and the baculovirus display system has proven to be an efficient tool for displaying foreign protein moieties on the surface of the virus. To develop baculovirus as a targeted vector for gene transfer to tumor vasculature the specific aims of this study were:

1. To study viral and receptor entry mechanisms into the cell and utilize the information in viral vector development.

2. To create baculovirus vectors displaying functional human cancer cell targeting motifs and encoding gene expression cassettes active in mammalian cells.

3. To enhance binding to and transduction of human cancer cells by using these baculoviral vectors.

4 SUMMARY OF MATERIALS AND METHODS

4.1 Cell lines

For cloning of plasmids, *E. coli* JM109 cells (III, IV, V) were used. The recombinant baculoviral genomes were propagated in *E. coli* DH10Bac cells (III, IV, V). The bacterial cells were grown in suspension cultures in Luria-Bertani medium at 37°C supplemented with appropriate antibiotics for the selection of desired clones. *Sf9* (ATCC CRL 1711) insect cells, used for baculovirus proliferation (I, III, IV, V), were maintained as monolayer or suspension cultures at 28 °C using serum free Insect-XPRESS culture medium (Cambrex, Walkersville, MD, USA) or HyQ®SFX-Insect medium (HyClone Inc, Logan, UT, USA) in the absence of antibiotics.

SAOS-α2β1 cells, stably transfected with α2 integrin expressing vector (Ivaska et al. 1999b), SAOS-pAW cells containing the empty expression vector and SAOS-α2/α1β1 cells, α2/α1 integrin mutant containing the intracellular tail from the α1 integrin subunit (Ivaska et al. 1999b) were all maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, 2 mM L-glutamate, 0.45% glucose, 10 mM Hepes pH 7.4. Geneticin (G-418; Sigma-Aldrich, Saint Louis, MO, USA) was added in order to select for α2 integrin or α2/α1 integrin chimera expressing cells only. The A549 human lung carcinoma cell line (ATCC CCL-185) was grown as monolayer cultures in DMEM supplemented with 10% inactivated FBS, 1% penicillin-streptomycin, and 2 mM L-glutamate (II, III). Chinese hamster ovary cells (CHO; ATCC) and CHO-α2β1 cells, stably transfected cell line expressing α2β1 integrin (Kapyla et al. 2000) were grown in monolayer cultures in α-Minimum Essential Medium, α-MEM, containing 10% inactivated FBS, 1% penicillin-streptomycin, and 2 mM L-glutamate. For CHO-α2β1 cells G-418 was added in order to select for α2 integrin-expressing cells only (IV). MDA-MB-435 human breast carcinoma cell line (kind gift from Pirjo Laakkonen, University of Helsinki, Finland) was maintained in monolayer culture in DMEM supplemented with 10% inactivated

FBS, and 1% penicillin-streptomycin (V). HepG2, human hepatocarcinoma cell line (ATCC HB-8065), was grown in monolayer culture using MEM, supplemented with 1% penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% FBS (I, V). All reagents used for cell culturing were from Invitrogen (Carlsbad, CA, USA) if not otherwise stated.

4.2 Antibodies

Table 1 describes the variety of primary and secondary antibodies utilized within this thesis work. Rabbit antiserum against EV1 (II) was produced in the research group of Timo Hyypiä by immunization of rabbits by primary subcutaneous injection containing 15 µg of the virus in Freund´s complete adjuvant, followed by booster doses of 10 µg in incomplete adjuvant 4 and 8 weeks later. The serum was collected 3 weeks after the last injection. Anti-EV1 antiserum had no crossreactivity with the SAOS cells.

TABLE 1 Antibodies utilized in this thesis

PRIMARY ANTIBODIES				
Antibody	**Target**	**Source**	**Provider and Reference**	**Article**
B12D5	gp64 envelope protein	mouse	Dr. Loy Volkman Keddie et al. 1989	I, III-V
p10C6	vp39 capsid protein	mouse	Dr. Loy Volkman; Whitt and Manning 1988	I, III, V
M1	FLAG epitope	mouse	Sigma Aldrich, MO	III, IV
EV1	echovirus 1	rabbit	Marjomäki et al. 2002 (II)	II
NAKG-4	αvβ3 integrin	rabbit	Dr. Merja Roivainen, Ylipaasto et al. 2004	III
MAB1950	integrin α2 subunit	mouse	Chemicon, CA, USA	II
12F1	integrin α2 subunit	mouse	BD Biocsiences, San Jose, CA	II
A-6455	GFP	rabbit	Molecular Probes, Eugene, OR	IV
anti-VSV-G	VSV-G protein, aa 497-511	rabbit	Sigma Aldrich	V
8873-HPEV1	human parechovirus 9	rabbit	Dr. Päivi Joki-Korpela, Joki-Korpela et al. 2000	III
8863-CAV	coxsackie A virus 9	rabbit	Dr. Päivi Joki-Korpela, Joki-Korpela et al. 2000	III
caveolin	caveolin	rabbit	Transduction Laboratories, Lexington, UK	II
caveolin-1	caveolin-1	mouse	Transduction Laboratories	II
caveolin	caveolin	mouse	Zymed Laboratories, San Francisco, CA	II
EEA-1	early endosomal antigen 1	mouse	Transduction Laboratories	I

(continues)

TABLE 1 (continues)

Antibody	Target	Source	Provider and Reference	Article
EEA-1	early endosomal antigen 1	rabbit	Dr. Harald Stenmark Mu et al. 1995	II
GM130	Golgi-matrix protein	mouse	Transduction Laboratories	I
Rab11	recycling early endosomes	rabbit	Zymed Laboratories	I
CD63	lysos. membr. glycoprotein late endosomes/lysosomes	mouse	Zymed Laboratories	I
CI-MPR	cation-independent mannose -6-phosphate receptor	rabbit	Dr. Varpu Marjomäki Marjomäki et al. 1990	I, II
TGN-38	trans-Golgi	mouse	Sigma Aldrich	I
TGN-46	trans-Golgi	rabbit	Dr. George Banting; Banting et al. 1998	II
p23	Transmembrane protein 23 cis-Golgi network	rabbit	Dr. J. Gruenberg; Rojo et al. 1997	II
ID3; PDI	protein disulfide isomerase ER	mouse	Dr. Stephen Fuller; Huovila et al. 1992	II
AB730	β2 migroglobulin	rabbit	Chemicon	II
BM-63	β2 migroglobulin	mouse	Sigma Aldrich	II
W6/32	class I HLA	mouse	Dr. Timo Hyypiä Barnstable et al. 1978 hybridoma cell line	II
Myc 9E10	Myc peptide	mouse	(ATCC CRL-1729)	II
HA tag	hemagglutinin (HA) tag	mouse	Santa Cruz, CA, USA	II
SECONDARY ANTIBODIES				
Antibody	**conjugate**	**Source**	**Provider and Reference**	
anti-mouse IgG	alkaline phosphatase	goat	Promega; Madison, WI, USA	III, IV, V
anti-rabbit IgG	alkaline phosphatase	goat	Promega	III, IV, V
anti-mouse IgG	Alexa Fluor® 546 nm red	goat	Molecular Probes, Inc, Eugene, OR	I-III, IV
anti-rabbit IgG	Alexa Fluor® 555 nm red	goat	Molecular Probes	V
anti-mouse IgG	Alexa Fluor® 488 nm green	goat	Molecular Probes	II, V
anti-rabbit IgG	Alexa Fluor® 488 nm green	goat	Molecular Probes	I, III
anti-mouse IgG	Nanogold®	goat	Nanoprobes, Yabhank, NY	I
anti-rabbit IgG	Nanogold®	goat	Nanoprobes	I
anti-rabbit IgG	horseradish-peroxidase	swine	Dako, Glostrup, Denmark	II

4.3 Viruses

4.3.1 Recombinant Baculoviruses

Recombinant baculoviruses constructed in this thesis work are listed in Table 2. In addition, wt *Ac*MNPV (I, III, IV) and previously constructed recombinant baculoviruses, vp39EGFP (Kukkonen et al. 2003) and *Ac*CMVEGFP (Gilbert et

al. 2005) (I), were utilized in the studies (Table 2). Vp39EGFP virus displays enhanced green fluorescent protein (EGFP) fused to the major capsid protein vp39. *Ac*CMVEGFP encodes EGFP as a marker gene under cytomegalovirus (CMV) immediate early promoter. Baculoviruses were propagated in *sf9* insect cells infected at multiplicity of infection (MOI) 1-10 and virus titers were determined by end-point dilution assay (O'Reilly et al. 1994a). For storage at 4°C, viral stocks were supplemented with 2.5% FBS. For electron microscopical studies (I), wt baculoviruses were sucrose gradient purified as follows: after standard infection infected cells and cell debris was removed by centrifugation (1000 g, 30 min, +4°C). Supernatant was further centrifuged through 25 % sucrose (7200 g, 8h, +4°C). Viral pellet was incubated on ice with phosphate buffered saline (PBS) (o/n, +4°C), resuspended and loaded onto stepwise 50/20% sucrose gradient prepared in PBS and ultracentrifuged (26 000 rpm, 1h, +4°C). Viral band was collected, diluted in PBS and subjected to ultracentrifugation (26 000 rpm, 1h, +4°C). Pellet was resuspended in PBS (o/n, +4°C, rocking) and stored at +4°C.

TABLE 2 Recombinant baculoviruses utilized in this thesis. See text for details.

Virus	Signal sequence	Displayed protein	Fusion partner	Expression cassette	Publication
vp39EGFP	-	EGPF	vp39	-	Kukkonen et al. 2003
***Ac*CMVEGFP**	-	-	-	CMV-EGFP	Gilbert et al. 2005
***Ac*CL**	GluR-D	CAV9RGD(GS)	gp64	-	III
***Ac*HL**	GluR-D	HPEV1RGD(GS)	gp64	-	III
***Ac*CL-luc**	GluR-D	CAV9RGD(GS)	gp64	SV40-luc	III
***Ac*-luc**	-	-	-	SV40-luc	III, V
***Ac*GFP(K)gp64**	GluR-D	GFP(IRKK)	gp64	-	IV
***Ac*GFP(H)gp64**	GluR-D	GFP(RKKH)	gp64	-	IV
***Ac*GFPgp64**	GluR-D	GFP	gp64	-	Mottershead et al. 1997
***Ac*LyP-1-luc**	gp64	LyP-1	VSVG TM	SV40-luc	V
***Ac*F3-luc**	gp64	F3	VSVG TM	SV40-luc	V
***Ac*CGKRK-luc**	gp64	CGKRK	VSVG TM	SV40-luc	V

Recombinant baculoviruses were designed to display cancer cell targeting motifs on the surface of the viruses utilizing baculovirus display system. The displayed targeting motifs included RGD motifs of human parechovirus 1 (HPEV1) and coxsackie A virus 9 (CAV9) (III), RKK motif from snake (*Bothrops jararaca*) venom disintegrin/metalloproteinase jararhagin (IV), and tumor homing peptides LyP-1, F3 and CGKRK (V) (Table 3). Targeting motifs were displayed either as a fusion to the second copy of the baculovirus major

envelope glycoprotein gp64 (III, IV) or to the membrane anchor of the VSV-G protein (V) (Table 2).

TABLE 3 Targeting motifs utilized in this study.

Motif	Amino acid sequence	Target	Source, reference	Article
HPEV1 RGD	SRAL**RGD**MANLTNQ	αvβ3 integrin	HPEV1 vp1 (aa 218-231) Hyypiä et al. 1992	III
CAV9 RGD	AQSRR**RGD**MSTLNTH	αvβ3 integrin	CAV9 vp1 (aa 285-299) Santti et al 2000	III
RKK	**RKK**	α2β1 integrin	jararhagin (aa 243-245) Ivaska et al. 1999a	IV
Lyp-1	**CGNKRTRGC**	tumor lymphatics	*in vivo* phage display, Laakkonen et al. 2002	V
F3	**KDEPQRRSARLSAKPA PPKPEPKPKKAPAKK**	endothelia of tumor blood vessel	*in vivo* phage display, Porkka et al. 2002	V
CGKRK	**CGKRK**	endothelia of tumor blood vessel	*in vivo* phage display, Hoffman et al. 2003	V

For viral constructs displaying RGD motifs (III), the RGD encoding sequences from C-terminus of VP1 of HPEV1 (Hyypiä et al. 1992) or CAV9 together with a GS linker were designed as complementary oligonucleotide sequences (TAG Copenhagen A/S, Copenhagen, Denmark). For assembly of the RKK displaying recombinant viruses (IV) the sequence encoding GFP was isolated from plasmid pK503-12 (Mottershead et al. 1997). Mutagenesis of GFP for introducing the integrin α2 specific RKK (IRKK or RKKH) sequence to corresponding sites in the GFP was achieved by the megaprimer method of site-directed mutagenesis (Sarkar & Sommer 1990). For the construction of gp64 fusion proteins (III, IV) the sequence encoding the GluR-D signal sequence, the FLAG epitope tag and gp64 (ssFLAG-gp64) was amplified from plasmid pFLAGgp64 (Mottershead et al. 1997). The corresponding sequences encoding targeting motifs/mutated proteins were inserted N-terminal to gp64 and cloned into the pFastBac1™ or pFastBac™ Dual (Invitrogen, Carlsbad, CA, USA) under the polyhedrin promoter. Nucleotide sequences of the fusion constructs were confirmed by sequencing.

The fusion proteins displaying LyP-1 (Laakkonen et al. 2002), F3 (Porkka et al. 2002) or CGKRK (Hoffman et al. 2003) tumor homing peptides (V) contained the N-terminal signal sequence (gp64ss), the sequence of the LyP-1/F3/CGKRK tumor-homing peptide, a linker region encoding twenty alanine residues (polyAla), and the reading frames of the TM and CT domains of the VSV-G. The codon usage of fusion genes was adapted to the codon bias of the *Sf* genes. A synthetic DNA sequence encoding the corresponding fusion protein was isolated from a commercial pPCR-Script-vector (GeneArt, Regensburg, Germany) and subsequently inserted into pFastBac™ Dual under the

polyhedrin promoter. Nucleotide sequences of the constructs were confirmed by sequencing.

