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Johanna Laakkonen

Intracellular Delivery of Baculovirus and Streptavidin-based Vectors *in vitro* – Towards Novel Therapeutic Applications

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Now This is Not the End.
It is Not Even the Beginning of the End.
But It is,
Perhaps,
The End of the Beginning.

W. Churchill

ABSTRACT

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Yhteenveto: Bakulovirus ja streptavidiini geeninsiirtovektoreina ihmisen soluissa

To achieve cell-specific delivery, gene therapy vectors must be able to internalize and express transgenes in their target cells. Despite of the high transduction efficiency of the viral vectors, their natural tropism rarely matches to required therapeutical needs. This thesis was focused on the development of two potential gene therapy vectors, the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) and a novel, non-viral vector TAT-streptavidin, by characterizing their underlying intracellular mechanisms in human cells. Baculovirus was shown to utilize a RhoA and Arf6-regulated phagocytosis-like mechanism for their cellular uptake. After endosomal release, the virus accumulated into nuclei near promyelocytic leukaemia nuclear bodies (PML NBs), a nuclear substructure involved in transcription and replication of viruses. Non-replicative baculovirus was also found to be capable of producing its immediate early proteins in human cells. In addition, virus transduction altered the size of PML NBs and the localization of the host chromatin.

Non-viral vector TAT-streptavidin was shown to internalize into all tested human cell lines. The vector was, however, unable to mediate efficient endosomal release and underwent lysosomal degradation. Co-treatment with the endosomal releasing agent (poly)propylacrylicacid significantly increased the nuclear delivery of the TAT-streptavidin and its cargo, indicating the potentiality of the vector to mediate efficient cellular delivery of biotinylated molecules. To conclude, the defining of the gene therapy vectors at the cellular level enables their more accurate developing towards targeting of specific cells and delivering of functional therapeutic macromolecules into human cells.

Keywords: Baculovirus; cellular delivery; cellular entry; gene therapy; human cells; streptavidin; TAT.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I Matilainen H.*, Rinne J.* (Laakkonen J.P.), Gilbert L., Reunanen H., Marjomäki V., Oker-Blom C., 2005. Baculovirus Entry into Human Hepatoma Cells. Journal of Virology 79(24):15452-15459.

II Laakkonen J.P., Mäkelä A.R., Kakkonen E., Peränen J., Kukkonen S., Ylä-Herttuala S., Airenne K.J., Oker-Blom C., Vihinen-Ranta M., Marjomäki V., Baculovirus Utilizes Phagocytosis-like Entry for Efficient Transduction in Human Cells. Submitted manuscript.

III Rinne J. (Laakkonen J.P.), Albarran B., Jylhävä J., Ihalainen T.O., Kankaanpää P., Hytönen V.P., Stayton P.S., Kulomaa M.S., Vihinen-Ranta M., 2007. Internalization of Novel Non-viral Vector TAT-streptavidin into Human Cells. BioMed Central Biotechnology 2;7:1.

IV Laakkonen J.P., Kaikkonen M.U., Ronkainen P.H.A., Ihalainen T.O., Niskanen E.A., Häkkinen M., Salminen M., Kulomaa M.S., Ylä-Herttuala S., Airenne K.J., Vihinen-Ranta M., 2008. Baculovirus-mediated Immediate Early Gene Expression and Nuclear Reorganization in Human Cells. Cellular Microbiology 10;3:667-681.

*Equal contribution

RESPONSIBILITIES OF JOHANNA LAAKKONEN IN THE THESIS ARTICLES

ARTICLE I: Heli Matilainen was responsible for planning the experiments for this paper. Matilainen and Gilbert performed the expression studies. I was responsible for the most of the confocal microscopy experiments with participation of Matilainen. Hilkka Reunanen primarily conducted the electron microscopy studies in collaboration with me and Matilainen. I wrote the article and Matilainen took part in finalizing it.

ARTICLE II: Varpu Marjomäki and I planned the experiments together with Anna Mäkelä. I conducted the experiments together with Mäkelä and Elina Kakkonen. I was responsible for the most of the confocal microscopy studies, including living cell imaging and defining the effects of various expressing constructs in baculovirus entry. I wrote the article together with Marjomäki and processed all figures.

ARTICLE III: I planned the article together with Maija Vihinen-Ranta. I was responsible for the most of the confocal microscopy studies. I wrote the article with Vihinen-Ranta and processed all figures.

ARTICLE IV: I planned the article and conducted the majority of the experiments. I was responsible for the most of the confocal microscopy studies and Minna Kaikkonen for the RT-PCR experiments. I wrote the article and processed the majority of the figures.

Study I was carried out under the supervision of Professor Christian Oker-Blom. Study II was supervised by Docent Varpu Marjomäki in a collaboration project with Docent Maija Vihinen-Ranta and Professor Christian Oker-Blom. Study III was carried out under the supervision of Docent Maija Vihinen-Ranta in collaboration with Professor Markku Kulomaa. Study IV was carried out under the supervision of Docent Maija Vihinen-Ranta.

ABBREVIATIONS

aa	amino acid
Ab	antibody
*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
AP180	clathrin assembly protein
Arf6	ADP-ribosylation factor 6, GTPase
BHK	baby hamster kidney cell line
BSA	bovine serum albumin
BV	budded virus
CA	constitutive active mutant
CAG	cytomegalovirus immediate early enhancer/ chicken β-actin promoter
CB	Cajal body
CCV	clathrin-coated vesicles
CCP	clathrin-coated pits
CD-63	lysosomal membrane glycoprotein
Cdc42	cell division cycle protein, GTPase
CI-MPR	cation-independent mannose phosphate receptor
CME	clathrin-mediated endocytosis
CMV	cytomegalovirus
DMEM	Dulbecco´s modified Eagle medium
DN	dominant negative mutant
E. coli	*Escherichia coli*
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EE	early endosome
eea-1	early endosomal antigen 1
EM	electron microscopy
FCS	fetal calf serum
GEEC	glycophosphatidylinositol-anchored protein enriched endosomes
gp64	baculovirus envelope glycoprotein
GPI-AP	glycophosphatidylinositol-anchored protein
GTP	guanosine triphosphate
HeLa	human cervical carcinoma cell line
HepG2	human hepatoma cell line
(HEK-)293	human epithelial kidney cell line
HIV-1	Human Immunodeficiency Virus type I
HRP	horse radish peroxidase
ie	baculovirus immediate early gene
IL-2R	interleukin-2 receptor
LAMP	lysosomal associated membrane protein
LE	late endosome
lef	baculovirus late expression factor gene

luc	firefly luciferase
MEM	Minimum Essential Medium
MOI	multiplicity of infection
NIH3T3	mouse fibroblast cell line
NLS	nuclear localization signal
NPM	nucleophosmin
ODV	occlusion derived virus
PAGE	polyacrylamide gel electrophoresis
Pak1	p21 kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
p.i.	post infection
PI3K	phosphatidylinositol-3-kinase
PLC	phospholipase C
PML NB	promyelocytic leukaemia nuclear body
PML	promyelocytic leukaemia protein
PPAA	(poly)propylacrylicacid
p.t.	post transduction
Rac1	Ras-related C3 botulinum toxin substrate 1 protein; GTPase
RE	recycling endosome
RhoA	Ras homolog gene family, member A protein; GTPase
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SA	streptavidin
SDS	sodium dodecyl sulfate
*Sf*9	*Spodoptera frugiperda* 9 insect cell line
siRNA	small interfering RNA
SV40	Simian virus 40
TAT	HIV-derived peptide, trans-activator of transcription
TRITC	tetramethyl rhodamine iso-thiocyanate
vATPase	vacuolar adenosine triphosphatase
vp39	baculovirus capsid protein
VSV-G	vesicular stomatitis virus G transmembrane glycoprotein

1 INTRODUCTION

Viruses, the small non-living, infectious agents, are able to internalize into human cells efficiently and hijack the cellular machinery for their own purposes. In gene therapy, virus-derived or synthetic vectors are exploited to deliver the therapeutic transgene(s) into target cells. Due to frequent immunogenic reactions induced by viral vectors of pathogenic human origin, various non-human viruses and non-viral vectors have been generated in attempt to circumvent the immune system.

In this thesis, the insect infecting *Ac*MNPV-baculovirus and bacterial protein streptavidin-based vectors were characterized in human cells. Both vector types were efficiently internalized into cells, but had unique characteristics of their entry pathways. Previously, baculovirus vectors have been shown to be prone to serum complement inactivation *in vivo* and proposed to be suitable for targeting of the immunopriviledged regions, such as the eye, nervous system and reproductive organs. To further characterize the suitability of baculovirus vectors for gene therapy, the mechanisms of baculoviral transduction in human cells were studied at the cellular level. Due to their non-replicating nature in mammalian cells, baculoviruses have been considered to be safe for gene therapy and classified as the lowest biohazard level. However, data presented here demonstrated that *Ac*MNPV-baculovirus transduction causes expression of viral proteins in human cells and alters the composition of the host chromatin and nuclear substructures. These cellular changes have to be considered in further *in vivo* studies. They also call into question the safety of baculoviral vectors for gene therapy applications.

Streptavidin and biotin have one of the strongest non-covalent binding affinities in the nature. The ease of functionalizing biomolecules via streptavidin-biotin system has created various biotechnological applications. In this study, the novel, non-viral vector TAT-streptavidin was analyzed in human cells and the proof-of-principle concept of the delivery of biotinylated cargos into living cells was presented. Apart from contributing to the cellular delivery, the Human Immunodeficiency Virus (HIV-1) derived TAT-peptides were used to enhance the nuclear delivery of the vector. To conclude, the utilization of

synthetic, bacterial and virus origin mediators in vector biology raise new possibilities for development of more suitable, cellular-specific vectors for gene therapy. To achieve successful and safe gene delivery, the intracellular machinery responsible for uptake, transportation and localization of the vector has to be well defined.

2 REVIEW OF THE LITERATURE

2.1 Gene Therapy – *En Route* from Cellular Level to Medicine

Gene therapy aims to treat a variety of genetic and acquired diseases through the transfer of functional genetic material into cells *in vivo* or *ex vivo*. Currently, totally 1347 gene therapy clinical trials are ongoing worldwide, including three in Finland (the database of the Journal of Gene Medicine, http://www.wiley.co.uk/genmed/clinical). Most of the trials have been developed against cancer and cardiovascular diseases by using adenovirus, retrovirus or naked plasmid/DNA as vectors. Totally, only 2.7% of all clinical trials have succeeded in entering the phase III, in which the efficiency of novel treatment is compared to existing one in large patient groups. Recent unsuccessful clinical trials, such as fatal outcome of adeno-associated virus trial, have further emphasized the importance of accurate knowledge of vector biology and pharmacology, both at the molecular and cell biological levels.

Various viral and synthetic, non-viral vectors have been developed for gene transfer. *In vivo* delivery is divided to systemic or locally applied methods, in which transferred genetic material is directly introduced into a patient. *Ex vivo* delivery method instead consists of isolation of cells from tissue, transfer of genetic material into the cells, and finally transplantation of the cells into a patient (Kay et al. 1997). At the cellular level, in order to succeed in gene transfer gene therapy vectors have to overcome numerous barriers, such as binding to specific target cells, efficient internalization, endosomal release, nuclear delivery and high-level expression of the therapeutic transgene (Cambell et al. 2005). Virus vectors, including vectors based on retroviruses, adenoviruses, adeno-associated viruses and herpesviruses, are so far the most efficient gene transfer method available for gene therapy (see reviews of Thomas, Ehrhardt & Kay 2003; Verma & Weitzman 2005 for extensive list of virus vectors available). Depending on the vector type used, different advantages and disadvantages have been arisen in clinical applications. These include manufactural production, persistence of the gene transfer, toxicity,

immunogenicity and packaging capability. Generally, one of the major disadvantages of the virus vectors is their inefficient targeting into specific cells (Thomas, Ehrhardt & Kay 2003).

Non-viral vectors are attractive alternatives for gene therapy due to their low toxicity, lack of pathogenicity and ease of pharmacologic production. Typically, non-viral vectors bind and internalize into the target cells efficiently but yield a relatively low transgene expression compared to viral vectors. Enhancement of the transduction efficiency by improvement of endosomal release, nuclear delivery and localization remains, therefore, a goal of continuous research. Non-viral vectors can be categorized according to their DNA carrier to liposome, polymer, peptide or protein-based vectors (Medina-Kauwe & Hamm-Alvarez 2005).

Current increased cell biological knowledge of endocytic and nuclear mechanisms enables more accurate approach for the development of suitable gene delivery vectors and their therapeutic applications. In the following chapters, endocytic routes and nuclear compartmentalization in mammalian cells are presented. Additionally, the vectors used in the present thesis, baculovirus and streptavidin-based vectors, are introduced.

2.2 Overview of Endocytic Pathways in Mammalian Cells

All mammalian cells exhibit various endocytic mechanisms, which are processes to carry macromolecules and particles from the external environment into cells. Endocytosis can be broadly categorized to pinocytosis (cell drinking) or phagocytosis (cell eating; Conner & Schmid 2003). It maintains cellular homeostasis through retrieval of proteins and lipids, delivered to the plasma membrane by secretion (Mellman 1996). Endocytosis is also crucial for development, immune response, signal transduction, neurotransmission and intercellular communication (Conner & Schmid 2003).

The endocytic route consists of different cellular organelles, known as endosomes, which have highly regulated mechanisms for communicating and fusing with each other. These membrane-bound vesicles typically invaginate or pinch-off from the cell membrane. For now the classification of the endocytic routes may differ depending on the source. According to Conner & Schmid (2003), pinocytosis is divided into clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin- and caveolin-independent pathways and macropinocytosis. To greater or lesser degrees, these various pinocytosis mechanisms and phagocytosis co-exist in a single cell type, and are either constitutive or triggered by ligand binding (Mayor & Pagano 2007, Fig. 1). Today, one of the major challenges of modern cell biological research is to define the various endocytic mechanisms at molecular level, and also characterize their regulation and integration with each other.

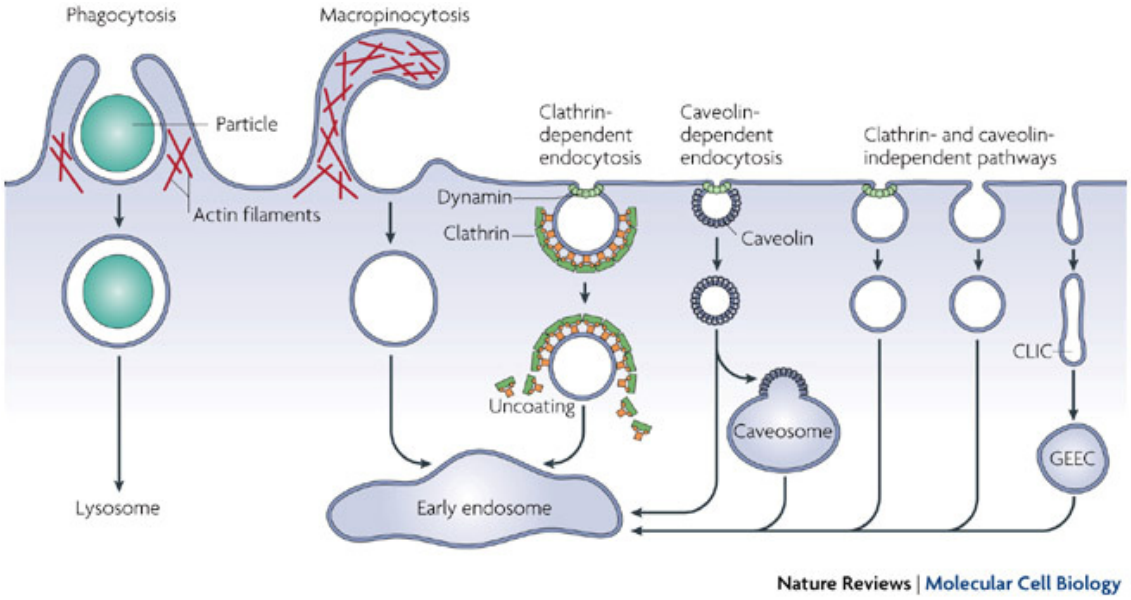

FIGURE 1 Entry pathways to mammalian cells (Mayor & Pagano, 2007). Phagocytosis is utilized in engulfment of large particles (>0.5 µm diameter), whereas macropinocytosis is a form of fluid-phase endocytosis leading to vesicular structures called macropinosomes (0.5-5 µm). Clathrin-dependent, caveolin-dependent and clathrin- and caveolin-independent pathways (<0.2 µm diameter) are forms of pinocytosis, which carry extracellular fluid and macromolecules into cells. Most of the cellular entry pathways lead to early endosomes via clathrin- or caveolin-coated vesicles or tubular clathrin-, caveolin-independent carriers (CLICs), some of using intermediate vesicles *en route* to early endosomes, such as caveosome or glycophosphatidylinositol-anchored protein enriched endosomes (GEECs). Reprinted by permission from Macmillan Publishers Ltd; Nature Reviews Molecular Cell Biology, copyright 2007.

To accomplish specific cell delivery, gene therapy vectors must internalize efficiently into their target cells. Typically, the natural tropism of viral vectors is modified by broadening or narrowing of their tropism to suit the therapeutical need (Hendrie & Russell 2005). Apart from pseudotyping i.e. altering the tropism of a virus by replacing its attachment proteins with that of another virus, various targeting methods have been encountered, e.g. directing vector towards clathrin-mediated endocytosis via incorporated transferrin receptor (Kreppel et al. 2005, Phillips 1995; Waehler, Russell & Curiel 2007). Adaptor proteins, such as receptor-ligand complexes or chemical conjugates, which have a dual specificity for the viral attachment protein and cellular receptor, have been extensively utilized as well. Additionally, avidin-biotin affinity-based systems, monoclonal antibodies and systems involving polypeptide ligands or small peptide motifs incorporated into the vector exist (Waehler, Russell & Curiel 2007). In next chapters, mammalian endocytic pathways and their regulators are outlined. The vector biology, especially transductional targeting, would significantly benefit from accurate cell biological knowledge of underlying endocytic mechanisms.

2.2.1 Pinocytosis

Pinocytosis, including fluid-phase, adsorptive and receptor-mediated endocytosis, typically refers to the constitutive formation of small vesicles (<0.2 μm diameter) carrying extracellular fluid and macromolecules specifically or non-specifically bound to the plasma membrane (Conner & Schmid 2003). Pinocytosis can occur by several different mechanisms, which are outlined in the following chapters. In this thesis, they are classified according to their requirement of clathrin, caveolin and dynamin (Table 1; Conner & Schmid 2003, Mayor & Pagano 2007).

TABLE 1 Classification of Different Pinocytosis Routes and Examples of Their Cargos*.

Endocytic Routes	Cargo
1) Clathrin-dependent Endocytosis	
A) Dynamin-dependent	Transferrin, low-density lipoprotein, EGFR, Shiga toxin, integrins
2) Caveolin-dependent Endocytosis	
A) Dynamin-dependent	Modified albumins, glyco-sphingolipid analogues, antibody-clustered GPI-APs, integrins, autocrine motility factor, alkaline phosphatase, Cholera toxin B, Shiga toxin
3) Clathrin- and Caveolin-independent Pathways	
A) Dynamin-dependent	
-RhoA-regulated	IL-2R-αβ, IL-2R-β, γc-cytokine and IgE receptors
B) Dynamin-independent	
-Cdc42-regulated	GPI-AP, Cholera toxin B, VacA toxin, aerolysin
-Arf6-regulated	MHC I, β1-integrin, E-cadherin, IL-2R-α(Tac), GPI-AP, carboxypeptidase E

Abbreviations: EGFR, epidermal growth factor receptor; GPI-AP, glycosyl phosphatidylinositol-anchored proteins; IL-2R, interleukin-2 receptor; VacA, *Helicobacter pylori* vacuolating toxin; MHC1, major histocombatibility complex 1. *Viruses are not included; see text for further details. Sources: Conner & Schmid 2003, Mayor & Pagano 2007, Pellinen & Ivaska 2006.

2.2.1.1 Clathrin-mediated Endocytosis.

The uptake of receptor-bound ligands and extracellular fluid into mammalian cells results mainly from the formation of clathrin-coated vesicles (CCVs). Constitutively active clathrin-mediated endocytosis (CME) is involved in cell and serum homeostasis, signal transduction and intracellular communication during tissue and organ development (Conner & Schmid 2003). CCVs (100-150

nm, ≤200 nm) are composed of three clathrin heavy chains, each complexed with a light chain (Mellman 1996, Rejman et al. 2004). Dynamin facilitates the budding of clathrin-coated pits (CCPs), leading to formation of coated vesicles (DeTulleo et al. 1998). Additionally, epsin, endophilin and amphiphysin may facilitate the process. Assembly of clathrin coat also requires monomeric assembly protein AP180C, clathrin recruiting eps15 and typically heterotetrameric adaptor complex AP2 (Conner & Schmid 2003, Motley et al. 2003). CCPs are typically concentrated into specific locations on the plasma membrane, constrained by actin (Conner & Schmid 2003).