Recombinant viruses (III, V) were equipped with the transcription unit of the firefly luciferase (luc) composed of the SV40-promoter, *luc* gene, SV40 late poly(A) signal and the SV40 enhancer sequence. The sequence of SV40-luc was amplified from the pGL3-Control Vector (Promega, Madison, WI, USA) and subcloned into pFastBacTM Dual downstream of the p10 promoter to be functional both in insect and mammalian cells. The luciferase-encoding control virus *Ac*-luc, possessing wild-type surface phenotype, was also prepared.

The recombinant viruses were generated using Bac-to-Bac® Baculovirus Expression System (Invitrogen) (III-V). Briefly, the respective fusion genes /expression cassettes in the pFastBacTM or pFastBacTM Dual vectors were transferred into the *Ac*MNPV genome by transposition in DH10Bac cells according to manufacturer's instructions.

4.3.2 Echovirus 1

Echovirus 1 (EV1; Farouk strain) was obtained from ATCC and propagated in the green monkey kidney (GMK) or monkey rhesus kidney (LLC-MK2) cells. To prepare the radioactively labeled EV1, infected GMK cells were incubated with 50 µCi/ml of [^{35}S]methionine (Pharmacia Biotech) in MEM depleted of L-methionine (GibcoBRL, Life Technologies). The virus was purified in sucrose gradients as described previously (Abraham & Colonno 1984) and the infectivity was determined by plaque titration. Purified HPEV1 was also used in some experiments (Joki-Korpela et al. 2001).

4.4 Characterization of the recombinant baculoviruses

4.4.1 Western blotting

Heat-denatured protein samples derived either from infected insect cells or concentrated viruses (III-V) were subjected to SDS-PAGE and immunoblot analysis. Proteins were probed with appropriate primary antibodies (Table 1) and detection was performed using alkaline phosphatase-conjugated secondary antibodies (Promega; Madison, WI, USA) (Table 1), NBT and BCIP (Sigma-Aldrich) according to manufacturer's instructions.

To analyze the ratio of the viral particle number with infectious virus (pfu) (III, V), similar pfus of each virus were concentrated and subjected to immunoblot analysis using an anti-vp39 monoclonal antibody and corresponding secondary antibodies (Table 1). Quantification of bands representing the vp39 capsid protein was performed by densitometry analysis software (Quantity One, Bio-Rad Laboratories, Hercules, CA, USA). The intensity of wild type virus (wt) was set as one. Average of total intensities of vp39 bands for each viral stock was used in the calculations (III).

4.4.2 Detection of protein expression in insect cells

To demonstrate the correct expression of RGD (III), LyP-1, F3 and CGKRK (V) displaying fusion proteins on the surface of *sf9* insect cells, infected cells were harvested at 36 or 38 h p.i. and stained with anti FLAG-M1 (4E11) (III) or anti-VSVG tag antibody (V). Alexa Fluor® 546 goat anti-mouse IgG or Alexa Fluor® 555 goat anti-rabbit IgG (Molecular Probes) was used as a secondary antibody, respectively. Stained cells were fixed with 4% PFA- 2% sucrose containing 1mM CaCl2 (III) and mounted on microscope slides with MOWIOL (Calbiochem) containing 25 mg/ml DABCO (Sigma-Aldrich). Samples were inspected under a laser scanning confocal microscope (Zeiss LSM510) using appropriate excitation and emission settings.

The fluorescence of the RKK displaying GFPgp64 fusion proteins (IV) expressed on the surface of *sf9* cells 72 h post infection with corresponding recombinant viruses was analyzed by fluorescent activated cell sorter (FACS) (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) and the CELLQUEST software.

4.4.3 Enzyme-linked immunosorbent assay (ELISA)

In vitro binding of the RKK displaying recombinant viruses to integrin α2I domain (IV) was analyzed by ELISA assay. 96-well plates were coated with the gluthatione-S-transferase-rα2I fusion protein in an overnight incubation at 4°C, followed by blocking and incubation with virus inoculum. The bound recombinant baculovirus was detected with mouse anti-gp64 mAb and alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Promega) (Table 1). The absorbance was measured with a Multiscan® (Labsystems, Helsinki, Finland) microtiter plate reader at 405 nm in the presence of a substrate solution.

4.5 Viral binding and internalization studies

To characterize the binding (I-V) and internalization (I, II, IV) of wt baculovirus, EV1 and recombinant baculoviruses to cancer cells, either confocal microscopy (III, IV), light microscopy (II), electron microscopy (I), FACS (V) or scintillation counter (II) was used. For viral binding and infection/transfection studies (exept a binding study with FACS, see 4.5.2) cells were plated on glass cover slips one or two days prior to the experiments. Virus binding (baculovirus: 50-200 pfu/cell for confocal microscopy, 500-1000 pfu/cell for electron microscopy; EV1: MOI 20) was performed for 1 hour on ice. Subsequent to washes, cells were either fixed immediately (0 h) or allowed to internalize virus at +37°C in complete medium. At set time intervals post transduction/infection, cells were fixed for confocal microscopy with either 4% PFA in PBS (I, V) or 4% PFA containing 2% sucrose and 1mM CaCl2 (III) for 20 min at RT or with methanol

for 6 min at –20 °C (II, III, IV). Fixed samples were further stained as described in 3.5.1. The handling of electron microscopical samples is described in 4.7. and [^{35}S]methonine-labeled EV1 binding assay in 4.5.2.

4.5.1 Immunofluorescence and confocal microscopy

Immunofluorescent stainings were performed depending on the fixation method and specific antibody used. In brief, infected/transfected fixed cells on cover slips (See 4.5) were probed with virus, receptor and cell organelle specific primary antibodies (Table 1), respectively, followed by addition of corresponding Alexa Fluor® 546 /555 or 488 nm IgG conjugated secondary antibodies (Molecular Probes). After final washes, samples were mounted on microscope slides with MOWIOL containing DABCO (I-V).

Immunostained cells were examined with a laser scanning confocal microscope (Zeiss LSM510) or Axiovert 100 M SP epifluorescence microscope (Carl Zeiss) by using appropriate excitation and emission wavelengths. For double-labeling experiments, multitracking for 488 nm and 546 nm laser lines (confocal microscope) was used to avoid false colocalization. Images were acquired at 512 x 512 resolution. The detector gain and offset values were set to appropriate levels for each labeling experiment using proper negative background controls. The dilutions of the secondary antibodies used gave neglible backround. Non-specific reactions between primary and secondary antibodies were not observed.

4.5.2 Quantitative binding studies

To study the binding efficiency of EV1 to SAOS-pAW and SAOS-α2β1 cells (II), [^{35}S]methonine-labeled EV1 (75 000 cpm) was added on the cells suspended into 30 µl of PBS containing 2 mM $MgCl_2$ and incubated for 1 h on ice. The cells were washed twice with $MgCl_2$-PBS and the pellet was suspended in PBS and analyzed for radioactivity in a scintillation counter (1450 Microbeta; Wallac, Turku, Finland).

In order to quantify RGD displaying baculovirus particles bound to the cell surface (III), viruses were allowed to attach and were stained with an anti-vp39 antibody as described in 4.5. and 4.5.1, respectively. Z stacks from individual cells were scanned (n=50) with confocal microscope at zoom 2.1. The laser power was adjusted low (HeNe laser threshold 9.8%) to avoid intensity values over the scale. The mean intensity was measured from z stacks, using the ImageJ program (batch statistics) (http://rsb.info.nih.gov/ij/). The background intensity value was subtracted. Results were presented as the mean value of the total fluorescence intensity in the cell population studied.

Binding efficiency of recombinant baculoviruses displaying LyP-1, F3 and CGKRK to MDA-MB-435 and HepG2 cells (V) was studied by incubating cells detached with trypsin-EDTA (Gibco-BRL) with each virus (100 pfu/cell) for 1.5 h at 4°C. Bound viruses were stained with mAb against gp64 for 1 h followed

by washes and incubation with Alexa Fluor®488-conjugated secondary antibody (Molecular Probes) (Table 1). Cells were analyzed with a FACS using CellQuest software.

4.5.3 Inhibition studies

To study the receptor usage of EV1 by antibody blocking (II), confluent SAOS-α2β1 cell monolayers were first incubated with antibodies against putative EV1 cell surface receptors: anti-α2 integrin (12F1), anti-β2 microglobulin and anti-HLA-I W6/32 or their combinations (15 min, RT). Purified EV1 (MOI 5) was added, and after 10 h incubation at 37°C the cells were methanol-fixed and immunoperoxidase stained (Ziegler et al. 1988) with EV1 rabbit antiserum and peroxidase-labeled goat anti-rabbit antibodies (Table 1). Finally, the proportion of infected cells was calculated under light microscope.

The blocking efficiency of methyl-cyclodextrin (Sigma-Aldrich) for EV1 infection (II) was studied by quantitating internalized fluorescently labeled EV1 particles (See 4.5. and 4.5.1 for infection and immunofluorescent labeling, respectively) with the help of a confocal microscope. Z slices from the center of the cell (2 μm in total) were selected and projected together and a histogram of intensity values was prepared with LSM510 program. A threshold value of 100 was selected for all the samples and the results were represented as a mean fluorescence value. Additionally, the colocalization of EV1/α2β1 integrin/caveolin-1 was studied with confocal microscope in the presence and absence of methyl-cyclodextrin.

In order to investigate the effect of phospholipase C (PLC; Sigma-Aldrich) on the binding and internalization of recombinant RKK displaying viruses (IV), CHO and CHO-α2β1 cells were incubated with 2.0 U of PLC/cover slip for 30 min at +37 °C before the viral binding, described in 4.5. Fixed samples were stained with an anti-gp64 antibody and corresponding secondary antibodies and inspected with confocal microscope (See 4.5. and 4.5.1).

The effect of the synthetic LyP-1, F3, or CGKRK peptides on the binding of the corresponding recombinant baculoviruses (V) (binding crossinhibition) was investigated by incubating (15 min, 4°C) cells with peptides (50, 200, 500, and/or 1000 μM) prior to virus exposure. After removal of the peptides virus binding, immunofluorescent stainings and detection with FACS was performed as described in 4.5.2.

4.5.4 Infectivity titration

The amount of the intracellular EV1 virus synthesized in the SAOS cell lines (II) after infection, described in 4.5., was determined by harvesting infected cells at different time periods p.i. Three freeze-thaw cycles were performed to disrupt the cells, and the supernatant containing the virus was collected after centrifugation. The amount of infectious virus was then expressed as end-point titers.

4.5.5 Sucrose gradient sedimentation

To analyze the conformational changes of EV1 during infection, sucrose gradient sedimentation was performed (II). SAOS-α2β1 cells grown in confluency were detached in 0.02% Versene in PBS, pelleted and washed twice with PBS. For virus attachment, cells were incubated for 1 h on ice with 150 000 cpm of [^{35}S]methionine-labeled EV1 in PBS containing 2 mM MgCl2. The cells were then washed twice with PBS-$MgCl_2$ and incubated at 37°C 0 to 2 h in DMEM, containing 1% FBS. After cell lysis (1% Triton X-100, 10 min on ice) and low speed centrifugation, the supernatant was layered on 5-20% (w/v) sucrose gradient and centrifuged 150 000 g for 2 h at 4°C. Collected fractions were analyzed for radioactivity in a scintillation counter. Samples containing only radiolabeled EV1 were used as a control.

4.5.6 Immunoisolation

For immunoisolation studies with EV1 (II) cells were infected with EV1 for different periods of time as described in 4.5. After infection, the cells were detached with a rubber policeman, washed and and homogenized by passing the pellet extensively through a 23G needle in a homogenization buffer (3 mM imidazole, 0.25M sucrose, 1 mM EDTA, pH 7). Homogenate was pelleted and the post-nuclear supernatant was then subjected to immunoisolation. M-450 Dynal beads (Dynal AS, Oslo, Norway), coated with mouse anti-caveolin-1 (clone 2234, Transduction laboratories) or non-specific mouse IgG (Sigma-Aldrich) together with 1% BSA overnight, were incubated with the homogenates for 1 h by rotating at 4°C. The homogenate, a sample from the unbound fraction and the beads were diluted in Laemmli sample buffer and subjected to SDS-PAGE and immunoblotting. The blots were visualized by rabbit anti-caveolin-1 or anti-EV1 antiserum, anti-rabbit HRP conjugate (BIO-RAD) and chemiluminescent Super Signal substrate (Pierce) (Table 1).

To measure the infectivity of the EV1 particles inside the caveola structures the immunoisolated material was treated with 0.5 % SDS for 15 min on ice to release the virus from caveola structures. After centrifugation (1 min, 16 000 g, 4°C) the collected virus was plaque titrated using GMK cells for 2 days, infected cells were stained with crystal violet and the average amount of plaques were counted from four parallel wells.

4.5.7 Transfection studies

SAOS-αβ21 or A549 cells grown on 8.8 cm^2 plate were transfected with 2 µg plasmid DNA encoding HA-tagged wt caveolin-3 or dominant negative mutant caveolin-3 (CavDGV) (Roy et al. 1999) using Fugene 6 reagent (Boehringer Mannheim) according to the manufacturer's instructions. After a 48 h incubation period the cells were infected with either EV1 or HPEV1 (used as a control virus), as described in 4.5. Ten or six hours post infection the cells were methanol-fixed and incubated with antibodies against the HA-tag (Santa Cruz)

and EV1 or HPEV1, followed by incubation with secondary antibodies (Table 1). Confocal microscopy was used to calculate the number of transfected and infected cells.

4.6 Transduction studies

The transduction efficiency of the recombinant baculoviruses was assessed either by luciferase activity measurement (III, V) or by analysis of the EGFP expression (I). For transduction experiments baculoviruses were allowed to attach to A549 (III), MDA-MB-435 (V) and HepG2 cells (I, V). Transduction was performed for 2-48 h at 37°C in the absence or presence of 5 mM sodium butyrate (Fluka Chemie AG, Buchs, Switzerland). For luciferase activity measurement (III, V), after appropriate transduction time, cells were detached, concentrated by centrifugation, resuspended and subsequently transferred into white-pigmented 96-well plate (CellStar, Greiner-Bio One, Frickenhausen, Germany) followed by addition of 1 mM D-Luciferin (Sigma-Aldrich) diluted in 0.1 M Na-citrate buffer. The enzymatic activity of luciferase was measured with a multilabel counter Wallac 1420 Victor TM 2 (Wallac Oy, Turku, Finland) utilizing Software version 2.00. For EGFP expression studies (I), transduced cells were fixed at appropriate time points with 4% PFA in PBS, mounted on microscope slides and inspected by confocal microscopy. Results were presented as EGFP expression percentage in the cell population studied.

4.6.1 Inhibition of transduction

The inhibitory efficiency of LyP-1, F3, CGKRK or RKK peptides on the transduction of recombinant baculoviruses (V) was investigated by preincubating HepG2 cells in the presence of the peptides (250, 500, and/or 1000 μM) for 30 min at 4°C followed by addition of the virus (100 pfu/cell) and further incubation for 1 h at 4°C. After washes, transduction was carried out for 24 h at 37 °C after which the cells were monitored for luciferase activity as described in 4.6.

The effect of ammonium chloride on the transduction of the recombinant baculoviruses (V) was assessed by incubating viruses with HepG2 cell for 1 h at 4°C. The virus-containing medium was replaced with warm (37 °C) medium followed by addition of ammonium chloride (1-10 mM). After 24 h transduction, the luciferase activity was measured. As a control, 10 mM ammonium chloride was added to control cells 1 h (37 °C) prior to harvesting.

4.7 Electron microscopy

4.7.1 Nanogold pre-embedding immunoelectron microscopy

For the baculovirus entry studies (I), HepG2 cells were transduced as described in 4.5. and fixed with PLP at +4°C o/n (4% PFA, 75 mM lysine-HCl-Na-phosphate buffer, 2.13 mg/ml $NaIO_4$). After fixation, cells were washed and permeabilized with phosphate and saponin buffer. Cells were incubated with the corresponding primary antibodies and the nanogold conjugated secondary antibodies (Table 1), at RT for 1 h. After the washes a postfixation by 1% glutaraldehyde and quenching with 50 mM NH_4Cl were performed, followed by silver enhancement (Nanoprobes) and gold toning. The cells were further postfixed with 1% osmium tetroxide containing 15 mg/ml $K_4Fe(CN)_6$ at +4°C for 1 h and dehydrated with 70% and 96% ethanol, stained with 2% uranylacetate, and embedded in LX-112 Epon (Ladd Research industries, Williston, VT). The samples were further stained with toluidine blue, cut with ultramicrotome (Reichert-Jung, Ultracut E) and stained with uranylacetate and lead citrate. The examination was performed by JEOL JEM-1200EX transmission electron microscope (JEOL LTD, Tokyo, Japan) operated at ~60 kV.

4.7.2 Ruthenium red staining

In order to stain cell surface carbohydrates, ruthenium red staining was performed for baculovirus transduced HepG2 cells (I). After transduction (described in 4.5.), cells were washed with cacodylate buffer (pH 7.3.) and fixed first with 1.3% glutaraldehyde for 1 h at RT followed by postfixation with 1.7 % osmium tetroxide for 3 h at RT. Both fixatives contained 0.07 mg/ml ruthenium red in cacodylate buffer. The specimens were dehydrated in ethanol, stained with uranyl acetate, and embedded in LX-112 Epon. Further preparation for electron microscopy was performed as described in 4.7.1.

4.7.3 Pre-embedding labeling with gold-conjugated protein A

To study the internalization of EV1 with electron microscopy (II), the virus was first allowed to bind to SAOS-α2β1 cells for 1 h on ice. After the washes, cells were incubated with EV1 antibodies for 1 h on ice, washed and treated with protein A-gold of 5 nm particles (G. Posthuma and J. Slot, Utrecht, The Netherlands), for 1 h on ice and washed. Cells were then either fixed immediately or allowed to internalize EV1 at 37°C in complete culture medium. Cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, then postfixed in 1% osmium tetroxide for 1 h in the same buffer, dehydrated in ethanol, stained with uranyl acetate, and embedded in LX-112. The cutting and further staining was performed as described in 4.7.1.

4.7.4 Post-embedding labeling for cryosections

EV1 infected SAOS-α2β1 cells were prepared for cryo-ultramicrotomy by fixaton with 8% PFA in PBS for 1h at RT. Infected cells were embedded in 10% gelatine at 37°C, and allowed to solidify on ice. Fixed blocks were infused with cryoprotectant (2.1 M sucrose), for at least 30 min. Blocks manipulated to make pyramids were placed on the aluminium specimen carrier, frozen in liquid nitrogen and cut using glass knives and a Reichert 4D ultramicrotome. Immunolabeling was carried out as described earlier (Griffiths et al. 1984) using protein A-gold particles of 5 and 10 nm (Slot & Geuze 1985). The non-specific binding was blocked using 10 % FBS in PBS throughout the staining. Free protein A in 5% FBS in PBS before secondary antibody treatment was used for blocking. Labeled sections were further contrasted and embedded as described earlier (Marjomäki et al. 1990).

5 REVIEW OF THE RESULTS

5.1 Characterization of the baculovirus entry route

5.1.1 General course of baculovirus transduction

Baculoviruses were shown to transduce HepG2 cells efficiently (I). EGFP expression started at 6h p.t., gene transfer efficiency being 3% at that time. A considerable increase in EGFP expression was observed after 12 h p.t. and maximum level of expression was reached at 22 h p.t., when approximately 30% of cells expressed EGFP (I, Fig. 1).

5.1.2 Binding and uptake of baculoviruses

The overview of baculoviral binding and internalization was obtained by transducing HepG2 cells with wt baculovirus for different periods of time. Viral particles, stained with anti-vp39 capsid antibody were visualised with confocal microscopy. Baculoviruses were seen to attach HepG2 cells efficiently (I, Fig. 2A and B), concentrating on certain areas of the plasma membrane. Baculoviruses were observed in cluster formations at 5-15 min p.t. (I, Fig. 2A). Starting at 30 minutes p.t., viruses were found in perinucear accumulations, which were most visible between 1 to 2 h p.t. Later (3-4 h p.t.) viral capsids were observed in the nucleus, although most remained in the cytoplasm (I, Fig. 2B).

Binding of baculoviruses to HepG2 cell surface was further studied by electron microscopy. Baculoviruses, with typical rod-shaped morphology surrounded with loose-fitting envelope, were clearly visible without specific labeling due to the large size of the viruses. Viruses were observed near the plasma membrane starting at 15 min p.t. Interestingly, viruses were frequently seen attached to microvilli-like structures on the cell surface by ruthenium red staining (I, Fig. 3D). Microvilli-like structures were more abundantly observed in transduced cells than in untransduced control cells, suggesting baculoviruses to induce the formation of these structures.

Although baculovirus has been suggested to use clathrin-mediated endocytosis in mammalian cells (Kukkonen et al. 2003, Salminen et al. 2005, van Loo et al. 2001), no direct evidence concerning the use of clathrin pits has been presented. In this study, some baculoviruses were seen at the cell surface in pits with an electron-dense coating resembling clathrin, starting at 30 min p.t., (I, Fig. 3B). At this time point, virus was also seen near the plasma membrane in small non-coated cytoplasmic vesicles (I, data not shown). However, no viruses in clathrin-coated vesicles were found. Additionally, virus particles were observed in larger non-coated plasma membrane invaginations and in larger vesicles near the cell surface at 30 min p.t. (I, Fig. 3C and D). To discriminate between internalized vesicles and membrane invaginations, ruthenium red staining was used (Henley et al. 1998, Marechal et al. 2001). Carbohydrate-specific ruthenium red staining confirmed that in addition to virus filled ruthenium red positive membrane invaginations on the plasma membrane, there were large cytoplasmic vesicles, not continuous with the plasma membrane, near the cell surface containing several virus particles (I, Fig. 3D).

5.1.3 Endocytic compartments in baculoviral entry

To study baculovirus internalization in more detail, different markers of intracellular organelles were exploited in immunofluorescent labeling studies performed with confocal microscopy and in nanogold pre-embedding immunoelectron microscopy. In confocal microscopy, baculovirus displaying EGFP as a fusion to the capsid protein vp39 (vp39EGFP) (Kukkonen et al. 2003), was shown to colocalize notably with an early endosomal marker, EEA-1, from 15 min up to 1 h p.t., maximum colocalization being at 30-45 min p.t. (I, Fig. 4A). Colocalization was not further observed after 1.5 h p.t. Nanogold pre-embedding immunoelectron microscopy further confirmed the result showing intact baculovirus particles inside structures positive for EEA1 from 30 min to 1.5 h p.t. (I, Fig. 4A).

The late endosomal-lysosomal marker, CI-MPR, showed partial colocalization with the wt baculovirus stained with either anti-vp39 or anti-gp64 starting at 45 min p.t. detected with confocal microscopy (I, Fig. 5). Maximum colocalization was estimated to take place at 1.5 h p.t. At later time points, colocalization with the viral capsid vp39 started to decrease, while the colocalization of the viral envelope glycoprotein gp64 and CI-MPR was still detectable. The presence of the virus in late endosomal/lysosomal structures was verified by nanogold pre-embedding immunoelectron microscopy and CD63 antibody specific for the late endosomal/lysosomal membrane glycoprotein. In electron micrographs, enveloped intact baculovirus particles were clearly visible in structures positive for CD63 at 2-4 h p.t. (I, Fig. 5).

The possible presence of baculovirus in other cell organelles was studied with confocal microscopy. However, no colocalization of baculovirus with recycling endosomes (anti-rab11; I, Fig. 4B) or the Golgi complex (anti-TGN-38,

anti-GM130; I, data not shown) was observed during the first four hours of transduction in the confocal double labeling studies.

5.2 Characterization of the echovirus 1 entry route

5.2.1 General course of EV1 infection

The overview of the EV1 infection in SAOS-α2β1 cells was obtained with confocal microscopy. EV1, stained with anti-EV1 antisera, was shown to bind to SAOS-α2β1 cells and the virus was observed in an intracellular location 2 h p.i. (II, Fig. 1A). The virus production, taking place at 5 to 10 h p.i. detected by end-point dilution assay (II, Fig. 2E), was observed by confocal microscopy as an intense fluorescence in the cytoplasm of the SAOS-α2β1 cells, but not in control SAOS-pAW cells (I, Fig. 1). In addition, immunoblotting of cell homogenates revealed a substantial increase in EV1 capsid proteins only in SAOS-α2β1 cells at 12 h p.i. (II, Fig. 2A). Furthermore, the binding assay performed with radioactively labeled EV1 showed clear binding to SAOS-α2β1 cells (70%) but not the control cell line (II, Fig. 2B). Together these results confirmed the previous observations that α2β1 integrin is needed for EV1 replication.

The structural changes in the EV1 virus particle during the early events of the infection were analyzed by sucrose gradient sedimentation of the cell-associated radiolabeled EV1 (II, Fig. 3). Prior to binding to the cells, the virus sedimented entirely as 160S form, known to be the form of native capsid particle. Incubation of SAOS-α2β1 cells for one hour on ice resulted in EV1 sedimentation as 160S and 135S particles, where the latter ones predominated (II, Fig. 3A). Particles representing empty capsids (80S) were observed between thirty minutes to 2 h incubation p.i. at 37°C (II, Fig. 3B).

As previous studies with another picornavirus, HPEV1, demonstrated the use of clathrin-dependent pathway in the virus entry (Joki-Korpela et al. 2001), the internalization of EV1 was further observed with the markers of the clathrin-dependent endocytic route. However, no colocalization of EV1 with early endosomal antigen (EEA1) was detected (II, Fig. 5). This was also the case with transferrin receptor, marker for early sorting and recycling endosomes (Gruenberg & Maxfield 1995) which was expressed as a myc-tagged construct (TF-myc) (Suomalainen & Garoff 1997) (II, Fig. 5). Likewise, no detectable co-localization of α2β1 integrin or EV1 (Fig. 5) with cation-independent mannose 6-phosphate receptor (CI-MPR; found primarily in the late endosomes), TGN-46 (trans-Golgi network) or p23 (cis-Golgi network) was observed (II, Fig. 5 and data not shown). Based on these studies EV1 does not seem to use clathrin-dependent endocytosis route.

5.2.2 Role of the α2β1 integrin in EV1 infection

To study the possible role of EV1 receptor candidates, the capability of specific antibodies to inhibit EV1 infection in SAOS-α2β1 cells was studied. Again, most of the SAOS-α2β1 cells were positive for the EV1 antigens, whereas only a small number of control SAOS-pAW cells were infected (II, Fig. 2C). Antibodies against α2β1 integrin reduced the infectivity by 56 ± 2% (12F1) and 86 ± 1% (MAB1950) (II, Fig. 2D). Antibodies against β2 microglobulin as well as combination of anti-α2 integrin and anti-β2 microglobulin antibodies resulted in the almost complete inhibition of infection (II, Fig. 2D). The antibody against HLA-1, expressed in association with β2 microglobulin on the cell surface, did not show any evident inhibition of infection (II, Fig. 2D). Results support previous findings that β2 microglobulin would have a role in EV1 infection (Ward et al. 1998).