In the entry process CCVs rapidly lose their coats and fuse with early endosomes (EE), a dynamic array of tubules and vesicles distributed throughout the cytoplasm. Slightly acidic pH (6.0-6.8) maintained by vacuolar adenosine triphosphatase (vATPase) is responsible for dissociation of many internalized ligand-receptor complexes (Klasse et al. 1998, Mellman 1996). Transit through EEs occurs in approximately 2-3 minutes, during which free receptors selectively accumulate into tubular extensions of EEs. Recycling endosomes (REs) budding from EEs transport receptors back to the plasma membrane or localize them into perinuclear region (Mellman 1996). Multivesicular bodies also pinch off from EEs, accumulate intralumenal vesicles and traverse to late endosomes (LEs; Gruenberg & Stenmark 2005).

Early endosomal antigen 1 (eea-1) and the two GTPases rab4 and rab5 are characteristic proteins of EEs, whereas REs are enriched in rab11 (Mellman 1996). LEs consist of lysobisphosphatidic acid, lgp/lamps, rab7, rab9, the cation-independent MPR and hydrolytically active lysosomal hydrolases. Following fusion of LEs with lysosomes, the final site of internalized macromolecules, tranported ligands are degraded by low pH (4.5) and lysosomal enzymes (Gruenberg & Stenmark 2005, Mellman 1996).

CME mediates constitutive uptake of ligands, such as transferrin and low-density lipoprotein as well as uptake of ligand-triggered epidermal growth factor receptor (Conner & Schmid 2003, Table 1). CME is also involved in entry of Shiga toxin and for uptake of many enveloped and non-enveloped viruses from *Retroviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Togaviridae, Rhabdoviridae, Adenoviridae, Papillomaviridae, Polyomaviriade, Reoviridae* and *Picornaviridae* families (reviewed in DeTulleo & Kirchhausen 1998, Marsh and Pelkchen-Matthews 2000). Size of the internalized particles has demonstrated to restrict the entry through CCPs (Klasse et al. 1998, Rejman et al. 2004). However, recently, bacteria *Listeria monocytogenes* (>1μm) was shown to internalize via CME in mammalian cells by recruiting clathrin (Veiga et al. 2005, 2007). Additionally, CCPs have been shown to assemble underneath the bound virus particles in the entry of influenza, reo- and Semliki Forest viruses (Ehrlich et al. 2004, Marsh & Helenius 2006, Rust et al. 2004).

2.2.1.2 Caveolin-mediated Endocytosis

Uptake of various endogenous plasma membrane molecules, including signaling receptors, sphingolipids, bacterial toxins, endothelin, growth

hormone, autocrine motility factor, albumin, folic acid, alkaline phosphatase and glycosylphosphatidylinositol (GPI)-linked proteins, occurs via caveolin-mediated endocytosis (reviewed in Nabi & Le 2003, Nichols 2003, Pelkmans & Helenius 2003; Table 1). Caveolae, with a diameter of 50-90 nm, are lipid-rich, non-coated, smooth, flask-shaped vesicles of the plasma membrane, which are frequently described in several cell types, particularly endothelial cells (Bishop 1997, Hewlett et al. 1994, Pelkmans & Helenius 2003). The shape and structural organization of caveolae are conferred by caveolin-1, a dimeric protein that inserts a loop into the inner leaflet of the plasma membrane and self-associates to form a striated caveolin coat on the surface of the membrane invaginations. Two other caveolin proteins also exist, caveolin-2, which often associates with caveolin-1, and caveolin-3, which is expressed predominantly in skeletal and cardiac muscle (Parton & Richards 2003). Unlike CME, caveolae endocytosis is a triggered event with activation of phosphorylation cascade (Pelkmans & Helenius 2003). Dynamin is required for the further vesicle budding of endocytic caveolar carriers, which can fuse with caveosomes, peripheral organelles with neutral luminal pH, or with endosomes (Henley et al. 1998, Oh et al. 1998, Pelkmans et al. 2002). Endocytic caveolar carriers may also fuse back to the cell membrane without other endosomal intermediates (Parton & Simons 2007). Depolymerization of cortical actin is required for closure of the caveolae and its further transport to the cytosol (Pelkmans & Helenius 2003). From caveosomes, the cargo can be further transported to endoplasmic reticulum, Golgi apparatus or endosomes (Parton & Simons 2007).

Caveolin-mediated endocytosis has been shown to be inhibited by kinase inhibitors, cholesterol depletion and disruption of actin cytoskeleton (Nabi & Le 2003). The route has also been demonstrated to be involved in the uptake of Simian virus type 40 (SV40; Kartenbeck et al. 1989, Pelkmans et al. 2002), Echovirus I (Karjalainen et al. 2008, Pietiäinen et al. 2004), Polyoma virus (Richterova et al. 2001), Respiratory syncytial virus (Werling et al. 1999), influenza virus (Nunes-Correia et al. 2004), Coxsackie B virus (Coyne & Bergelson 2006), as well as mediating the transcytosis of HIV-1 (Campbell et al. 2001) and the entry of FimH-positive strains of *E. coli* (Baorto et al. 1997) and *Chlamydia trachomatis* (Norkin et al. 2001).

2.2.1.3 Clathrin- and Caveolin-independent Pathways

Until recent years, clathrin- and caveolin-independent pathways were difficult to study due to lack of identifiable markers. Common characteristics of these routes have been outlined by the absence of coats and adaptor proteins in the entry and the dependence/independence of their vesicle fission on dynamin. Although somewhat controversial, these pathways have also been classified according to their usage of other cellular regulators such as actin regulating ArfGTPase Arf6 and RhoGTPases RhoA and Cdc42 (Table 1; reviewed in Donaldson 2003, Ridley 2006, Mayor & Pagano 2007). These GTPases serve as molecular switches cycling between two conformational states; an active GTP-bound and an inactive GDP-bound forms.

For cargo selection in clathrin- and caveolin-independent endocytosis multiple suggestions have been presented, including the association of the cargo with plasma membrane lipid domains (Fivaz et al. 2002), clustering of lipid-tethered cargo proteins (Sharma et al. 2004) and specific internalization sequences in the cytoplasmic tails of cargo proteins (Arnaoutova et al. 2003). The uptake of lipid-raft associated interleukin-2 (IL-2) and also γc-cytokine receptors have been shown to be mediated via a RhoA-regulated, dynamin-dependent, clathrin- and caveolin-independent mechanism (Mayor & Pagano 2007). GPI-APs have been suggested to utilize the Cdc42-dependent, dynamin-independent pathway leading to glycophosphatidyl-inositol-anchored protein enrinched endosomes (GEECs). Furthermore, Carboxypeptidase E has been shown to internalize via an Arf6-regulated, dynamin-independent pathway (Arnaoutova et al. 2003) and the recently found flotillin-1 via a Cdc42- and/or Arf6-regulated, dynamin-independent route (Glebov et al. 2006). The clathrin- and caveolin-independent pathways have also been proposed e.g. in the uptake of bacterial cholera toxin B, ricin and *Helicobacter pylori* vacuolating toxin (Mayor & Pagano 2007), as well as in the entry of SV40 into cells devoid of caveolae (Damm et al. 2005) and influenzavirus (Sieczkarski et al. 2002).

2.2.1.4 Macropinocytosis

Actin-mediated cell membrane ruffling frequently leads to the formation of large, heterogeneous and dynamic vesicular structures called macropinosomes (0.5-5 μm; Bishop 1997, Hewlett et al. 1994). Macropinocytosis is a form of fluid-phase endocytosis, utilized in efficient, non-selective uptake of extracellular solute macromolecules e.g. in epithelial cells, fibroblasts, neutrophils and macrophages (Nichols & Lippincott-Schwartz 2001, Swanson & Watts 1995). Circular ruffles on the cell membrane, which are formed by inward curving of peripheral ruffles, are suggested to be precursors of forming macropinosomes.

Unlike phagocytosis, cellular ruffles in macropinocytosis can form without guidance of any particle surface (Araki et al. 2003). However, they are both dependent of actin, RhoGTPases Rac1 and Cdc42 and Arf6 (Nobes & Marsh 2000, Radhakrishna et al. 1999, West et al. 2000). Macropinocytosis also requires p21-kinase Pak1, phosphoinositide 3-kinase, phospholipase C and Rab34 GTPase (Dharmawardhane et al. 2000, Jones 2007). Uncoated macropinosomes do not concentrate receptors and they are capable of fusing with each other (Racoosin et al. 1992). The fate of these large endocytic vesicles, however, appears to vary within a cell type, being dependent on the type of receptors in the engaged particle (Falkow et al. 1992, Hewlett et al. 1994). Vesicles may recycle back to plasma mebrane or decrease in size, acidify and eventually fuse with lysosomes (Bishop 1997). Maturation of macropinosomes occurs by recruitment of late endosomal and lysosomal markers, such as rab7 and lysosomal glycoprotein A, into vesicles (Racoosin & Swanson 1993).

In macrophages and dendritic cells, macropinocytosis is utilized in major histocombatibility complex class I and II antigen presentation (Norbury et al. 1995, Nobes & Marsh 2000). Macropinocytosis can also be transiently induced

by growth factors, phorbol esters or mitogenic agents in most cell types (Johannes & Lamaze 2002). Bacteria, such as *Shigella flexneri*, *Salmonella typhimurium*, *Haemophilus influenzae*, as well as Vaccinia and adenoviruses have been shown to rely on macropinocytosis for their entry (Francis et al. 1993, Ketterer et al. 1999, Meier et al. 2002, Mercer et al. 2008, Nhieu et al 1999). Evidence also implicates a role for macropinosomes in the uptake of HIV-1 and Epstein-Barr virus (Maréchal et al. 2001, Miller & Hutt-Fletcher, 1992).

2.2.2 Phagocytosis

Phagocytosis, which refers to recognition and engulfment of large (>0.5 µm diameter) particles into cells, is the most prominent in specialized mammalian cells, such as macrophages, monocytes and neutrophils (Conner & Schmid 2003). Phagocytosis is an active, receptor-mediated, and highly regulated process involving Rho-GTPase mediated signaling cascades, which lead to reorganization of filamentous actin (Klasse et al. 1998, Conner & Schmid 2003). Internalized particles may be directly recognized by receptors, but frequently recognition is mediated by opsonins e.g. antibodies, which coat the particles and bind to specific receptors (Rabinovitch 1995). After engulfment, phagocytosis further leads to formation of phagosomes, which depending on the nature of the phagocytic cell and the ingested particle, have been suggested to obtain membranes from plasma membrane, early endosomes or endoplasmic reticulum (Stuart & Ezekowitz 2005).