To study further the role of α2β1 integrin in EV1 infection, SAOS-α2β1 cells were double labeled with antibodies against EV1 and α2 integrin (II, Fig. 4A). The distribution of the α2β1 integrin was not altered by the attachment of EV1 to the plasma membrane. Colocalization of EV1 and α2β1 integrin was most obvious on the cell boundaries (II, Fig. 4A). Echovirus 1 was seen in the perinuclear accumulations in some (12%) SAOS-α2β1 cells already after 5 min p.i. Thirty minutes p.i. α2β1 integrin and EV1 co-localized in the perinuclear accumulations in 30% of SAOS-α2β1 cells and at 2 h p.i., already in 70% of the cells (II, Fig. 4A). Although α2β1 integrin and β2 microglobulin co-localized to some extend on the cell surface after EV1 attachment (II, data not shown), no significant co-localization of β2 microglobulin with α2 integrin subunit was detected at 2 h p.i. (II, Fig. 4B).

The role of the intracellular domain of α2 integrin subunit in EV1 infection was studied with the α2/α1 integrin chimera (SAOS-α2/α1β1 cells). In line with previous studies (Kawaguchi et al. 1994), the mutation in the cytoplasmic domain of α1 integrin had only minor effect on the replication of EV1 (II, Fig. 2E).

5.2.3 Role of caveolin-1 in EV1 infection

Electron microscopic studies revealed that the surface of SAOS-α2β1 cells is rich in caveolae (II, Fig. 6A). After 30 min p.i. EV1, labeled with protein A-gold, was detected with electron microscopy in vesicular structures with characteristics of caveolae (spherical vesicles, approximately 60 to 90 nm in diameter, lacking visible coat) (II, Fig. 6B and C). These caveolae-like vesicles were observed close to the plasma membrane in the cell periphery, as well as occasionally near the nucleus in large accumulations. After 2 h of incubation at 37°C, some of the internalized structures seemed to be surrounded by a limiting membrane (II, Fig. 6D). Double immunolabeling of the cryosections verified colocalization of caveolin-1 and EV1 (II, Fig. 6E, F).

In confocal microscopy, caveolin-1 was observed in scattered, small vesicular structures in uninfected SAOS-α2β1 cells and in infected SAOS-α2β1

cells immediately after virus attachment (II, Fig. 7A). Only weak colocalization of caveolin-1 with α2β1 integrin was observed at that time. However, between 30 min and 2 h p.i., caveolin-1 showed substantial accumulation in perinuclear vesicles (II, Fig 7B) and colocalized markedly with EV1 and α2β1 integrin (II, Fig 7C, D), suggesting that the EV1 capsid polypeptides, the integrin, and caveolin-1, were co-distributed to perinuclear structures as a consequence of EV1 infection.

As SV40 is known to enter the endoplasmic reticulum (ER) at 3-5 h p.i. (Kartenbeck et al. 1989, Pelkmans et al. 2001), double-labeling with PDI, a marker for the ER, was performed. EV1 showed no colocalization (II, data not shown), indicating that EV1/integrin/caveolin complex did not enter PDI-positive areas of ER during the first 2 h p.i.

To verify the association of EV1 with caveolae or caveosomes, immunoisolation of caveoli structures was performed (II, Fig. 8). A substantial amount of EV1 was isolated already after viral receptor binding with anti-caveolin-1 beads. The amount of EV1 in caveolae increased after 15 min incubation at 37°C (II, Fig. 8). A considerable amount of α2β1 integrin could be isolated with anti-caveolin-1 antibodies 15 min p.i., suggesting that also integrin is recruited to caveolae shortly after virus binding (II, data not shown). Immunoisolated caveola structures contained infective virus, which was demonstrated by plaque titration.

Expression of the dominant negative caveolin caused an approximately 66% inhibition of infection (II, Table 1), while expression of the wild type caveolin had no effect on EV1 infection. The clathrin mediated entry pathway was unaffected by the dominant negative caveolin, as shown by the HPEV control virus infection (Joki-Korpela et al. 2001). Methyl β-cyclodextrin, known to affect cholesterol distribution and destroy caveolae, inhibited the EV1 infection significantly as observed by double labeling experiments in confocal microscopy. It consequently reduced the percentage of cells showing perinuclear colocalization of EV1, caveolin-1, and α2β1 integrin approximately from 70 % to 5% (II, Table 2).

5.3 Characterization of the recombinant baculoviruses

5.3.1 Expression of transgenes in infected insect cells

In order to get an overview of the recombinant virus production in insect cells, expression of the fusion proteins on the surface of *sf9* cells was studied. For this infected cells were subjected to immunofluorescent staining and immunoblotting. Confocal microscopy analysis demonstrated the presence of anti-FLAG antibody stained RGD-gp64 fusion proteins on the *sf9* cell surface at 36 h p.i. showing somewhat clustered distribution (III, Fig. 2A-C). Likewise, the fusion proteins composed of a tumor homing peptide (LyP-1, F3 or CGKRK)

and the VSVG anchor, were clearly detectable and uniformly distributed on the surface of infected insect cells at 38 h p.i., as demonstrated with the anti VSVG tag antibody staining (V, Fig. 2A). The expression of corresponding fusion proteins on the infected *sf9* cells was confirmed by SDS-PAGE and western blotting using appropriate antibodies (III, IV, V, data not shown). For unknown reason, the GFP-fusion proteins displaying RKK showed dramatically reduced fluorescence detected both with confocal microscopy and FACS (IV, Fig. 3). Cells infected with control virus expressing nonmutated GFPgp64 showed high GFP fluorescence, indicating that the generated mutations in GFP loops (RKKH/IRKK), rather than the fusion of GFP to gp64, have a negative impact on the GFP fluorescence. In addition, strong luciferase marker gene expression was demonstrated at 38 h p.i. by immunoblot analysis (V, data not shown) using *Sf9* cells infected with *Ac*LyP-1-luc, *Ac*F3-luc or *Ac*CGKRK-luc (V, Fig. 2B).

5.3.2 Western blot analysis of the recombinant viruses

To demostrate the expression of the fusion proteins on the viral particles, SDS-PAGE and immunoblotting were performed. Results of the RGD displaying baculoviruses, *Ac*CL and *Ac*HL, detected with anti-FLAG antibody showed succesful incorporation of the fusion proteins into the recombinant virus particles (III, Fig. 2D). The presence of the fusions in gp64 trimers was clearly seen from nonreducing samples (III, Fig. 2E). The expression of RGD as a part of the fusion proteins was confirmed with HPEV1 and CAV9 antisera (III, Fig 2F and G). The successful incorporation of the RKK displaying GFPgp64 fusion proteins on the viral particles as well as in gp64 trimers was demonstrated with anti-gp64, anti-FLAG and anti-GFP antibody (IV, Fig. 2A-C). Both RGD and RKK displaying viruses expressed wt gp64 protein more effciciently than the fusion protein itself (III, IV). The incorporation of the LyP-1, F3 or CGKRK displaying VSVG fusion proteins on the surface of the corresponding viruses was confirmed with anti-VSVG tag antibody (V, Fig. 2C). The fusion proteins were mainly visible in dimeric or trimeric forms despite of reduction. Post-translational modifications of the proteins were likely to be present as well.

The ratio of total particle number versus the amount of infectios virus particles was determined by immunoblotting similar pfus of recombinant viruses. The amount of infectious particles and quantity of immunostained vp39 correlated well indicating rather similar total particle/infectious particle ratio between the recombinant viruses (III, Fig. 3; V, Fig. 2D).

5.3.3 Ligand binding studies *in vitro*

To study the ability of RKK displaying fusion proteins to bind to the the recombinant α2I-domain, the ligand binding domain of α2 integrin, ELISA was performed (IV). The results demonstrate that both RKK displaying recombinant viruses, *Ac*GFP(IRKK)gp64 and *Ac*GFP(RKHH)gp64 showed more efficient

binding to α2I-domain than the control viruses, suggesting functional expression of the RKK motifs on the surface of the virions (IV, Fig. 4).

5.4 Binding and entry of recombinant viruses to mammalian cells

The ability of the cancer cell targeting motifs, RGD, RKK, LyP-1, F3 and CGKRK to enhance the binding of the recombinant viruses to their target cells was assessed with the confocal microscope (III, IV) and FACS (V).

The A549 human lung carcinoma cell line was shown to express abundant levels of αvβ3 integrin, the receptor molecule for HPEV and CAV9 RGD when stained with NAKG-4 antibody (III, Fig. 5A). This was consistent with the earlier report (Triantafilou et al. 2000a). The binding of the recombinant RGD displaying baculoviruses, *Ac*CL and *Ac*HL, to A549 cells was more efficient than the binding of the control virus (wt), as observed by confocal imaging using anti-vp39 antibody (III, Fig. 4A). Quantitative fluorescence intensity measurement confirmed the results and demonstrated 2-3 times higher binding capacity of the recombinant viruses over the wt virus to the A549 cells (III, Fig. 4B). Interestingly, the recombinant viruses showed extensive clustering on the cell surface. This phenomenon was not observed with wt virus to this extent (III, Fig. 5B).

The binding of the RKK displaying viruses, *Ac*GFP(RKKH)gp64 and *Ac*GFP(IRKK)gp64 to their target cells CHO-α2β1 was analyzed with anti-gp64 antibody and confocal microscopy. Recombinant viruses did not, however, show improved binding when compared to the control virus *Ac*GFPgp64 (IV, Fig. 5). Preincubation with phospholipase C, known to inhibit BV transduction in mammalian cells (Tani et al. 2001), seemed to diminish both wt and recombinant virus binding and entry. However, there was some indication of the *Ac*GFP(K)gp64 virus entry to PLC treated cells at 60 min supporting the functionality of RKK (IV, Fig. 5).

The binding ability of LyP-1, F3 and CGKRK displaying recombinant baculoviruses, *Ac*LyP-1-luc, *Ac*F3-luc and *Ac*CGKRK-luc, to the MDA-MB-435 and HepG2 cells was measured by flow cytometry. Recombinant viruses were incubated with MDA-MB-435 or HepG2 cells in suspension, stained with anti-gp64 and analyzed with FACS. Results showed that the recombinant viruses exhibited 2-5 fold higher binding to both MDA-MB-435 and HepG2 cells when compared to the control virus (V, Fig. 3), suggesting that the tumor homing peptides enhanced the binding.

5.4.1 Inhibition of virus binding

The ability of the synthetic LyP-1, F3 and CGKRK peptides to inhibit the binding of *Ac*LyP-1-luc, *Ac*F3-luc and *Ac*CGKRK-luc viruses to MDA-MB-435 cells was measured with flow cytometry (V). Cells were preincubated with

different concentrations of soluble peptides, followed by incubation with recombinant viruses, immunofluorescent staining and flow cytometric analysis. LyP-1 peptide was the strongest inhibitor of all recombinant viruses and the control virus. All peptides self- and cross-inhibited the binding of each peptide displaying virus and the control virus, F3 and CGKRK peptides to a lesser extend than LyP-1 (V, Fig. 6). Results demonstrate the complexity of the binding of both control and recombinant viruses to the MDA-MB-435 cells.

5.5 Transduction efficiency of recombinant baculoviruses

To quantify the efficiency of ligand directed gene delivery, the enzymatic activity of luciferase was measured from cells transduced with *Ac*CL-luc (III), *Ac*LyP-1-luc, *Ac*F3-luc and *Ac*CGKRK-luc (V). Control virus, *Ac*-luc, with wild-type surface characteristics served as a control.

With the RGD displaying *Ac*CL-luc, 3-fold and 2-fold increase in the transgene expression was achieved with αvβ3 integrin expressing A549 human lung carcinoma cells at 24 h p.t. with MOIs 20 and 50, respectively (III, Fig. 6). No increase in luciferase activity was observed in HepG2 cells.

Up to 7- and 24-fold increase in the luciferase activity was obtained with the tumor homing peptide displaying baculoviruses in MDA-MB-435 and HepG2 cells, respectively. The luciferase activity was dose-dependent and the increase in the transduction efficiency was most significant at low pfu/cell (V, Fig. 4A). The highest transduction efficiency was detected at 34 h in HepG2 cells (V, Fig. 4B). MDA-MB-435 cells were rather non-permissive to viral transduction as luciferase expression was only weakly detectable before 48 h. Sodium butyrate did not affect the level of transgene expression.

5.5.1 Inhibition of transduction

The ability of LyP-1, F3 and CGKRK peptides to inhibit the transduction of *Ac*LyP-1-luc, *Ac*F3-luc and *Ac*CGKRK-luc in HepG2 cells was assessed with the luciferase activity assay (V). LyP-1 reduced the transduction of the corresponding LyP-1 displaying virus by 50% but had no effect on the transduction of the control virus Ac-Luc. In contrast, F3 peptide inhibited almost completely both AcF3-luc and *Ac*-luc transduction. CGKRK peptide inhibited the transduction of *Ac*CGKRK-luc by 25%, but did not affect the transduction of the control virus. The cationic control peptide, RKK, had no effect on the viral transduction (V. Fig. 7).

Ammonium chloride, which is known to prevent the endosomal acidification, was used to study whether the recombinant viruses, *Ac*LyP-1-luc, *Ac*F3-luc, *Ac*CGKRK-luc and *Ac*-luc, use the endosomal entry route as has been suggested for wt virus in HepG2 cells (Kukkonen et al. 2003, Salminen et al. 2005, van Loo et al. 2001). The luciferase activity was gradually declined with

increasing concentrations of ammonium chloride (V, Fig. 5). The transgene expression was completely inhibited at 6 mM ammonium chloride, suggesting the use of similar entry mechanism by the control and the surface modified viruses (V, Fig. 5).