Phagosomes fuse with endosomes and lysosomes and mature to phagolysosomes, which are acidic, rich in hydrolytic enzymes, vATPases and anti-microbial agents (Klasse et al. 1998, Vieira et al. 2002). The factors mediating the maturation process are so far poorly characterized, however, a role for Rab GTPases and phosphatidylinositol 3-kinases have been suggested (Vieira et al. 2001, 2002). Rab5 proteins have been shown in early phagosomes and Rab7 proteins in later stages of maturation process (Desjardins et al. 1994, Duclos et al. 2000). Additionally, Rab2, Rab3, Rab10, Rab1 and Rab14 proteins have been identified as components of the phagosomal membrane (Garin et al. 2001).

Multiple modes of phagocytosis exist, which are determined by the ingested cargo and the recognizing receptor (Conner & Schmid 2003) and are controlled by different Rho GTPases. In macrophages type I phagocytosis, used e.g. by the immunoglobulin receptor, activation of Cdc42 and Rac1 is required, whereas RhoA activation has been linked to complement-activated type II phagocytosis (Caron et al. 1998). Rho induces assembly of contractile actin-myosin filaments, whereas Rac and Cdc42 mediate actin reorganization into cellular protrusions (Nobes & Hall 1995). Notably, phagocytosis is not restricted to "professional phagocytes", since cell types including dendritic cells, polymorphonuclear leucocytes, fibroblasts and epithelial cells, are able to engulf material by a phagocytic mechanism (Niedergang et al. 2005, Rabinovitch 1995). These "non-professional phagocytes" have, however, more limited range of uptaken particles due to their lack of the efficient phagocytic

receptors. Unlike professional phagocytes they also do not produce microbicidal reductive products in response to particle uptake or secrete all identical cytokines (Rabinovitch 1995).

Numerous phagocytic tracers, such as latex beads, opsonized or denaturated erythrocytes, yeast derivatives or killed/live bacteria exist (Rabinovitch 1995). Phosphoinositide 3-kinase inhibitors, wortmannin and LY294002, have been demonstrated to inhibit the closure of phagosomes in type I phagocytosis (Araki et al. 1996). Phagocytosis has been proposed to be strictly ATP dependent and can be blocked by tyrosine kinase and actin inhibitors (Klasse et al. 1998). Additionally, cholesterol, as well as myosins I, II, V, IX and X have been shown to be present in maturing phagosomes (Diakonova et al. 2002). The role of dynamins in phagocytosis has been controversial (Tse et al. 2003). In macrophages, dynamin2 has been reported to be required for formation of membrane extension around the ingested particle (Gold et al. 1999). However, in the phagosomal entry of *Chlamydia trachomatis* into epithelial cells, dynamin1 was not involved (Boleti et al. 1999).

Phagocytosis is crucial in clearing of apoptotic cells in inflammation, tissue damage and during development (Conner & Schmid 2003). Paradoxically, phagocytosis is also a common mechanism for microorganisms to invade host cells, either by blocking the phagosomal maturation or by escaping them, and thus avoiding destruction by serum antibodies and complement system (Rabinovitch 1995). Several other bacteria such as *Salmonella, Listeria* and *Shigella* have been shown to utilize phagocytosis as an entry pathway to non-professional phagocytes (reviewed in Pizarro-Cerda & Cossart 2006). These entry pathways may be mediated via fibronectin or laminin receptors or by heparan sulfates (Rabinovitch 1995). In macrophages, *Leishmania major* and *Mycobacterium leprae* have been suggested to utilize type II phagocytosis in their invasion (Caron et al. 1998). The role of phagocytosis in virus entry has not been clear. Influenzavirus has been seen in vesicles reminiscent of phagosomes, and Equine herpesvirus 1, as well as mimivirus has been suggested to enter cells via phagocytosis (Frampton et al. 2007, Ghigo et al. 2008, reviewed in Klasse et al. 1998). Additionally, Herpes Simplex virus type 1 (HSV-1) has been demonstrated to use RhoA-regulated, phagocytosis-like uptake (Clement et al. 2006).

2.3 Nuclear Compartmentalization in Mammalian Cells

Nuclear envelope is one of the most efficient barriers for successful gene delivery. Nuclear pore complex has been shown to allow free diffusion of 10 nm-sized molecules and active transport of 39 nm-sized particles (Pante et al. 2002, Stewart 2007). Nuclear localization of viruses and cellular proteins are mediated by specific nuclear localization signals (NLSs) recognized by cytosolic importins, which further translocate their cargo through nuclear pore complex (Matera 1999). Non-viral vectors instead lack the natural nuclear delivery

mechanisms. Virus-derived, synthetic or cellular DNA-binding proteins containing NLS signals have been utilized to carry gene therapy vectors into the nucleus (Pouton et al. 2007).

Nuclear architecture of the mammalian cell consists of highly organized and dynamic substructures (Fig. 2). In addition to chromatin and nucleoli, the nucleoplasm contains numerous nuclear compartments, such as Cajal bodies (CBs), splicing speckles and promyelocytic leukaemia bodies (PML NBs). In contrast to cytoplasmic organelles, nuclear bodies do not have surrounding membranes. High local concentration of specific nuclear factors is, however, characteristic of these compartments, possibly regulating the access of enzymes or receptors to their substrates and physically separating active and inactive factors (Lamond & Sleeman, 2003). Thus far, the classification of some of the nuclear bodies is not definitive and may overlap, as many nuclear factors are able to move between the structures.

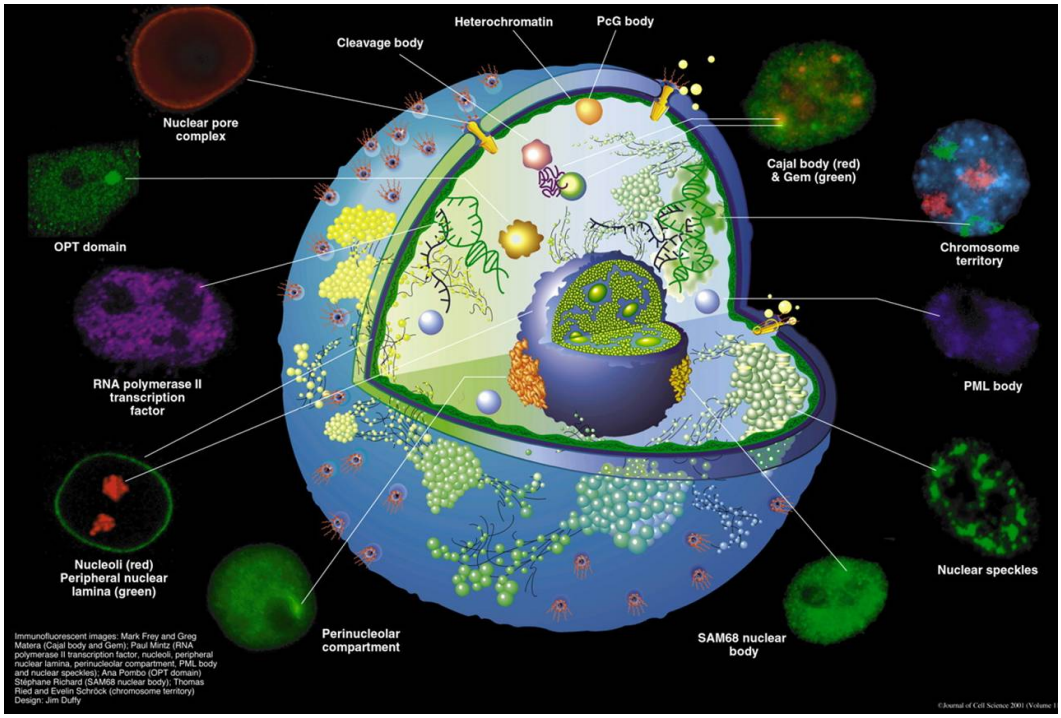

FIGURE 2 Nuclear compartments in mammalian cells (Spector 2001). Nucleus is bound by nuclear envelope and besides chromatin contains numerous nuclear bodies (diameters 0.1-5 µm). See text for details (2.3.1). Reproduced with permission of the Company of Biologists, copyright 2001.

Many viruses, such as herpesvirus, adenovirus, and papovavirus have been shown to interact with nuclear components and subsequently induce alterations in the nuclear architecture during their infection (Carvalho et al. 1995, Everett & Maul 1994, Guccione et al. 2004). Nuclear localization of gene therapy vectors and their effects on the transgene expression efficiency and stability is not well

defined. Next chapters outline the generally observed nuclear bodies, chromatin territories and their function in mammalian cells.

2.3.1 Subnuclear Structures

Most evident nuclear body in interchromatin space is the nucleolus (0.5-5.0 μm, 1-5/cell, Table 2), a dynamic substructure, which is involved in the assembly of ribosomal subunits and in rRNA synthesis and processing (Spector 2001). The structure contains three layered regions: inner fibrillar centers, dense fibrillar component and outer granular region. Approximately 700 proteins have been identified in purified nucleoli, 30% of which are encoded by previously uncharacterized genes. Nucleolus has been shown to be surrounded by heterochromatin and interact with Cajal bodies (Lamond & Sleeman 2003, Thiry & Lafontaine 2005). Association of nucleolus with perinucleolar compartment and Sam68 bodies, frequently located near nucleolus, has also been reported (Lamond & Sleeman, 2003). Nucleolus has been suggested to function in DNA damage repair, RNA editing, telomere metabolism, tRNA processing and regulation of protein stability (Lam et al. 2005).

TABLE 2 Nuclear compartments in mammalian cells.

Nuclear Substructure	Size/Quantity per Nucleus	Composition
Nucleolus	0.5-5.0 μm/1-5	rRNA, >700 identified proteins
Perinucleolar compartment	0.25-1.0 μm/1-10	hnRNPs, RNA binding proteins
SAM68 nuclear body*	0.25-1.0 μm/1-10	RNA binding proteins
PML NB	0.2-1.0 μm/5-30	constitutive residents: PML and sp100 proteins; various transiently localized components
Nuclear speckles	<1.8 μm/20-50	pre-mRNA splicing factors
Paraspeckles	N/A	RNA binding protein, paraspeckle protein 1 and 2
Cajal body	0.1-2 μm/1-10	small nuclear and nucleolar RNPs, coilin, SMN
Gems	0.2-0.4 μm/N/A	small nuclear RNPs, SMN, gemin2
Cleavage bodies	0.3-1.0 μm/1-4	RNA cleavage factors
OPT domain	1.0-1.5 μm/1-3	transcription factors
PcG body	0.2-1.5 μm/>2	polycomb group proteins

Abbreviations: RNP, ribonucleoprotein particle; PML NB, promyelocytic leukaemia nuclear bodies; N/A; not available; SMN, survival of motor neuron protein; OPT, Oct1/PTF/transcription domain; PcG, polycomb group. Sources: Dellaire & Bazett-Jones, 2004, Lamond & Spector, 2003, Ogg & Lamond, 2002, Spector 2001, Fox et al. 2002.