6 DISCUSSION

Baculovirus is a new fresh vector candidate for therapeutic gene transfer. The virus is regarded as safe with low or no pathogenic potential (Airenne et al. 2004). The baculovirus display strategy allows expression of targeting motifs on the viral surface (Oker-Blom et al. 2003). To this end, the main goal of this thesis work has been to develop an efficient targetable gene delivery vehicle using baculovirus display technology. This was implemented by displaying targeting motifs specific for tumor vasculature on the surface of the virus. Recombinant viruses were further used to monitor the binding and transduction efficiency of the target cells (III, IV, V). Moreover, the baculovirus entry route to human hepatocarcinoma cells was studied to obtain a better understanding of the internalization pathway leading to transduction (I). Additionally, the entry route of α2β1 integrin together with EV1 was assessed in study (II). This integrin is the target molecule of RKK targeting motif utilized in this study, thus information about the internalization mechanism of this molecule was of particular interest. Together, the understanding of cell entry mechanisms, viral entry steps as well as natural receptor interactions, among several factors, are needed in order to develop a virus vector that is able to specific and targeted gene transfer.

6.1 Viral entry

In this thesis work, the entry of two very different viruses was studied. Baculovirus, *Ac*MNPV, is a large, rod-shaped, enveloped insect virus (Blissard 1996). In contrast, echovirus 1, belonging to the enteroviruses family *Picornaviridae,* is a tiny nonenveloped icosahedral virus particle. As humans are hosts for EV1, the natural entry pathway in human cells leads to infection and production of progeny virus particles (Racaniello 2001). The internalization mechanism of EV1 was unknown. Since the virus is a human pathogen (Pallansch & Roos 2001), the study is also of clinical importance. The role of

α2β1 integrin, the echovirus 1 receptor (Bergelson et al. 1992), in infection, was also of particular interest. The insect cells are the natural hosts of the baculovirus (Blissard & Rohrmann 1990), but viruses have been shown to enter and transduce mammalian cells (Hofmann et al. 1995, Volkman & Goldsmith 1983). The entry of baculoviruses to mammalian cells, transduction, could be regarded as "imitation of an infection", in the sense that the virus delivers its genome for expression of transgenes without viral replication and production viral progeny. The development of baculovirus gene transfer technology urges knowledge of internalization of the baculovirus in mammalian cells.

Viral structure as a determinant of the entry pathway. The "main goal" of the viruses is to deliver their genome to the host cell for progeny production. Baculovirus must deliver its genome to the nucleus for replication /expression of the transgene. Echovirus 1, in contrast, replicates in the cytoplasm, inside virus-induced vesicles, and hence do not need nuclear transportation (Bienz et al. 1992). In order to deliver the genome to the correct place for replication/transcription, viruses must gain access to the cell, and uncoat their genome (Smith & Helenius 2004).

Outside the cell, viruses are stable structures. However, due to the interaction with the target cell, they must be able to break up and release their genome. This striking ability is a result of ingenious structural design, where the entire virus particle or specific proteins are locked in metastable conformation, that is able to undergo major structural changes due to interactions with the cell (Marsh & Helenius 2006). During the viral entry, the cell provides "cues", e.g. receptor interaction or exposure to low pH that trigger the programmed structural changes leading to uncoating of the virus (Smith & Helenius 2004). The stucture of the virus hence determines prerequisites for the successful entry pathway. Binding of the virus to the cell surface is known to be the first step of virus entry. Many viruses are known to utilize several receptors, some of which are attachment factors while others are true receptors that are needed to guide bound viruses to endocytic routes. (Marsh & Helenius 2006, Smith & Helenius 2004). Thus, binding of the virus to the specific receptor(s) determines its fate. Viruses have evolved to utilize receptor(s) that offer the most suitable "cues" and guide them inside to a route that best fits for their structural demands.

For baculovirus, the receptors on mammalian (and insect) cells are not known. The wide range of mammalian cells that BV enters (Kost et al. 2005, Kost & Condreay 2002) suggests that the virus interacts with conserved molecules present on many cell types or that interactions are quite non-specific (Hefferon et al. 1999, Shoji et al. 1997). The use of negatively charged molecules, especially heparan sulphate, by baculovirus in mammalian cells has been suggested by Duisit and colleagues (1999). The clear preference of baculoviruses for human hepatocytes is, however, an interesting issue, and might reflect, among other things, the use of a specific receptor in these cells (Hofmann et al. 1995). It is not sure if gp64 participates in the binding of the

virus to the mammalian cell surface. However, Markovic and colleagues (1998) have suggested that the binding of gp64 to its putative receptor would be a prerequisite for the membrane fusion (Blissard & Wenz 1992, Charlton & Volkman 1993, Leikina et al. 1992, Volkman & Goldsmith 1985, Wang et al. 1997) mediated by this molecule in insect cells. For membraneous viruses, such as baculovirus, viral membrane must fuse with cellular membrane, in order for the virus capsid to penetrate into cytoplasm. This membrane fusion is triggered by low pH and involves structural changes in the viral fusion protein (Marsh & Helenius 2006, Smith & Helenius 2004). Hence, although gp64 dependent membrane fusion is acid-induced (Blissard & Wenz 1992, Leikina et al. 1992), the binding of the molecule to the receptor could induce the process. It would be surprising if the same would not apply in mammalian cells, since the capsid release mechanism seems to be the same.

In contrast to baculovirus, EV1 is acid-stable, though it must have other means for uncoating. For picornaviruses, receptor-mediated conformational changes are known to be important for internalization and genome release. In this study (II), EV1 structural changes were observed shortly after receptor binding by saccharose gradient centrifugation, suggesting that EV1-receptor interactions lead to this conformational change. However, it is not sure whether conformational change is due to binding to α2 integrin. The study of EV1 entry confirmed the necessity of α2β1 integrin in EV1 infection; virus did not bind nor infect the α2β1-deficient control cells (II). The differences in the degree of blocking of the EV1 infection by different α2β1-mAbs likely reflects binding of different mAbs to their specific epitopes on the integrin molecule. Additionally, the earlier suggestion for the role of β2-migroglobulin in EV1 infection (Ward et al. 1998) was confirmed. However, based on the antibody blocking experiment, it cannot be stated if the protein directly interacts with EV1.

Viral binding to the cell surface receptor(s) may trigger signalling that leads to the internalization of the receptor and viral particle. Clustering of the receptors may be needed for this activity (Smit & Helenius 2003). Gp64 molecules have been suggested to form a multimeric structure, the fusion machine, during the entry of the virus to insect cells (Markovic et al. 1998). It may be that the process is dependent on the activity of the baculovirus receptor and that the clustering of the receptor-gp64 -complexes on the cell surface would trigger signaling cascades as well. Baculovirus accumulation on the cell surface after binding was clearly seen by confocal microscopy staining and live cell imaging in this study (III). As the virus was seen in clathrin coated pits in electron microscopy and later in early endosomes (I), these regions of virus aggregation might represent specific receptor areas or regions of high endocytic activity (Gaidarov et al. 1999).

Interestingly, EV1 has also been later found to induce clustering of α2β1 integrin on the cell surface, leading to entry of the virus (Upla et al. 2004). EV1 might mimic natural clustering of this integrin, which with natural ligands leads to caveolin-1 oligomerization (Wei et al. 1996) and, thus, leads the virus to the caveolae route. Other evidence of virus-receptor interactions leading to

clustering comes for example from adenovirus and its receptor αvβ3, the clustering of which leads to virus internalization (Nemerow & Stewart 1999). The internalization of α2β1 integrin with the EV1 into caveolae and further to caveosomes (II) is unique among integrins. Some integrins, like αvβ5 (Memmo & McKeown-Longo 1998) and α5β1 (Gao et al. 2000, Vignoud et al. 1994) are known to internalize via the receptor-mediated endocytosis, after which they are rapidly recycled from the early endosomes to the cell surface. In adenovirus internalization, integrin possibly internalizes, but if so, it is likely recycled immediately back to the cell surface (Nemerow 2000).

Viruses utilize different endocytic pathways. The majority of viruses need endocytic processes in order to successfully enter the cells. The utilization of endocytosis offers a convenient way to "free ride" deep into the cytoplasm and at the same time escape from immune defences (Marsh & Helenius 2006). The choice of the endocytosis route depends on both structural demands of the virus and the final destination of the virus in the cell.

The baculovirus internalization into early endosomes observed in this study (I) was in line with Kukkonen and colleagues (2003). As clathrin-mediated endocytosis is known to lead to early endosomes, baculovirus likely used this pathway in the entry. The large size of baculovirus, which is somewhat above the suggested upper size limit (200 nm) of the clathrin coated pit (Rejman et al. 2004), possibly restricts the use of this route, as only few viruses were seen in numerous electron microscopy samples. Based on electron microscopic studies, also macropinocytosis seems to be a possible entry route for baculovirus. Supporting data comes from adenovirus, which due to binding to αv integrin, is internalized via macropinocytosis in concert with clathrin-mediated endocytosis. As a consequence, the virus is released simultaneously from both endosomes and macropinosomes to cytoplasm (Meier et al. 2002, Meier & Greber 2004). Baculovirus might use this route similarly to adenovirus to enhance the internalization and acid-induced penetration to cytosol. The possible involvement and even inducement of microvilli by baculovirus gives further support to the use of macropinocytosis. Actin-induced membrane ruffling is known to be the prerequisite for the macropinocytosis (Swanson & Watts 1995). Similar phenomena, involving microvilli, has been reported to take place during adenovirus (Meier et al. 2002, Meier & Greber 2004) and HIV (Liu et al. 2002) entry by macropinocytosis. Interestingly, baculovirus entry to hepatocytes has been earlier reported to require contact with the basolateral surface (Bilello et al. 2001) that is known to contain numerous microvilli.

After transport to early endosomes baculovirus is likely to penetrate endosomal membrane and enter the cytosol as has been suggested based on the dynamitin overexpression studies (Salminen et al. 2005). In this study (II), intact baculoviruses were observed also in the late endosomes and lysosomes indicating that all viruses are not able to escape from endosomes, but are transported futher the endosomal route. Most likely these viruses will be degraded in lysosomes. Escaped capsids are then further transported to the

nucleus most likely using the actin network (Salminen et al. 2005) after which capsid probably enters the nucleus trough the nuclear pore (van Loo et al. 2001).

Echovirus was found to utilize a different entry route than most other viruses. The use of caveolae route by EV1 was supported by several facts (II). SV40 virus, earlier suggested to utilize caveolae in its entry, is also acid-stabile and its entry is inhibited with the same dominant negative caveolin used in this study (Anderson et al. 1996). The recent study by Upla and colleagues (2004) further confirmed the use of the caveolae route by live imaging. According to this study EV1 was shown to be transported inside caveolae and end up in perinuclear structures composed of vesicles containing caveolin-1, possibly representing caveosomes (Pelkmans et al. 2001). In fact, the more recent studies have suggested that EV1 enters caveosomes very efficiently also via clathrin- and caveolin-independent pathway (Paloranta et al. unpublished).

SV40 is known to be transported from caveosomes to smooth ER and further to the nucleus for replication (Pelkmans et al. 2001, Pietiäinen et al. 2005). EV1 in turn was not seen in the ER in this study (II) and thus likely uses different internalization route from SV40. This would be presumable, as EV1 replication has been reported to take place in cytoplasm (Bienz et al. 1992). The use of similar early entry steps by both EV1 and SV40, however, emphasizes the variability of internalization routes. Thus, this same mechanisms can be used to achieve viral entry leading both to cytoplasm and nucleus. After the presence of EV1 in putative caveosomes the fate of the virus is unknown. However, the virus RNA seems to be associated wih the capsid proteins in the caveosomes, until the start of replication (Pietiäinen et al. 2004).

Altogether, results from baculovirus (I) and echovirus 1 (II) entry studies demonstrate the versality of virus entry mechanisms and the evolved elegant mechanisms of individual viruses to exploit cell entry mechanisms in order to gain access to cells.

6.2 Baculovirus display

Targeting can be obtained by either chemical linking of targeting moieties on the viral surface or by using genetical approach. As chemically linked motifs can possess insufficient stability, the genetic modification is a more attractive alternative (Verma & Weitzman 2005). Baculovirus display offers a versatile tool for the display of complex proteins on the viral surface and hence broadens the applicability of display-technique from phage display (Mäkelä and Oker-Blom 2006). In baculovirus display, fusion to the N-terminus of a second copy of the gp64 major membrane glycoprotein has enabled display of motifs and proteins of varying sizes in a fuctional form (Boublik et al. 1995, Grabherr et al. 1997, Mottershead et al. 1997, 2000, Ojala et al. 2001). Although the structure of the gp64 is not known, the N-terminus apparently is exposed to allow succesful

presentation of the fusion partners. The use of a second copy of gp64 for display allows native gp64 to mediate budding and entry of the virus (Blissard & Wenz 1992, Hefferon et al. 1999, Monsma et al. 1996, Oomens & Blissard 1999). In this study, both small RGD motifs of viral origin (III) and GFP protein carrying RKK motif (IV) was displayed utilizing this technique. As RGD motifs of CAV9 (Chang et al. 1989, Hendry et al. 1999) and HPEV1 (Hyypiä et al. 1992) naturally are situated on the flexible C-terminus of the vp1 protein being exposed on the viral surface, the display as a fusion to the gp64 N-terminus was thought to mimic this situation to some extent. For better accessibility, a short GS-linker was added, although the study of Ojala and colleagues demonstrated the functionality of small fusion partners without a linker (Ojala et al. 2001).

The successful incorporation of RGD motifs of CAV9 and HPEV1 (III) as well as RKK-GFP fusion protein (IV) on the viral surface as a gp64 fusion was in line with earlier studies (Boublik et al. 1995, Grabherr et al. 1997, Mottershead et al. 1997, 2000, Ojala et al. 2001). The more abundant presence of the wild-type gp64 protein in viral particles compared to the fusion protein has also previously been reported (Chapple & Jones 2002, Mottershead et al. 1997, 2000, Ojala et al. 2001). The presence of fusion proteins in gp64 trimers imply that the fusion partner does not, at least to a large extent, impede the oligomerisation of gp64, which is known to be essential for the transportation of gp64 to the insect cell surface (Monsma & Blissard 1995, Oomens et al. 1995, Oomens & Blissard 1999). Nevertheless, wild-type gp64 may be favored over the recombinant one in the trimerization and budding processes. The size of the fusion partner likely plays a role as larger fusion partners, such as GFP, have been suggested to interfere with oligomerization of gp64 and, thus, incorporation on the viral particles (Mottershead et al. 1997). On the other hand, despite the strong polyhedrin promoter used, the amount of fusion protein produced or available, can simply be smaller than that of the wt gp64 resulting in competition for access to the viral surface (Boublik et al. 1995, Chapple & Jones 2002).

A prerequisite for functional display of the RKK motif (IV) was a specific three dimensional structure that is needed for the RKK to bind to its target, α2I-domain (Ivaska et al. 1999a, Pentikäinen et al. 1999). As the structure of the gp64 is not known, the design of a specific scaffold with it was not possible. Based on the studies of Mottershead and colleagues (1997), GFP was known to be functional when displayed as a GFP-gp64 fusion. Additionally, results of Abedi and collegues demonstrated the suitability of solvent exposed GFP loops as a scaffold for peptide display (Abedi et al. 1998). Therefore, most optimal loops for the RKK display were chosen based on the computer modeling. Mutations to create the RKK motif were implemented with minimal changes in the genetic code of GFP, e.g. KRDH (aa 214-217) in loop 10 of GFP resembles the structure of RKKH loop in jararrhagin model (Pentikäinen et al. 1999). Thus, the strategy was to create RKK by minimal point mutations without inserting additional codons. Mutations, however, turned out to dramatically impair the GFP fluorescence (IV), as the control virus with GFP-gp64 fusion showed normal GFP fluorescence. Based on an earlier studies (Abedi et al. 1998, Peelle et al.

2001) the loop 6 that IRKK was placed in, was the most suitable for peptide display without loss of fluorescence. In contrast, in the case of RKKH, placed in loop 10 of GFP, at least a decrease of fluorescence was expected according to an earlier study (Abedi et al. 1998). However, in both reports (Abedi et al. 1998, Peelle et al. 2001), peptides have been inserted into loops rather than created by mutagenesis. Hence, it seems that insertions into GFP loops better preserve the GFP fluorescence. The lack of fluorescence does not, however, impede the use of this display-strategy, as long as the GFP scaffold retains its overall structure. This seemed to be the case in this study, since the activity of RKK motif in meditating binding to the α2I domain of integrin α2β1 was demonstrated *in vitro* using an ELISA assay (IV). Sequences flanking RKK (RKKH/IRKK) did not seem to affect the binding to the I domain, which is in line with the results of Ivaska and coworkers (1999a). Similarly, the localization of the RKK containing loop in GPF did not seem to have an influence on the binding efficiency as the theoretically better located RKKH mutation showed as strong binding as IRKK located near the fusion part of the recombinant protein.

The other display strategy, utilized in study (V), was based on studies of Chapple and Jones (2002) as well as of Ojala and colleagues (2004), who demonstrated the use of the VSV-G anchor in the baculovirus display. This strategy allows more efficient display due to nonpolar distribution of fusion partners and leaves the gp64 molecule unoccupied, to assist the viral entry. As the 21-amino acid truncated ectodomain was known to mediate unspecific binding and enhanced transduction of cells (Kaikkonen et al. 2006, Ojala et al. 2004), it was replaced with a 20 alanine- residue linker. Thus the anchor in the study V consisted of the alanine linker attached to the transmembrane and cytoplasmic domains of the VSV-G protein. This inert anchor was designed to provide distance from the viral membrane and increase the flexibility for more optimal display. Using this strategy, the tumor homing peptides, LyP-1, F3 and CGKRK were designed to be displayed uniformly on the viral surface (Chapple & Jones 2002, Ojala et al. 2004). While LyP-1 is a cyclic peptide that has an internal disulphide bridge (Laakkonen et al. 2002), F3 and CGKRK are linear ones (Hoffman et al. 2003, Porkka et al. 2002). Based on the earlier studies (Akerman et al. 2002, Christian et al. 2003, Hoffman et al. 2003, Laakkonen et al. 2002, 2004, Porkka et al. 2002), these peptides can be attached to different payloads, such as the T7 phage via genetic fusion to the capsid protein 10b, and chemical coupling to inorganic nanostructures or fluorescein, demonstrating the adaptation potential of the peptides to different systems. The strategy used in this study turned out to be successful for incorporation of the tumor homing peptide –VSV-G fusion proteins on the viral surface. Based on the western blot analysis, the VSV-G fusions were likely present as oligomers on the viral surface, indicating that the cytoplasmic and transmembrane domains might also be sufficient for oligomerization of the VSV-G protein. Additional support comes from an earlier report showing the necessity of trimerization for the transport of the VSV-G to the cell membrane (Doms et al. 1987). Additionally,

Ojala and coworkers (2004) suggested that their fusion proteins carrying 21-amino acid ectodomain form trimers.

The incorporation mechanism of the VSV-G fusion protein on the baculovirus membrane is not known. During baculovirus budding, most cellular proteins on the plasma membrane are excluded, therefore VSV-G either interacts with baculoviral nucleocapsid or is passively taken in, being too small to be excluded (Chapple & Jones 2002). The small sizes of the VSV-G fusion proteins used in this study, would allow incorporation according to this theory. The study of Mangor and coworkers (2001) showed that the VSV-G protein possibly also facilitates baculoviral budding, although with lower efficiency than gp64. Therefore, the cytoplasmic and transmembrane domains of VSV-G present in the fusion construct might assist the incorporation on the baculoviral surface.

In concert with earlier studies (Ojala et al. 2001, 2004, Räty et al. 2004, Yoshida et al. 2003) our studies herein (III, V) further confirm the feasibility of baculovirus display as a strategy to display targeting motifs on the viral surface. The use of a foreign anchor protein, such as VSV-G, seems to be a feasible system for display of both small (V, Ojala et al. 2004) and large (Chapple & Jones 2002) fusion partners, whereas the larger size of the fusion partner may likely affect the incorporation efficiency of the gp64 fusions proteins (IV, Toivola et al. 2002) and at the same time disturb the internalization of the virus.

6.3 Display of targeting motifs to obtain enhanced gene delivery

The efficiency of the expression of the transgene is one of the major keynotes in therapeutic gene delivery. Although many vectors exhibit high gene delivery *in vitro*, the efficacy *in vivo* has been modest (Lundstrom 1993). Thus, enhancement of the transduction is one of the prerequisites for successful gene delivery. Although baculovirus seems to enter a great variety of mammalian cell lines, the transduction efficiency varies substantially, cell lines of hepatocyte-origin being the most susceptible to transduction (Boyce & Bucher 1996, Condreay et al. 1999). The reason for this is not straightforward. In order for baculovirus to express the transgene, DNA has to be transported to the nucleus. After this the transcription and translation needs to be performed. In theory, the "block" could lie anywhere from viral attachment to post-translational modifications for the expressed protein. Few explanations for the differencies in transgene expression level have been suggested. The block has been suggested to exist in the penetration of the capsid from the endosomes (Boyce & Bucher 1996, Barsoum et al. 1997, Park et al 1997). This suggestion has been based on experiments performed with more susceptible cell lines e.g. HepG2 with ammonium chloride and the observation that the amount of virus internalized is similar regardless of the transduction efficiency (Boyce & Bucher 1996). Additional proof has been obtained from pseudotyping experiments, where

VSV-G on the surface of the virus has facilitated the release of the capsid and led to increased transgene expression even in cell lines that the virus without VSV-G had only marginal expression (Barsoum et al. 1997, Park et al 1997). Kukkonen and colleagues (2003) have reported the general block of transduction to be in the nuclear entry of the capsids using EAHY, MG63 and NHO cells. It may well be that the nuclear entry through nuclear pores in mammalian cells (van Loo et al. 2001) is the limiting step due to the large size of the baculovirus capsid. As more viral capsids would access the cytosol after penetration of the endosomal membrane, nuclear entry might succeed more probably, thus leading to expression.

Interestingly, the MDA-MB-435 cells used in this study turned out to be rather nonpermissive to wild-type baculovirus transduction (V). The fact that the histone deacetylase inhibitor, sodium butyrate, was unable to enhance the transduction efficiency could mean that the virus might not reach the nucleus of these cells efficiently. This would be in line with results of Kukkonen and coworkers (2001). On the other hand, Spenger and colleagues (2004) have reported histone deacetylase inhibitor to have only a moderate effect on the transgene expression mediated by SV40 promoter, used in our study. The association of baculoviral DNA with proteins sensitive for deacetylation in the nucleus is known to restrict efficient transgene expression (Spenger et al. 2004) and the use of butyrate or trichostatin A, histone deacetylases, has usually enhanced the transduction (Kost et al. 2005). Thus, it is also possible that SV40 promoter is not optimally working in this cell line. According to earlier studies, the choice of the promoter suitable for the target cell has been reported to affect gene transfer efficiency susbtantially (Shoji et al. 1997, Spenger et al. 2004). Without further studies the precice reason for the nonpermissivity in MDA-MB-435 cells with wild type baculovirus in our experiments can only be speculated.

Viral entry is a complicated process and thus the enhancement of transduction by the display of targeting motifs is not an easy task. There are two ways that displayed motif can enhance baculoviral transduction i) Motif may enhance the binding, known to be the limiting step in the baculovirus entry at least in insect cells (Wickham et al. 1992). After attachment augmented with the displayed motif, the virus would be capable of binding its own receptor/receptors and enter the cell using its "natural" entry pathway. ii) After binding with the help of displayed motif, the receptor would direct the virus to the entry route used by the targeted receptor. The use of natural entry route certainly more likely leads to the prospective outcome. Thus, the best choice would probably be the efficient targeting motif that would internalize using the same pathway as the virus. In order to design strategies possibly utilizing other entry mechanisms, caution should be taken. As reported also using other viruses, like adenovirus and retrovirus, the development of functional targeted viruses through modifications in the viral entry-related proteins may reduce or impair viral entry (Nicklin & Baker 2002, Peng & Russell 1999, Sandrin et al. 2003). Thus, the information on both the natural entry mechanism of the virus

and properties of the targeted receptor are of utmost importance in developing successful targeting strategies leading to efficient transduction.

The trend has been the same also with baculovirus. The enhancement of baculovirus binding due to the display of targeting moieties seems to be succesful as reported in several studies (Ojala et al. 2001, 2004, Räty et al. 2004, Yoshida et al. 2003) in addition to our studies (III, V). Ojala and coworkers (2001, 2004) were the first to show increased binding of target cells mediated by single chain antibody against carcinoembryonic antigen. Another targeting strategy involved the display of the synthetic IgG binding domains (ZZ) of *Staphylococcus aureus* protein A. Targeting was mediated by antibodies specific for the target cell. These display strategies were, however, not successful in enhancing the transgene expression. According to Ojala (2004), the barrier in the transgene expression was not in endosomal release. This was a conclusion from the fact that leaving the gp64 intact by using VSV-G anchor showed no increase in the efficiency of transduction (Ojala 2001 and 2004). Although enhancing the binding to target cells, these display-strategies apparently were not able to direct attached viruses either to natural entry route/routes of baculovirus or other routes and thus enhancement of transduction was not observed. Successful attempts to increase the transgene delivery by baculovirus have certainly been made e.g. by displaying the VSV-G or its truncated form or additional gp64 on the surface of the virus (Barsoum et al. 1997, Kaikkonen et al. 2006, Tani et al. 2001). However, these applications increase the host cell range rather than restrict it.

Räty and colleagues were the first to show both enhanced and targeted approach *in vitro*, using avidin-displaying baculovirus, Baavi, coated with biotinylated paramagnetic particles targeting the cells with the aid of an external magnetic field. Additionally they demonstrated enhanced transduction of biotinylated cells with Baavi (Räty et al. 2004). These attempts showed promise for baculovirus as a targetable therapeutic vector. In this system, binding/targeting of the virus was mediated by specific interactions with the target cell, a process that itself likely enhances transduction. Displayed avidin as a cationic molecule, additionally enhanced the cellular uptake of the viruses likely by adsobtive endocytosis. The avidin-biotin –technology adds more value to this system, enabling flexible targeting strategies (Räty et al. 2004). The recent study of Ernst and colleagues (2006), displaying FMDV RGD sequence on the baculovirus surface, showed increased transgene delivery to target cells and, thus, confirms the results (III) of the capability of viral RGD sequences to mediate targeted and enhanced baculovirus transduction.

6.4 Towards baculovirus targeting to tumor vasculature

As viral-mediated therapeutic gene transfer has become an extensively investigated form of experimental therapy, the urge for targeted vectors has

become apparent. Targeted vectors would be especially beneficial for cancer treatment using intravascular delivery, as the uptake by normal vasculature could be minimized and at the same time maximizing the homing to the targeted site (Trepel et al. 2000, Wilson 2002). Additionally, for example, treatment of metastasis would require targeted systemic therapy (Trepel et al. 2000). Targeting to tumor vasculature is particularly tempting, as tumor vasculature has been shown to express several distinct markers that can be utilized in targeting. Some of these markers, including αvβ3 integrin utilized in this study (III), are angiogenesis related, and are thus shared by all angiogenic vessels (Ruoslahti 2002a, 2002b). However, *in vivo* phage display has revealed unique molecular signatures in tumor vasculature that can be utilized in targeting (Ruoslahti 2002a, 2002b). Some of the peptides targeting to these novel signatures, namely LyP-1, F3 and CGKRK, have been employed in this thesis work (V).

Figure 10 exhibits a shematic representation of recombinant baculoviruses targeting to tumor vasculature using targeting motifs utilized in this study. The potential entry routes of recombinant viruses into tumor endothelial cells as well as the ability of the targeting motifs to enhance the transduction of the target cells are discussed below.