PML NBs (0.2-1.0 μm, 5-30/cell) are identified in nearly all cell lines and are suggested to be involved in growth suppression, transcription, cell cycle control, apoptosis, DNA repair and interferon-mediated cell defense (Borden

2002, Dellaire & Bazett-Jones 2004, Maul et al. 2000). Diseases such as promyelocytic leukaemia and hepatocellular carcinoma have been demonstrated to cause disruption of PML NB morphology (Terris et al. 1995, Weis et al. 1994). PML NBs are also modulated during stress and virus infection (Stensdorf et al. 1997). Two constitutive residents of these structures, PML and sp100 proteins have been shown to be covalently modified by the small ubiquitin-like protein SUMO-1, being important for their accumulation into PML NBs (Dellaire & Bazett-Jones, 2004, Duprez et al. 1999, Stensdorf et al. 1997).

Nuclear speckles are irregularly shaped (varying size; <1.8 μm, 20-50/cell) and scattered, dynamic structures that are enriched with various transcription and splicing factors. The structures are involved in mRNA transcription, mRNA maturation and nuclear export (Carter et al. 1993, Lamond & Spector 2003, Xing et al. 1995). Speckles are often found in the interchromatin regions close to the actively transcribing genes (Spector 2001, Lamond & Sleeman 2003). Changes in the shape of the structure occur in interphase as splicing factors move between speckles and the site of gene transcription, and in mitotis, in which speckles disassemble and reform (Lamond & Sleeman 2003).

CBs are relatively large nuclear structures (0.1-2 μm, 0-10/cell), which participate in RNA biogenesis, RNP maturation and assembly of spliceosomal subcomplexes (Ogg & Lamond 2002). CBs have suggested to deliver newly assembled factors for nucleoli and nuclear speckles (Spector 2001). CBs contain coilin proteins and most of them have also the survival of motor neurons (SMN) protein, which gene defect causes spinal muscular atrophy (Lamond & Sleeman 2003). Cleavage bodies, which operate in cleavage and polyadenylation steps of pre-mRNA processing, either overlap or are localized adjacent to CBs having newly synthesized RNA (0.3-1.0 μm, 1-4/cell). Additionally, gems, a nuclear substructure involved in assembly and/or maturation of small nuclear RNPs has been found adjacent to CBs (Spector 2001).

Other nuclear substructures include for example paraspeckles, which contain RNA binding nuclear and paraspeckle proteins; PcG domains, consisting of chromatin-associated polycomb group proteins; and Oct1/PTF/transcription domains, which have suggested to facilitate the gene expression (Fox et al. 2002, Pombo et al. 1998, Zimber et al. 2004). Also clastosomes, which have suggested to function as the nuclear sites for ubiquitin-mediated protein degradation have been identified (Lafarga et al. 2002).

2.3.2 Chromosome Territories

In addition to nuclear substructures, the nuclear volume of mammalian cells is crowded by genome, which is packaged in DNA-histone complexes i.e. the chromatin. Within nucleoplasm, chromosomes consisting of chromatin fibers (10-30 nm) are arranged into chromosome territories. Each territory extends from the gene-rich interior to more gene-poor peripheral space, and has a predominantly distinct interchromatin space (Cremer 2001). Chromosomes

have two chromatids, consisiting of telomeres (end of the chromatid), centromere (the joining portion of the two chromatids) and chromatid arms.

Each chromosome can have regions with different level of compaction, the loosely packed, active euchromatin and more condensed, inactive heterochromatin. Generally, the tightly packed heterochromatin is frequently seen lining the inner nuclear membrane, and is associated with the nuclear lamina and surrounds nucleoli. In contrast, the euchromatin is located in the interior of the nucleoplasm. Heterochromatin tends to replicate later in cell cycle than euchromatin. It also represses gene transcription, suppresses recombination, and is responsible for long-range chromatin interactions and maintenance of genomic integrity (Grewal & Elgin 2007).

Nucleosomes, the subunits of chromatin, are composed of four histone core proteins (H2A, H2B, H3 and H4). Their post-translational modification has previously been linked with large chromatin structure changes (Lachner & Jenuwein 2002). Two chromatin remodelling protein complexes have been established; ATP-dependent and histone modifying enzyme complexes. ATP-dependent complexes use the energy of ATP hydrolysis to change localization or stabilization of the nucleosome (Fan et al. 2003). Histone modifying enzymes instead regulate various post-translational modifications, including acetylation, phosphorylation, methylation and ubiquitination of the histones (Fischle et al. 2003). In addition to chromatin changes during transcription, chromatin condensation and H2B phosphorylation has been demonstrated in apoptotic cells (Ajiro 2000, Studitsky et al. 2004).

DNA viruses have been shown to marginalize the host cell chromatin and disaggregate the heterochromatin lining the nucleoli prior to their transcription and replication (Monier et al. 2000). Additionally, nuclear enlargement and nuclear lamina disruption has been demonstrated for instance in HSV-1 infection (Simpson-Holley et al. 2005). Also the insect infecting baculoviruses, outlined in next chapter, have been shown to alter the host chromatin by unknown mechanisms during their replication (Granados et al. 1981, O´Reilly et al. 1994).

2.4 Baculovirus Vectors

Baculovirus vectors derived from *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV, *Baculoviridae*) have emerged a promising vector for gene delivery into mammalian cells of different origin, specifically human hepatocytes (Hofmann et al. 1995). Baculoviruses are rod-shaped (~54-68 nm width x ~256-266 nm length), enveloped, double-stranded DNA (134 kb, 154 predicted genes) viruses that replicate only in arthropods, mainly in insects (*Lepidoptera*; Blissard, & Rohrmann 1990, Ihalainen, Laakkonen et al. unpublished; Transfiguracion et al. 2007). In addition to contributing to gene transfer and the production of various proteins, baculoviruses act as pesticides (Kost, Condreay & Jarvis 2005; Szewczyk et al. 2006). Baculoviruses are also

utilized in vaccine production, including the commercially available Cervarix (GlaxoSmitheKline Plc) against human papillomaviruses and FluBlok (Protein Sciences Corporation) against influenza. In next chapters, the virion structure, infection and transduction routes, as well as baculovirus-mediated gene transfer, are outlined.

2.4.1 Infection *in vitro* and *in vivo*

Baculoviruses can produce two virion phenotypes, budded virus (BV) and occlusion-derived virus (ODV), which are structurally and functionally distinct and produced at different locations in the cell, at different times in the infection cycle (Blissard 1996). The ODV transmits infections from insect to insect, whereas the BV spreads the infection from cell to cell within an infected insect (Lanier & Volkman 1998). However, the two viral forms have identical DNA, and differ only in their protein composition (Blissard & Rohrmann 1990). Both forms of baculoviruses have a major capsid protein vp39, minor capsid proteins p80, p24 and pp78/83 as well as DNA-binding protein p6.9, but BV alone has a major envelope glycoprotein gp64 (Funk et al. 1999). The phenotypes also differ in source of viral envelopes, relative infectivity to cultured insect cells and cell types infected in the insect (Blissard 1990).

The ODVs, also called polyhedra, are enveloped nucleocapsids embedded in a crystalline protein matrix, polyhedrin. As the insect larvae ingest the viruses as a contaminant of its food, the polyhedrin matrix is solubilized in the alkaline midgut of the insects, releasing embedded virions. In this primary phase of infection, ODV virions enter the midgut cells by plasma membrane fusion of microvilli in a highly alkaline environment. Infection results in BV production and virus spread via the hemolymph to the other tissues of the insect such as hemocytes, hypodermis, and fat body cells (Volkman 1997). The gp64 protein is required for propagation of the budded virus from cell to cell and for systemic infection. Both BV and ODV are produced in most tissues during this secondary phase. Finally, after five to seven days, the virus destroys the larvae and the ODVs are released into the environment (O´Reilly et al. 1994). The structure of BV, the most used phenotype of the virus in biotechnological applications, is presented in figure 3.

Kinetics of the BV infection has been characterized using *Spodoptera frugiperda* (*Sf*) cell lines. Viruses have been proposed to enter insect cells via adsorptive endocytosis (Volkman & Goldsmith 1985, Wang et al. 1997). The involvement of bivalent cations (Wang et al. 1997), phosphatidic acid and phosphatidylinositol of the cell membrane on the viral attachement has been suggested (Tani et al. 2001). Additionally, the role of baculoviral membrane protein gp64 in viral binding has been demonstrated (e.g. Hefferon et al. 1999, Jarvis et al. 1994, Oomens et al. 1999, Zhou et al. 2008). The uptake of virion on the endocytic vesicles have shown to occur at 10-20 min post infection (p.i.; Blissard 1996). Recently, chlorpromazine, CME inhibitor, was shown to block the baculovirus infection (Long et al. 2007).

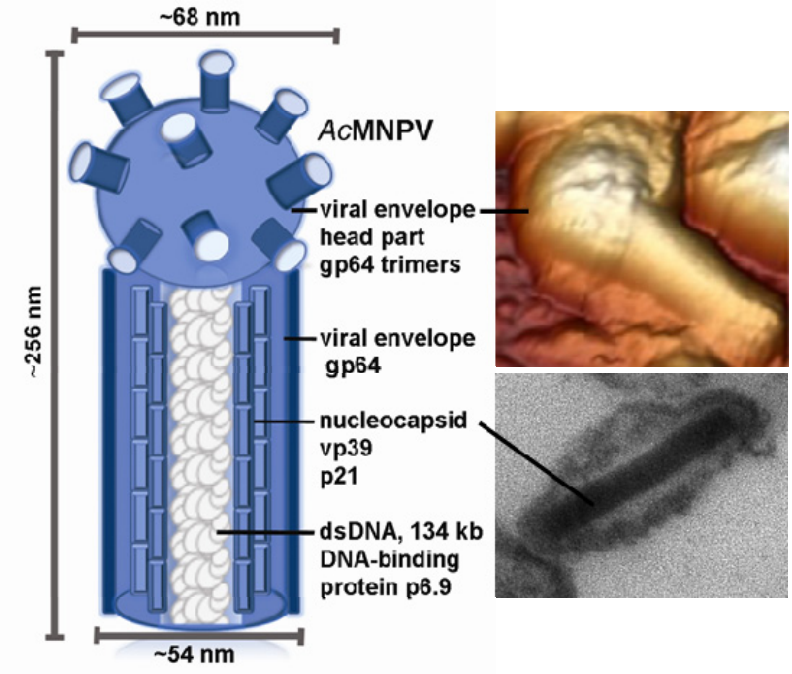

FIGURE 3 Schematic structure of the budded form of the *Ac*MNPV-baculovirus (Laakkonen, 2008). Atomic force microscopy (provided by T. Ihalainen) and electron microscopy images (provided by M. Salminen) of the enveloped viruses are presented. The diameters of the virus are based on atomic force microscopy measurements (Ihalainen, Laakkonen et al. unpublished).