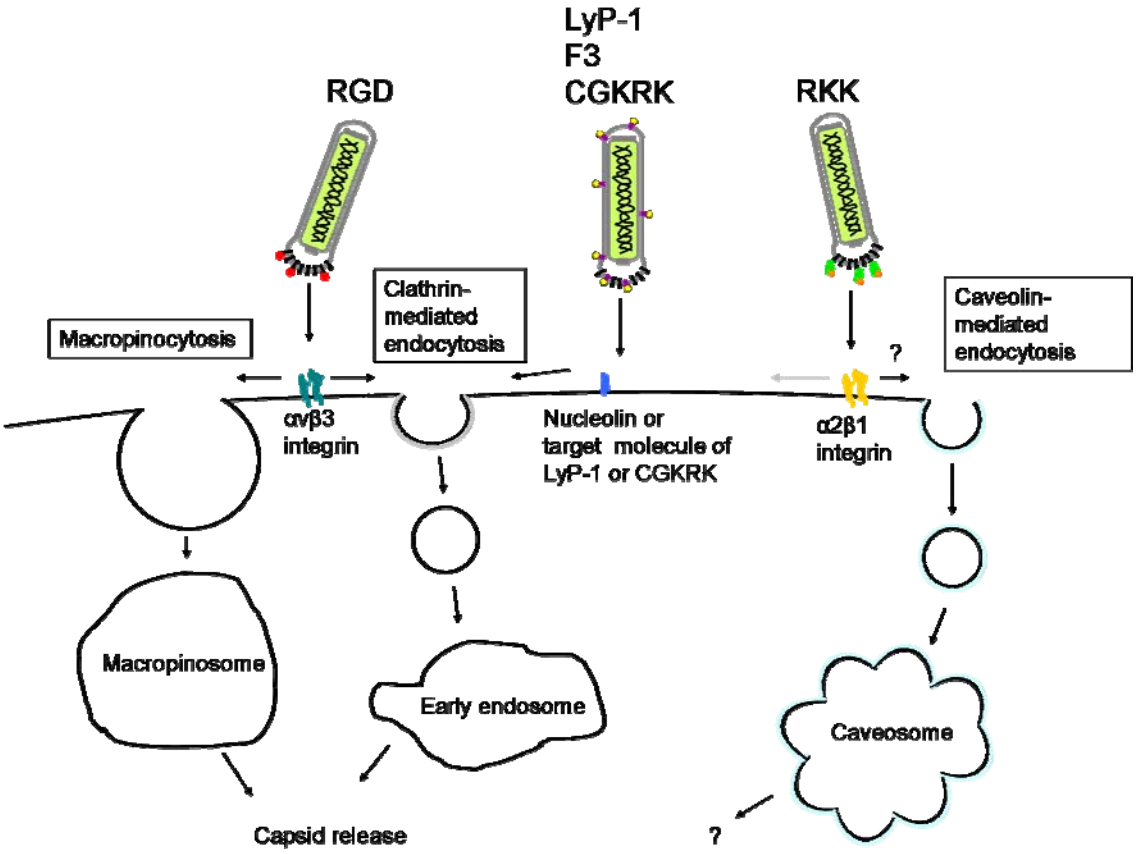

FIGURE 10 Towards baculovirus targeting to tumor vasculature. Putative entry routes of recombinant baculoviruses into tumor and tumor endothelial cells. See text for details.

RGD motifs have been vastly utilized in targeting (Temming et al. 2005), e.g. by incorporation of the RGD into different viruses, such as the filamentous phage (Hart et al. 1994) and human adenoviruses (Temming et al. 2005, Wickham et al. 1997). However, the RGD sequenses from CAV9 or human HPEV1 have not been tested in this context before. Virus-derived RGD sequences have not been utilized vastly in targeting (Temming et al. 2005). A recent paper by Ernst and collegues nonetheless describes successful baculovirus targeting strategy with FMDV RGD loop inserted at position 283 in the second copy of the gp64 glycoprotein (Ernst et al. 2006). Thus, viral derived RGD sequences seem to offer a feasible way to enhance the transduction as well.

The RGD motifs carrying payloads have been demonstrated to be able to mediate αvβ3-dependent entry to cells via an energy-dependent process, possibly integrin-depedent receptor-mediated endocytosis (Boturyn et al. 2004, Schraa et al. 2002). This suggests that RGD displayed on the baculovirus surface could be able to enhance the internalization of the virus, which in turn could increase the transduction efficiency (III). The occurrence of RGD displaying viruses in distinct clusters on the surface of A549 cells could, hence, be a result of binding to αvβ3 integrin on the cell surface, since this integrin has been demonstrated to form clusters after stimulation with αvβ3 integrin-specific antibodies mimicking the natural ligand binding (Upla et al. 2004). Integrin clustering has also been described with adenovirus, the binding of which to several αv integrins to the penton base pentamer, possessing five RGD protrusions, induces clustering and subsequent integrin signaling leading to virus internalization (Nemerow & Stewart 1999). Also CAV9 RGD-mediated binding has been suggested to cluster αvβ3 integrins, as multiple integrin heterodimers are able to accommodate at adjacent binding sites (Xing et al. 2004). The finding that αvβ3-mediates adenovirus internalization not only via the clathrin mediated endocytosis route, but also via macropinocytosis (Meier et al. 2002) is particularly intereresting because of the putative use of macropinocytosis of baculovirus suggested in this thesis work (I). Thus, RGD displayed on the baculoviral surface (III) could possibly augment virus entry via both internalization mechanisms naturally used by the virus leading to enhanced transduction (Figure 10).

RKK. The reason for the lack of RKK-mediated enhancement of cell binding is not clear. Given that binding activity of the RKK displaying viruses to the α2I domain of the α2β1integrin was demonstrated *in vitro*, binding to cell surface expressing α2β1 integrin would have been presumable. According to the binding and inhibition experiments, binding of the cyclic RKK motif to the α2I domain is known to be strong (Ivaska et al. 1999a). One reason might be the low amount of GFP-gp64 fusion proteins present on the viral surface reported by Toivola and coworkers (2002). Based on fluorescence correlation spectroscopy (FCS) studies they stated that the amount of GFP displayed as a GFP-gp64 fusion can be as low as three proteins per virus (Toivola et al. 2002). Another possibility is that the GFP scaffold does not offer the most optimal display for

efficient binding of the RKK to the α2β1 integrin. The RKK display strategy was based on the computer modeling of the RKK-GFP and the I domain, as structure of the integrin was at that time not available. Therefore it is possible that there are structural constraints in the binding of bulk RKK-displaying GFP molecule to the I domain of the α2β1 integrin. To achieve effective binding with the RKK motif, the display should be further optimised.

Based on the results from work II, binding of a ligand, EV1, to the α2β1 integrin leads to α2β1 integrin (and EV1) recruitment to caveolae and internalization of the ligand and integrin using caveolae-mediated endocytosis. RKK binding to α2I domain has been suggested to induce structural change in the protein, increasing the affinity to echovirus 1 (Ivaska et al. 1999a). As RKK is able to bind α2I domain also simultaneously with EV1 (Xing et al. 2004), and EV1 binding has later been shown to induce specific signaling that activates α2β1 integrin leading to its internalization through caveolae (Upla et al. 2004), it is possible that RKK binding also could induce the same pathway. Moreover, it has been suggested that natural ligands could activate the internalization via caveolae (Upla et al. 2004). Therefore, binding of the displayed RKK motif to the cell surface α2β1 integrin could in theory lead the virus to the caveolae-mediated endocytosis route. Due to the large size of baculovirus it is though unlikely that the virus could be internalized through this route. Moreover, baculovirus entry is dependent on acid-induced fusion of the virus membrane (Blissard & Wenz 1992, Charlton & Volkman 1993, Volkman 1986, Volkman & Goldsmith 1985, Wang et al. 1997), and caveosomes have neutral pH (Pelkmans & Helenius 2002), hence the natural internalization mechanism could not be utilized. As a consequence, the only possibility for RKK to augment baculovirus entry would be by binding to α2β1 integrin on the cell surface after which baculovirus would use its own preferred entry route (Figure 10).

LyP-1, F3 and CGKRK. The enhancement of transduction due to display of the tumor homing peptides demonstrates the ability of baculovirus display to increase the transduction of also rather nonpermissive cell types, like MDA-MB-435. LyP-1, F3 and CGKRK have been demonstrated to internalize and end up in the nuclei of their their target cells (Hoffman et al. 2003, Laakkonen et al. 2002, Porkka et al. 2002). Given the fact that they also have been reported to carry various cargos, including phage, fluorescein and inorganic nanoparticles into target cells *in vivo* (Akerman et al. 2002, Christian et al. 2003, Hoffman et al. 2003, Laakkonen et al. 2002, 2004, Porkka et al. 2002), they might well be able to assist baculovirus in the entry process. They have not been evaluated earlier in viral gene transfer context, thus, this study demonstrates for the first time enhancement of transduction augmented by these motifs.

According to the ammonium chloride inhibition results (V), tumor homing peptide-displaying baculoviruses enter HepG2 cells primarily by endocytosis, like the wild-type virus (Kukkonen et al. 2003, Salminen et al. 2005, van Loo et al. 2001). This indicates that these tumor homing peptides likely augment viral binding and possibly enhance the entry via the natural entry route of the virus

(Figure 10). Given the fact that F3, CGKRK and baculovirus might all bind heparan sulphate and/or phosphatidyl serine (Christian et al. 2003, Duisit et al. 1999, Hoffman et al. 2003, Tani et al. 2001), the enhancement of natural entry route of the virus seems likely. The target molecules of LyP-1 and CGKRK have not been identified and the entry mechanisms of these tumor homing peptides are currently unknown. It has, however, been suggested that CGKRK might internalize with the help of heparan sulphate (Hoffman et al. 2003). The fact that the heparan sulfate molecule is known to internalize using endocytosis during its normal turnover fits well in this picture (Iozzo 1987). Heparan sulphate-bound molecules, e.g. basic fibroblast growth factor, are also known to be carried along during this turnover (Roghani & Moscatelli 1992). The putative binding of F3 to heparan sulphate, however, does not lead to internalization (Christian et al 2003). F3 is known to utilize cell surface nucleolin as a receptor molecule. The internalization mechanism of F3 is known to be energy-dependent (Christian et al. 2003). Nucleolin has been reported to employ several entry mechanisms, depending on the ligand. Lactoferrin-bound nucleolin enters the cell by clathrin-mediated endocytosis through early endosomes (Legrand et al. 2004). In contrast, binding of cytokine midkine to cell surface nucleolin seems to recruit midkine-nucleolin -complex to lipid rafts, and possibly activate lipid raft -mediated endocytosis (Hovanessian 2006). Nucleolin likely transports midkine all the way to the nucleus (Shibata et al. 2002) and same mechanisms may be employed with F3. The putative use of endocytosis route by both CGKRK and F3 may thus actively support the baculovirus internalization.

Interestingly, heparan sulphate (El-Sheikh et al. 2002, Qiao et al. 2003, Smetsers et al. 2003) and phosphatidylserine (Ran et al. 2002), are accessible or preferentially expressed on the tumor blood vessels. Heparan sulphate seems to be the target molecule for an endothelial growth factor-derived peptide that exhibits a tumor-homing activity *in vivo* (El-Sheikh et al. 2002). Phosphatidylserine might be, due to its negative charge, a target molecule for cationic liposomes reported to selectively home tumor vasculature (Thurston et al. 1998). Thus, the preference of CGKRK and F3 for these molecules is not a non-specific feature but targets them to tumor vasculature as well. Moreover, the baculovirus tendency to bind these molecules (Duisit et al. 1999, Tani et al. 2001) may be of use in the targeting of the virus to the tumor vasculature.

6.5 Selectivity as a prerequisite for successful targeting

The ideal targetable vector for gene transfer should specifically accumulate in the target organs or tissues harboring the target cells and deliver the transgene efficiently and selectively to these cells, but not the neighboring cells. In the real world, the lack of targetable vector systems has been the major draw-back in systemic gene therapy *in vivo* (Trepel et al. 2000, Verma & Weitzman 2005,

Wang & Liu 2003). A prerequisite for successful targeting is the targeting motif possessing efficient binding to its receptor (Wickham et al. 1997). Also for baculovirus, efficient motif used in targeting might overcome the relatively low affinity interactions of virus with its "receptors" in mammalian cells and redirect the virus to cells of interest. However, as also seen in this work with the RKK display, high affinity of the targeting motif is not enough. Thus, high avidity, correct conformation as well as efficient internalization are also required (IV).

As being composed of strongly basic and positively charged residues, all targeting motifs used in this thesis work might in principle bind to the negatively charged cell surface by the virtue of charge. Cell-penetrating peptides, such as Tat peptide, with high content of basic amino acids are known to bind and enter the cell by an energy-independent process (Zorko & Langel 2005). Especially LyP-1, F3 and CGKRK resemble basic cell-penetrating peptides. However, the prominent difference is that these tumor homing peptides enter only certain target cells (Hofmann et al. 2003, Laakkonen et al. 2002, 2004, Porkka et al. 2002). Additionally, the uptake of F3 is known to be energy-dependent (Porkka et al. 2002). Despite of the reported specificity of the motifs used in the display, the selectivity of each should be confirned experimentally. In this aspect, studies (III) and (IV), displaying RGD and RKK, respectively, are incomplete and do not show that the enhancement of binding/transduction would be sequence-specific. Thus, proper inhibition experiments with corresponding peptides or antibodies inhibiting the binding would add more value to the statements.

To study the selectivity of the binding in the case of the tumor homing peptide –displaying baculoviruses an inhibition experiment was performed (V). The results might reflect the complicated binding mechanism of wt baculovirus suggesting the use of several binding moieties. As for the peptides, the superiority of the LyP-1 peptide in mediating degree and perhaps also the specificity of binding, was observed. The finding that all peptides cross inhibit binding of all viruses to some extent could partly be explained by the use of similar cell surface molecules, such as heparan sulphate and phosphatidyl serine by both wt baculovirus (Duisit et al. 1999, Tani et al. 2001) and tumor homing peptides (Christian et al. 2003, Hoffman et al. 2003). The reason for the strong inhibition of binding of all viruses by LyP-1 peptide is not clear, as the receptor molecule of LyP-1 is unknown. However, it is also possible that the complicated results might reflect secondary binding of tumor-homing peptide -displaying viruses to the cell surface. This could be a result of the detaching method used, trypsin-EDTA. Trypsin is a serine protease that catalyzes peptide bond hydrolysis (Radinsky et al. 2006). During detachment of cells, it thus may split membrane proteins (Baumann & Doyle 1979) and consequently affect the result of the experiment. Hofmann and colleagues have suggested the number of baculovirus receptors to diminish due to typsin treatment (Hofmann et al. 1995). Hence, using some other detachment method e.g. EDTA might have given more reliable results.

In this respect the inhibition of transduction of tumor-homing peptide-displaying viruses likely give a more consistent result (V). Inhibition of the transduction of LyP-1 and F3 displaying baculoviruses by corresponding peptides suggests that displayed peptides have at least a partial effect on transduction. Inhibitory effect of the F3 peptide on the transduction of wt virus might indicate the use of similar protein moieties in the binding and/or entry of F3 and wt virus. One potential target molecule mediating binding of both wt virus and F3 would be heparan sulphate, although the internalization of F3 has been reported not to need this molecule (Christian et al. 2003, Duisit et al. 1999). As a nonrelevant cationic RKK peptide showed no inhitory effect, the tumor homing peptides likely mediate transduction by using more specific interactions than solely cationic in nature.

The selectivity of the displayed peptides is a prerequisite for specific targeting and transduction of target cells expressing appropriate receptor molecules. However, the broad cell-type range of the virus vector can also affect nontarget tissues in the gene therapy attempts (Wilson 2002). This could be a concern also with baculovirus, known to exhibit a broad cell type preference (Condreay et al. 1999). Adenovirus has been successfully modified to ablate the natural virus binding by mutating fiber knob and RGD in the penton base and replacing fiber shaft domain, thereby ablating binding to CAR, αv integrin and heparan sulphate, respectively. By this method, the virus exhibited reduced tropism to organs studied (the liver, spleen, kidney, heart, and lung) *in vivo* (Koizumi et al. 2003). When specific targeting moieties will be displayed on the surface of this virus, truly targeted viral vector might be at hand. Thus, in addition to displaying a targeting motif, it would be feasible to be able to block the wild-type virus binding. It has been suggested that interaction of gp64 with cell surface phospholipids may be important in baculovirus during mammalian cell transduction (Tani 2001). The inactivation or removal of the gp64 from baculovirus, would however result in a block of the internalization as well. For baculovirus, ablation of tropism by mutatiting gp64 would need more details of the structure of the gp64 and virus-receptor-interactions. Demonstration of replacement of gp64 molecule with other ligands of interest, pseudotyping, has certainly been performed. The incorporation of VSV-G protein to the surface of gp64-null virus (Mangor et al. 2001), however, does not markedly restrict the cell type specificity. The use of a more specific molecule, such as CD46, reported by Kitagawa and colleagues (2005), in this context, could enable more specific delivery of transgenes. Thus, in addition to displaying selective targeting motifs on the baculovirus surface, it would be beneficial to be able to reduce the tropism of baculovirus by using this kind of approach.

6.6 The potential of baculovirus in therapeutic gene transfer

Baculovirus holds a great potential for a therapeutic gene transfer vector. The many attractive features of the virus include easy cloning and propagation to high titers. The extremely large insertion capacity of the virus also enables the design of more complicated therapeutic strategies. The capability of the virus to enter a variety of mammalian cell types and express the transgene further broadens the possibilities (Cheshenko et al. 2001, Ghosh et al. 2002, Kost & Condreay 2002). Baculovirus is generally regarded as safe due to the lack of replication and low transcription of its genes in mammalian cells (Airenne et al. 2004). The lack or low level of the cytotoxicity induced by baculovirus transduction in host cells is also a credit (Cheshenko et al. 2001, Ghosh et al. 2002, Kost & Condreay 2002). The mild immune responses, such as cytokine production has, however, been detected (Airenne et al. 2000, Beck et al. 2000, Gronowski et al. 1999) and integration of the parts of viral genome has been reported under selective pressure (Condreay et al. 1999, Merrihew et al. 2001). Despite this, after futher studies in this area, baculovirus will likely be attractive alternative for the use of pathogenic mammalian virus based vectors (Thomas et al. 2003).

However, intravenous delivery of baculovirus is hampered because of inactivation of baculovirus vector by the complement system (Hofmann et al. 1998, Hofmann & Strauss 1998, Sandig et al. 1996). Complement inactivation is a general phenomenon that has been observed with many types of gene delivery vehicles e.g. liposomes (Szebeni et al. 1994) and retrovirus (Takeuchi et al. 1994). In the case of baculovirus, several attempts to solve this problem have been taken, for example using molecules blocking the complement or generating complement-resistant viruses (Hoare et al. 2005, Hofmann et al. 1999, Hofmann & Strauss 1998, Huser et al. 2001, Tani et al. 2003). Successful baculovirus gene transfer *in vivo* to human derived liver tumors in nude mice suggest that if complement lysis will be avoided, baculovirus could be used for gene transfer *in vivo* (Hofmann et al. 1998).

With the help of the baculovirus display –system, targeting motifs can be successfully displayed on the viral surface in the correct form. Importanty, based on this (III, V) and other studies (Räty et al. 2003, Grabherr et al. 2006) the display of targeting motifs seems to enhance the binding and transduction of target cells *in vitro*. The next step – accomplishing the targeting using baculovirus display *in vivo*- will reveal the real potential of the virus.

Taken together, targeted baculovirus gene transfer to tumor vasculature has taken the first small step. However, several others have to be taken until this ambitious goal will be achieved.

7 CONCLUSIONS

The main conclusions of this thesis are:

1) Baculovirus most likely utilizes endosomal entry route in human hepatocarcinoma cells and may enter cells via clathrin coated vesicles. Additionally, the virus may exploit macropinocytosis as an alternative mechanism of entry.

2) α2β1 integrin internalizes in concert with EV1 into cells using the caveosomal pathway.

3) The binding and transduction of human cancer cells can be enhanced *in vitro* by displaying specific targeting motifs on the baculoviral surface.

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YHTEENVETO (Résumé in Finnish)

Bakulovirukset ovat hyönteisiä infektoivia viruksia, jotka ovat vaarattomia ihmisille. Bakuloviruksilla on DNAsta koostuva genomi, joka on pakattu sauvamaisen kapsidin sisään. Lisäksi kapsidia ympäröi lipidikalvo. Tässä lipidikalvossa on virukselle elintärkeä pintaproteiini, gp64, jonka avulla virus tunkeutuu isäntäsoluunsa. Lisäksi uudet syntyvät viruspartikkelit käyttävät gp64-proteiinia päästäkseen ulos solusta. Bakulovirus display -menetelmän avulla gp64 proteiiniin voidaan liittää erilaisia vieraita proteiineja tai polypeptidejä, jotka näin ilmentyvät viruksen pinnalla. Tätä display-systeemiä on käytetty onnistuneesti useissa erilaisissa sovelluksissa, esimerkiksi peptidikirjastojen teossa ja rokotetutkimuksessa. Viime aikoina kiinnostuksen kohteena on ollut liittää viruksen pinnalle peptidejä, jotka spesifisesti tarttuvat tiettyihin reseptoreihin solujen pinnalla. Tätä bakuloviruksen kohdennusta tiettyihin solutyyppeihin suunnitellaan käytettäväksi virusvälitteisessä geeniterapiassa. Bakuloviruksen mahdollista käyttöä geeninsiirroissa puoltaa niiden kyky pystyä tunkeutumaan myös nisäkässoluihin. Koska bakulovirukset eivät lisäänny nisäkässoluissa, ovat ne melko turvallinen vaihtoehto hoidollisissa geeninsiirroissa, jossa tavallisesti käytetään ihmisille patogeenisista viruksista johdettuja kuljettimia. Bakuloviruksen on havaittu pystyvän kuljettamaan haluttu geeni nisäkässolun tumaan, jossa geeni voidaan saada ilmentymään käyttämällä nisäkässoluissa toimivaa promoottoria.

Bakulovirusten sisäänmenon yksityiskohdat nisäkässoluihin ovat kuitenkin vielä suurelta osin hämärän peitossa. Niinpä tämän tutkimuksen yhtenä päämääränä oli tutkia viruksen sisäänmenoa maksasyöpäsoluissa. Bakulovirus näytti käyttävän mahdollisesti kahta reittiä sisäänmenossaan. Ensimmäinen, jo aikaisemmin ehdotettu reitti, kulkee mahdollisesti klatriinikuopakkeiden kautta reseptorivälitteiselle endosomaaliselle reitille. Tämän lisäksi virusten arveltiin tunkeutuvan soluun mahdollisesti makropinosytoosia hyväksikäyttäen. Adenoviruksen on aiemmin raportoitu tehostavan klatriinivälitteistä endosomaalista sisäänmenoaan makropinosytoosin avulla ja näinollen on mahdollista, että bakulovirus toimisi samalla tavalla.

Kohdentuvat geeninsiirtomenetelmät ovat tärkeitä esimerkiksi syövän hoidossa. Syöpäkasvaimeen kohdennettu geeninsiirtovektori saisi aikaan tehokkaamman vasteen halutussa paikassa, samalla vähentäen sivuvaikutuksia muissa kudoksissa. Tehokas kohdennus myös mahdollistaisi virusvektorien käytön suonensisäisesti, jolloin vektori pystyisi hakeutuvan tehokkaasti syöpäkasvaimeen ja tunkeutumaan sen sisään, siten lisäten hoidon tehoa. Syöpäkasvaimen verisuonista viimeaikoina löydetyt spesifiset markkerit sekä niihin kohdentuvat peptidit mahdollistavat kohdennuksen käytön syövän hoidossa tulevaisuudessa.

Tämän tutkimuksen päätarkoituksena oli kehittää bakulovirus display-systeemin avulla syövän verisuonien markkeriproteiineihin kohdentuvia viruksia. Tämä aikaansaatiin liittämällä bakuloviruksen pintaan syövän verisuoniin

kohdentuvia peptidejä. Spesifiset peptidit, RGD (III) ja RKK (IV) tunnistavat syöpäkasvaimen verisuonissa yleisiä integriinejä. LyP-1, F3 ja CGKRK peptidien (V) on puolestaan havaittu *in vivo* faagidisplay-tekniikan avulla kohdentuvan spesifisti tiettyjen syöpätyyppien veri- ja lymfasuoniin. Integriineihin kohdentuvat peptidit liitettiin bakuloviruksen pintaan gp64-molekyylin fuusioina. RKK-peptidin oikeaa konformaatiota edesautettiin liittämällä peptidi vihreän fluoresoivan proteiinin (GFP) silmukkarakenteeseen ja aikaansaamalla GFP-gp64 fuusioproteiini. LyP-1, F3 ja CGKRK peptidien kohdalla display-menetelmänä käytettiin uudempaa sovellusta, jossa peptidi liitetään vesicular stomatitis viruksen (VSV) G-proteiinin osaan. Lisäksi virusten genomiin liitettiin nisäkässoluissa ilmentyvä merkkigeeni, lusiferaasi, jonka avulla voitiin monitoroida geeninsiirron tehokkuutta (III, V).

Virusten tuottaminen onnistui hyvin ja kaikki kehitetyt virukset ilmensivät onnistuneesti pinnallaan haluttua peptidiä. RGD, LyP-1, F3 ja CGKRK-peptidit lisäsivät viruksen kiinnittymistä kohdesoluihin, mikä voitiin havaita fluoresenssikvantitaatiomittauksin. Myös virusten geeninsiirtokyky parani huomattavasti peptidien liittämisen seurauksena; lusiferaasi-geenin aktivisuusmittaukset osoittivat, että peptidejä kantavat virukset pystyvät ilmentämään merkkiigeeniä jopa 24-kertaisesti varrattuna kontrollivirukseen. Vaikka RKK peptidiä ilmentävä virus pystyi kiinnittymään spesifisesti kohdeproteiiniinsa *in vitro,* ei virus kuitenkaan sitoutunut tehokkaammin kohdesoluunsa. Tähän saattaa olla syynä edellisissä tutkimuksissa raportoitu vähäinen GFP fuusioproteiinien määrä viruksen pinnalla. On myös mahdolllista, että RKK ei ollut optimaalisesti esillä tarttuakseen kohdeproteiiniinsa.

Koska virusta kohdennettaessa on tärkeä tietää kohdemolekyylin kulkureitti solussa, tutkittiin tässä työssä myös RKK peptidin kohdemolekyyliä, α2β1 integriiniä, echovirus 1:n (EV1), tätä integriiniä reseptorinaan käyttävän viruksen avulla. Tutkimuksissa selvisi, että kyseinen integriini mahdollisesti ohjaa EV1:n kaveolireitille, jota käyttäen sekä integriini että virus menevät solun sisään. Kaveolireitti on hiljattain tunnistettu sisäänmenoreitti, ja integriinin sisäänmeno soluihin kyseistä reittiä pitkin on ainutlaatuista.

Tämän väitöskirjatyön tulokset osoittavat, että kohdennuspeptidit bakuloviruksen pinnalla parantavat sekä virusten kiinnittymistä että geeniensiirtoa kohteena oleviin syöpäsoluihin *in vitro*. Näin ollen kyseisiä peptidejä pinnallaan ilmentävät virukset ovat lupaavia kandidaatteja jatkotutkimuksiin, joissa päämääränä on kehittää bakuloviruksesta toimiva geeniterapiavektori.

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