After uptake, the viral gp64 protein mediates the acid-induced endosomal escape of the viruses at 15-30 min p.i. (Hefferon et al. 1999, Kingsley et al. 1999, Tani et al. 2001). In the cytoplasm, the nucleocapsid further induces formation of cytoplasmic actin filaments and cytoplasmic trafficking towards the nucleus (Charlton et al. 1993). Finally, nucleocapsids interact with the nuclear pore (0.5-4 h p.i.), enter the nucleus and uncoat (Granados et al. 1981, Wilson & Price 1988). Viral mRNA has been detected as early as 30 min p.i., indicating rapid transcription of viral immediate early genes in infected cells (Friesen 1997).

For replication in *Spodoptera frugiperda* cell culture, infectious cycle of the virus has been considered to occur in three basic phases; early, late and very late. In early phase (0-6 h p.i.) infected cells undergo significant changes, such as cytoskeletal rearrangements, host chromatin dispersion within the nucleus and nuclear enlargement, which all precede viral DNA replication (Granados et al. 1981). Four early viral gene expression proteins have been identified, namely IE-0, IE-1, IE-2 and PE-38. IE-1, the principal transregulator of transcription, activates various early gene promoters, some of which are required for viral replication (reviewed in Friesen, 1997). The following late phase (6-20 h p.i.) is a period of extensive viral DNA replication, late gene expression, and BV production. In the nucleus, an electron-dense structure known as the virogenic stroma forms, in which the capsid sheat assembles and is filled with a nucleoprotein core (Blissard 1996, O´Reilly et al. 1994). During the assembly, filamentous actin colocalizes with the capsid protein p39 within nuclei, being a

unique feature among intracellular pathogens (Charlton & Volkman 1993, Goley et al. 2006, Kasman & Volkman 2000, Ohkawa et al. 2002, Ohkawa and Volkman 1999). Progeny BV nucleocapsids have suggested to egress further from the nucleus by budding through nuclear membrane and travel to the cell membrane in double-membrane transport vesicles. *En route* to cell membrane, however, BVs have suggested to loose these membranes, since intact nucleocapsids have seen to bud from the cell membrane regions having trimeric forms of gp64 (Jarvis et al. 1998, Tani et al. 2001, reviewed in Williams and Faulkner, 1997). Finally, in the very late phase of infection (>20 h p.i.), by unidentified mechanisms, virions start their intranuclear maturation i.e. ODV production. In the nucleus, ODVs are enveloped by membrane envelope segments either individually or in groups. After nucleus is filled with ODVs, a fibrous protein p10 begins to accumulate in cell, having a role in further cytolysis and controlled disintegration of larvae. See reviews of Blissard 1996, Okano et al. 2006 and Volkman 1997 for more comprehensive details of the infection cycle.

2.4.2 Transduction in Mammalian Cells

Baculovirus transduces efficiently various mammalian cell lines *in vitro*, leading to stable gene expression (Carbonell et al. 1985, Hoffman et al. 1995). The first successful transduction studies were performed in human hepatoma Huh7, HepG2 cells and primary hepatocytes with luciferase transgene under cytomegalovirus (CMV) or Rous sarcoma virus (RSV) promoters (Boyce & Bucher 1996, Hofmann et al 1995). Later, high-levels of expression were also shown in human hepatic stellate cells and hepatoma FLC4 cells (reviewed in Hu et al. 2006). Various other human cell lines have also been proven to be efficient gene transfer targets for baculovirus vectors, e.g. human epithelial kidney (HEK-293), human cervix carcinoma (HeLa), human neuroblastoma (SHSY-5Y), breast carcinoma (BGC-223), prostate cancer (PC3) and bone marrow derived mesenchymal stem cells, just to mention a few (see reviews of Hu 2006, Kost and Condreay 2002 for extensive list of cells).

Analogous to insect cells, cell surface molecule(s) that interacts with baculovirus during uptake mammalian cells are not known. Involvement of widely distributed and heterogeneous cell surface motifs has been suggested in viral binding including heparan sulfate, phospholipids and electrostatic charges (Duisit et al. 1999, Shoji et al. 1997, Tani et al. 2001). Also the role for viral protein gp64 in binding has been suggested (Westenberg et al. 2007). Additionally, baculovirus transduction of primary hepatocytes has proposed to require contact with the basolateral surface (Bilello et al. 2001). However, detailed mechanisms of the mode and kinetics of entry have not been revealed. The inhibition studies with chloroquine, bafilomycin A1 and ammonium chloride, led to hypothesis that an endocytic pathway was involved (Pieroni et. al 2001, van Loo et al. 2001). In pig kidney epithelial cells and later in HepG2 cells, acid-induced endosomal escape was shown to occur at 1 h p.t., leading to uncoating of the virus (Mäkelä et al. 2008, van Loo et al. 2001). Importance of

EEs in baculovirus transduction were demonstrated in HepG2 and endothelial cell lines (EaHy) by colocalization of the virus capsid protein with early endosome antigen marker at 30 min p.t. Transduction was also shown to be inhibited in monensin-treated cells, which causes EE acidification (Kukkonen et al. 2003). Fluorescent labeling of REs with rab11 antibody, LEs and lysosomes with CD-63 antibody showed no colocalization with the viral capsid at 24 h p.t in EaHy cells (Kukkonen et al. 2003).

In further cytoplasmic transport of nucleocapsids, the involvement of actin filaments was suggested due to inhibition studies with actin disrupting agents (Salminen et al. 2005). As in insect cells, viruses are suggested to enter the nucleus through nuclear pores (Granados et al. 1981, van Loo et al. 2001, Wilson & Price 1988). Unlabelled as well as capsid-labelled viruses have previously been detected in the nucleus of HepG2 cells at 4-6 h p.t. (Kukkonen et al. 2003, Salminen et al. 2005). Interestingly, no nuclear localization of viruses was detected in EaHy or MG-63 human osteosarcoma cells, known to lead to unefficient baculovirus-mediated transgene expression (Kukkonen et al. 2003).

2.4.3 Biomedical Applications – Gene Transfer

Baculoviruses have many advantages as a gene delivery vector. First of all, they are incapable to replicate in mammalian cells (Volkman & Goldsmith 1983), thereby avoiding any risk of outbreak of a replication-competent virus in clinical trials. Additionally, they are non-toxic and non-allergic *in vivo* and are classified into the lowest biohazard level according to long-term carcinogenicity and teratogenicity tests in various mammalian species, including humans (reviewed in Airenne et al. 2008, Kost & Condreay 2002). Moreover, baculovirus vectors are able to transduce both dividing and non-dividing mammalian cells (Hofmann et al. 1995, van Loo et al. 2001). They are also easy to produce, and can accommodate large inserts enabling multigene strategies (Davies 1994, Hartley et al. 2007, O´Reilly et al. 1994).

Targeting of baculovirus into specific cellular receptors via expressing foreign proteins or peptides on the viral membrane, i.e. via baculovirus display system, has been examined in many *in vitro* studies. Typically, viral glycoprotein gp64 surface display strategy has been used, in which the foreign protein is attached to the copy of full gp64, a type I integral membrane protein, or to its membrane anchor region (reviewed in Oker-Blom et al. 2003). Alternatively, vesicular stomatitis virus G transmembrane glycoprotein (VSV-G) or measles virus receptor CD46 mediated display strategies have been utilized, since they have shown to compensate the gp64 on the viral surface and produce infective virions in insect cells (Chapple et al. 2002, Kitagawa et al. 2005, Mangor et al. 2001). The targeting ligands, such as the mouse hepatitis virus S protein and extra copy of gp64 (Tani et al. 2001), avian influenza hemagglutinin (Yang et al. 2007), RGD/RKK-motifs (Matilainen et al. 2006, Riikonen et al. 2007), tumor-homing peptides (Mäkelä et al. 2006), single-chain antibody fragment and antibody binding protein (Mottershead et al. 2000), transmembrane of neuraminidase (Borg et al. 2004) and avidin (Räty et al. 2004)

have also been used. Recently, truncated VSV-G, lacking the cytotoxicity of VSV-G, was shown to produce high titers in insect cells and transduce mammalian cells efficiently *in vitro* and *in vivo* (Kaikkonen et al. 2005). For more comprehensive details of the baculovirus display strategies, please see reviews of Mäkelä and Oker-Blom (2007) and Oker-Blom, Airenne and Grabherr (2003).

Transient transgene expression of baculovirus vectors has shown to peak at 3-5 days after administration (Airenne et al. 2000, Lehtolainen et al. 2002). Typically, the transgene is situated under cytomegalovirus (CMV; Spenger et al. 2004) or chicken beta actin promoter (CAG; Shoji et al. 1997). Additionally, astrocyte- or neurone-specific promoters have been utilized in targeting transgene expression into specific cell types (Li et al. 2005, Wang et al. 2006). Various enhancers have been shown to boost the promoter activity, such as cytomegalovirus enhancer homologous region sequence of *Ac*MNPV, and woodchuck hepatitis virus post-transcriptional regulatory element (Mähönen et al. 2007, Ong et al. 2005, Li et al. 2005, Wang et al. 2006, Viwanatham et al. 2003). Transduction efficiency in various cells can be further enhanced by optimizing the cell culture conditions, medium types and its supplements (Airenne et al. 2008, Mähönen et al. 2007, Shen et al. 2007).

The first pre-clinical trials of baculovirus vectors were performed into liver parenchyma of the rat and mice (Hofmann et al. 1998, Sandig et al. 1996). Later, the vectors were, however, shown to be inactivated by the serum complement system, which represents the first-line host defense of the innate immune system (Hofmann et al. 1998). Additionally, baculovirus transduction caused interferon and cytokine production in cells, including IL-1α/β, IL-6 and tumor necrosis factor (TNF-α; Beck et al. 2000, Abe et al. 2003, 2005, Gronowski et al. 1999). Absence of serum (e.g. by collar device mediated delivery) or injections to the sites lacking the complement system, as well as pegylation of the vectors has shown to increase transduction efficiency in immunocompetent animals (Airenne et al. 2000, Hofmann et al. 1998, Kim et al. 2006). Additionally, enhanced transgene expression has been obeserved in liver parenchyma of neonatal Wistar rats by using decay-acceleration factor-displaying complement-resistant baculovirus (Hüser et al. 2001). Recently, biodistribution of baculoviruses in immune competent rats was demonstrated by single-photon emission computed tomography. Viruses were shown to spread via the lymphatic system and accumulate in the kidneys (Räty et al. 2007). Systemic delivery of baculovirus into complement deficient Neuro2a tumor-bearing A/J mice instead resulted in transgene expression primarily in liver, spleen, and kidneys (Kircheis et al. 2001).

Successful baculovirus-mediated transgene expression has been shown in intervertebral disc of rabbit, cerebral cortex and testis of mice, as well as in corneal endothelium, retinal pigment epithelial cells, lens and retina (Haeseleer et al. 2001, Liu et al. 2006, Tani et al. 2003; Table 3). In brains, baculovirus transduced efficiently the choroid plexus cells of BDIX and Wistar rats (Kaikkonen et al. 2006, Laitinen et al. 2005, Lehtolainen et al. 2002, Räty et al. 2006). Baculoviruses have also been shown to deliver short hairpin RNA into cells *in vitro* (Nicholson et al. 2005) and in rat brain *in vivo* (Ong et al. 2005).

Baculovirus hybrid vectors, incorporated with genes from adeno-associated virus, have been constructed to pro-long the transgene expression (Palombo et al. 1998). In C5-deficient immunocompromised mice injection into quadriceps femoris muscle resulted in sustained gene expression (~178 days; Pieroni et al. 2001). Notably, baculovirus-mediated transgene expression levels have been shown to be comparable with the efficiency of adenovirus vectors, yielding much lower cytotoxicity levels than adenovirus vectors with high virus doses (Airenne et al. 2000, Lehtolainen et al. 2002, Shoji et al. 1997). For comprehensive list of *in vivo* strategies applied in baculovirus vectorology, please see the review of Airenne et al. (2008).

TABLE 3 Baculovirus vectors *in vivo*.

Transduced Organs	Tissues/Cell Types *in vivo*
Eyes[1,2]	cornea/endothelial cells; lens/epithelium retina/pigment epithelial and inner nuclear layer cells, Müller cells, ganglion cells, photoreceptor cells
Brain[1,2]	striatum/astrocytes; choroid plexus/ cupoid epithelium cells; cerebral cortex/neurons; pyramidal cells; microvessels/endothelial cells; substantia nigra; visual cortex; lateral ventricle/epithelial cells; corpus callosum; ependymal layer; superior colliculus
Lungs[1,2]	not determined
Heart[1]	not determined
Liver[1,2]	hepatocytes, parenchymal cells
Kidneys[1,2]	tubular epithelial cells
Spleen[1,2]	not determined
Testis[1]	seminiferous tubule/Sertoli cells, spermatogenic cells
Muscle[3]	quadriceps femoris/myofibers, smooth muscle cells
Adventitia[3]	fibroblasts, smooth muscle cells

in vivo studies performed in [1]mice, [2] rats, [3] rabbit. Modified from Airenne et al. 2008.

2.5 Streptavidin-based Non-viral Vectors

Streptavidin is a tetrameric protein (60 kDa) derived from bacteria *Streptomyces avidinii*. It is known to possess one of the strongest non-covalent binding affinities with biotin vitamin (244 Da), having dissociation constant of ~10^{-14} mol/l (Green 1990; Fig. 4). The ease of functionalizing biomolecules via streptavidin-biotin system has created various biotechnological applications in diagnostics, biomolecular imaging, affinity separations and therapeutic delivery (Mcdevitt et al. 1999, Stayton et al. 2000, Wilbur et al. 1999, Yu et al. 2000). Cancer therapy has utilized streptavidin-biotin system by employing tumor-antigen binding to streptavidin-linked monoclonal antibodies. Currently, phase I and II clinical trials towards prostate cancer, metastatic colon cancer, malignant gliomas and non-Hodkin´s lymphomas are ongoing via systemic

delivery of streptavidin linked monoclonal antibody and radioactive 90Y-biotin (Forero et al. 2004, Goldenberg et al. 2006, Knox et al. 2000, Paganelli et al. 1999, Weiden & Breitz 2001). Streptavidin conjugates are also utilized to deliver toxins or imaging agents to tumor cells (reviewed in Boerman et al. 2003 and Stayton et al. 1999). In next chapters, the structure of streptavidin as well as its use as a gene transfer vector is outlined.

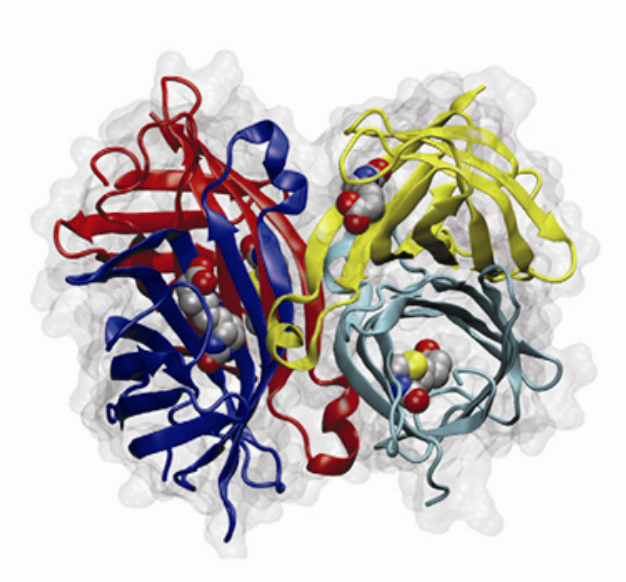

FIGURE 4 The x-ray christallography structure of the streptavidin tetramer (60 kDa; modified from Laitinen et al. 2007). The molecular surface (indicated as grey), four monomers (indicated as different colours), as well as four biotin binding sites (indicated as spheres) are presented. Reprinted by permission from Elsevier Ltd, copyright 2007.

2.5.1 Streptavidin – Structure and Function

Streptavidin-biotin complex has been extensively studied by rational site-directed mutagenesis and by biochemical and biophysical methods (Laitinen 2006, Wilchek 2006). Streptavidin (pI 6.04) shares a homologous structure to chicken avidin (pI 10.5), having similar quaternary structure with four identical polypeptide chains arranged as tetramer and similar relevant amino acids in its biotin-binding pockets. In comparison with avidin, streptavidin is not glycosylated and is devoid of cysteine or methionine containing amino acids (Green 1990). In bacteria, streptavidin is produced first as a preprotein (159 aa) and then cleaved proteolytically N- and C-terminally to a core form of 125-127 aa (Bayer et al. 1989, Pähler et al. 1987, Weber et al. 1989). Each streptavidin monomer (14-18 kDa) is a barrel of eight antiparallel β-strands, having six tryptophan residues per subunit (Chaiet & Wolf 1964, Hendrickson et al. 1989). Biotin-binding sites are located at end of each β-barrel subunit and are formed of hydrophobic aromatic residues Trp79, Trp92 and Trp108 from one subunit and Trp120 from the neighboring subunit. Hydrophilic residues, such as Ser45,

Asn49 and Ser88 form further hydrogen bonds with biotin (Chilkoti et al. 1995, Pazy et al. 2002, Pähler 1987).

Streptavidin has been shown to be very stable and functional at high temperatures, pH extremes, and in the presence of denaturants and proteolytic enzymes (Wilchek 2006). The natural function of streptavidin in bacteria is not well known but it has been suggested to have antibiotic properties (Chaiet & Wolf 1964). Recently, streptavidin with single biotin binding site was generated to enhance the delivery properties of biomolecules by preventing multimerization of the conjugates (Howarth et al. 2006). To gain more details of the streptavidin structure please see reviews of Laitinen *et al.* (2006) and Wilchek *et al.* (2006).

2.5.2 Biomedical Applications – Gene Transfer

Protease-resistant core streptavidin (125-127 aa, Sano et al. 1995) has been utilized to target and deliver variety of biotinylated biomolecules into mammalian cells, such as DNA and monoclonal antibodies (Garcia-Espana et al. 1999, Sato et al. 2000, Wilbur et al. 1998, Kalofonos et al. 1990). As a vector, streptavidin is very feasible due to its stability, non-cytotoxicity and ease large-scale production (up to 230mg/l, Humbert et al. 2007; Alon et al. 1993). It has, however, shown to be immunogenic (Knox et al. 2000, Weiden & Breitz 2001).

At the cellular level, internalization and delivery of streptavidin has not been defined in detail. Uptake has been proposed to occur via receptor-mediated endocytosis, involving lysine residues and RYD sequence (Arg-Tyr-Asp; Alon et al. 1993, Wilbur et al. 1998). In Balb/c mice intravenously injected intact streptavidin showed slow clearance from the bloodstream and high organ retention, having affinity to liver, spleen, kidney and bladder tissues. Protease-resistant core streptavidin, however, accumulated mainly into kidney (Schechter et al. 1990). Biodistribution in the kidneys has been reduced by pre-infusion of basic amino acids prior to core streptavidin injection or by succinylation/mutation of its lysine residues (Wilbur et al. 2004). Additionally, core streptavidin has successfully been targeted into hepatic cells of New Zealand white rabbits by adding galactose sugars to streptavidin (Rosebrough et al. 1996).

Synthetic or virus-derived peptides have been used to enhance the delivery of core streptavidin into mammalian cells (Albarran et al. 2005, Hussey et al. 2002). Cell penetrating peptide, TAT derived from HIV, was recently covalently linked into streptavidin and demonstrated to carry biotinylated alkaline phosphatase efficiently into human Jurkat T lymphocytes (Albarran et al. 2005). Transgene delivery via streptavidin-biotin system has also been demonstrated *in vitro* and *in vivo* (Table 4). For example successful targeting of metastasized k562 tumors of severe combined immunodeficient mice has been reported, in which transgenes were conjugated to transferrin by biotin-streptavidin linkage (Sato et al. 2000). In the same study, transduction efficiency of streptavidin-based system *in vitro* was also shown to be superior to

lipefection or retrovirus-mediated expression, being only slightly lower than expression with adenovirus vectors.

TABLE 4 Examples of Streptavidin-based Vectors in Gene Transfer.

Target Cells/Animal	Vector
HeLa, cervical carcinoma	DNA-streptavidin-biotinylated transferrin*
	streptavidin, poly-L-lysine-DNA*
A431, epidermoid carcinoma	EGF-PEG-biotin–streptavidin–PEI–DNA
	biotinylated DNA, streptavidin-TGFα-fusion
	biotinylated EGF-streptavidin, poly-L-lysine-DNA
Jurkat T cells,	CD3/CD19 Ab-streptavidin-PEI-DNA
Granta 519 cells (B cells)	
CPH 54A, small cell lung carcinoma	SLN-DNA-streptavidin"
HREC, retinal endothelial cells	biotinylated-TAP, streptavidin-coated nanoparticles
Huh7, human hepatoma	
Metastized k562 tumor/SCID mice	biotinylated DNA, streptavidin-transferrin

Abbreviations: PEG, polyethylene glycol; PEI, polyethylenimine; TGF, transforming growth factor; Ab, antibody; SLN, solid lipid nanoparticle; TAP, transcriptionally active PCR products; SCID, severe combined immunodeficiency. *high transduction efficiencies only in the presence of chloroquine; "no successful gene transfer observed.
Sources: Garcia-Espana et al. 2000; Lee et al. 2002; Pedersen et al. 2006, Prow et al. 2006, Sato et al. 2000; Schoeman et al. 1995, Xu et al. 1998, Guillem et al. 2002.

In addition to gene transfer, (strept)avidin-biotin interaction has been utilized in virus vector targeting with biotinylated baculoviruses, adenoviruses, vaccinia viruses, retroviruses and adeno-associated viruses (Kaikkonen et al. 2008, Pereboeva et al. 2007, Ponnazhagan et al. 2002, Purow et al. 2005, Zhong et al. 2001). Recently, biotinylated antisense oligonucleotides were successfully delivered into cells via streptavidin attached gold nanoparticles (Liu et al. 2008). Streptavidin-based applications are further presented in the reviews of Boerman et al. (2003) and Wilbur et al. (1999).

3 AIM OF THE STUDY

In this thesis, two potential gene therapy vectors were studied; insect infecting *Ac*MNPV-baculovirus and bacterial protein streptavidin-based vectors. Baculovirus, which has been shown to internalize efficiently into various mammalian cell lines and lead to stable transgene expression, has been hampered by serum complement system *in vivo*. To develop baculovirus-mediated gene transfer and transductional targeting, as well as to define the cellular mechanisms of the novel streptavidin-based non-viral vector, the objectives of the thesis were following:

1. To define the functional entry route of *Ac*MNPV-baculovirus in human cells.

2. To test the potentiality of novel TAT-streptavidin vector to internalize and deliver biotinylated cargos in human cells.

3. To determine the nuclear localization of *Ac*MNPV-baculovirus in human cells and evaluate its transcription capability, as well as its effects in the nuclei during transduction.

4 SUMMARY OF MATERIALS AND METHODS

The materials and methods are described in more detail in the corresponding original publications (I-IV).

4.1 Viruses (I, II, IV)

Wild type (wt), *Autographa californica* nucleopolyhedrovirus (AcMNPV, E2 strain) and recombinant baculoviruses were produced in *Spodoptera frugiperda* insect cells (*Sf*9; CRL 1711, ATCC) grown in suspension cultures at 27°C in HyQ®SFX-Insect medium (HyClone Inc). To prepare concentrated batches of the viruses, *Sf*9 cells were inoculated with wt or recombinant viruses at a multiplicity of infection (MOI) of 0.1. At four days p.i. the progeny viruses were collected from the insect cell medium followed by sucrose gradient centrifugations as previously described (Airenne et al. 2000). Virus titers gained from end-point dilution assay (O´Reilly 1994) acted as a reference point to the virus doses (MOI 80-1000) used in mammalian cell transductions. Recombinant viruses utilized in this thesis project are presented below (Table 5).

TABLE 5 Recombinant baculoviruses used in the thesis.

Virus	Displayed protein/ Fusion partner	Exp. Cassette	Reference/Thesis article	
vp39EGFP	EGFP/vp39	-	Kukkonen et al. 2003	I-II,IV
AcCMVEGFP	-/-	CMV-EGFP	Gilbert et al. 2005	I
Ac-luc	-/-	SV40-luc	Mäkelä et al. 2006	II
Ac-EGFP	-/-	CMV-EGFP	Mäkelä et al. 2008	II
AcVP39	-/-	SV40-luc and CMV-EGFP	Mäkelä et al. 2008	II
P24mCherry	mCherry/p24	CMV-dsred2	unpublished	II
BA-CAG-EGFP	-/-	CAG-EGFP	Mähönen et al. 2007	II, IV

4.2 TAT-Streptavidin and Biotinylated PPAA (III)

The design and construction of the TAT-streptavidin (SA 140 aa; $TAT_{47-57;}$ YGRKKRRQRRR) gene using core streptavidin in pUC18 plasmid and overhang primer polymerase chain reaction (PCR), as well as T7 expression system isolation, refolding and purification have previously been reported by Albarran *et al.* (2005, Fig. 5). TAT-SA (60 kDa) and SA (60 kDa) were labeled with Alexa-488 (A488) according to the manufacturer's protocol for amine-reactive probes (Molecular Probes).

Amine-terminated poly(propylacrylic acid) (PPAA, 11 kDa) synthetisation and end-biotinylation were described previously (Lackey et al. 1999, Murthy et al. 2003). The biotin-binding ability and stability of protein constructs were detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis, in which the samples were preheated to 22°C, 37°C or 68°C.

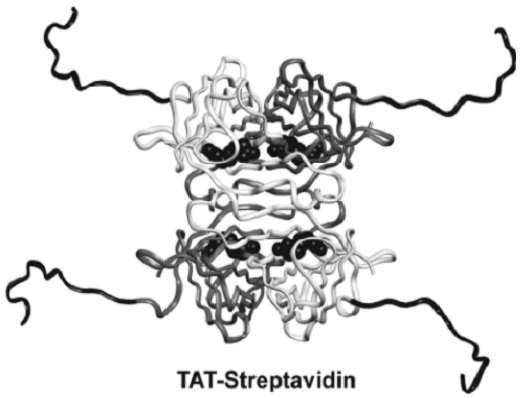

FIGURE 5 The schematic structure of TAT-SA (Albarran et al. 2005). Four streptavidin monomers (15 kDa/each) with N-terminal TAT_{47-57}-peptides, as well as biotin binding sites (indicated as spheres) are presented. Reprinted by permission from Oxford University Press, copyright 2005.

4.3 Transduction in Mammalian Cells (I-IV)

For baculovirus transduction studies, human hepatoma (HepG2, ATCC HB-8065; I-IV), human epithelial kidney (293, ATCC CRL-1573; III, IV) and human epithelial carcinoma (HeLa, III) cell lines were used. All human cells were cultured in monolayers in Minimum Essential Medium (MEM, HepG2 and 293; II-IV) or Dulbecco´s modified Eagle medium (DMEM, HepG2 and HeLa; I, III). After reaching the subconfluency (60-80%), the cells were transduced with appropriate baculovirus (MOI 80-1000) in medium containing 1% heat inactivated fetal calf serum (FCS) for 1 h at 4°C or 37°C followed by incubation in complete culture medium containing 10% FCS at 37°C.

For TAT-SA transduction studies (III), HeLa, HepG2, human lung carcinoma (A549, DMEM) and embryonic human lung (MRC-5, DMEM) cell lines were used. The cells were grown to subconfluency (60-80%) and transduced with TAT-SA, TAT-SA-A488 or SA-A488 (2μM). For the delivery of biotinylated molecules, TAT-SA-A488 was conjugated with Biotin-DY-633 (1–2 μM) and/or biotinylated-PPAA (4 μM, TAT-SA-PPAA).

4.4 Cellular Markers and Inhibitors (I-IV)

For transfection experiments (II-IV), electroporation (HepG2; IV) or lipofection (293/HeLa; II-IV) of appropriate plasmids were performed (Table 4). After transfections the cells were transduced with baculovirus, paraformaldehyde (PFA) fixed, immunolabeled and observed by confocal microscopy (see 4.6 for further information).

TABLE 6 Plasmids used in the thesis.

Plasmid	Expressed Protein/Tag	Provider	Article
Pak1T423E	DN Pak-1/myc	Dr. J. Chernoff	II
Pak1 AID	CA Pak1/FLAG	Dr. E. Manser	II
Cdc42 wt	wt Cdc42 GTPase/EGFP	Dr. L. Pelkmans	II
Cdc42 17N	DN Cdc42 GTPase/EGFP	Dr. J. Peränen	II
Cdc42 12V	CA Cdc42 GTPase /EGFP	Dr. J. Peränen	II
Rac1 17N	DN Rac1 GTPase /EGFP	Dr. J. Peränen	II
Rac1 12V	CA Rac1 GTPase /EGFP	Dr. J. Peränen	II
Arf6 WT	wt Arf6GTPase/EGFP	Dr. J. Peränen	II
Arf6-T27N	DN Arf6/-	Dr. J. Peränen	II
Arf6-Q67L	CA Arf6/-	Dr. J. Peränen	II
RhoA WT	wt RhoA GTPase/EGFP	Dr. J. Peränen	II
RhoA-G14V	DN RhoA GTPase/EGFP	Dr. J. Peränen	II
RhoA-T19N	CA RhoA GTPase/EGFP	Dr. J. Peränen	II
Rab34	wt and DN, CA Rab34/EGFP	Dr. W. Hong	II
Clathrin	wt light chain clathrin/tomato	Dr. T. Kirchhausen	II
IL-2R beta-chain	wt Ntb-domain of IL2-receptor/-	Dr. A. Dautry-Varsat	II
AP180-C	DN AP180-C	Dr. D. Blaas	III
H2B-EYFP	wt H2B/EYFP	Dr. J. Langowski	IV
EGFP-sp100	wt sp100 nuclear protein /EGFP	Dr. G. Dellaire	IV
EGFP-PAB2	wt nuclear poly(A)-binding protein PAB2/EGFP	Dr. M. Carmo-Fonseca	IV
NPM-EGFP	wt nucleophosmin/EGFP	Dr. M. Laiho	IV

As controls, immunolabeling with antibodies (Abs) and dextran or transferrin internalization into the transfected cells were used. For entry studies (II), GTPase functions were studied using expression of constitutively active (CA) and dominant negative (DN) mutants, both of inhibiting the normal cycle of GTP binding and hydrolysis. In brief, CA mutants were unable to hydrolyze the bound GTP and accumulated a large pool of activated GTPase in cells. In contrast, DN mutants did not bind GTP and were as inactive form in cells. Wild-type (wt) forms of GTPases were used as a control.

For immunofluorescence microscopy (I-IV), the cells were fixed at set time intervals post transduction (p.t.) with PFA at RT (I-IV) or with methanol on ice (I) or at RT (III, IV). The fixed cells were further permeabilized with Triton-X (in BSA-PBS) or washed with BSA-PBS, immunolabeled (Table 7) and embedded to MOWIOL-DABCO or Pro-longed ProLong® Gold antifade reagent with DAPI (Molecular Probes) according to standard protocols.

TABLE 7 Primary and secondary antibodies utilized in the thesis.
A. MOUSE MONOCLONAL ANTIBODIES

Antibody	Target	Source	Article
B12D5	*Ac*MNPV membrane glycoprotein gp64	Dr. L. Volkman/	I
p10C6	*Ac*MNPV capsid protein vp39	Dr. L. Volkman/	I-IV
eea-1	early endosomal antigen 1	BD Transduction Lab., USA	I, II
Rab11	recycling endosomal protein	Zymed Laboratories, USA	I, III
CI-MPR	cation-independent mannose phosphate receptor	Dr. V. Marjomäki/	I
CD-63	lysosomal protein	Zymed Laboratories, USA	I
GM130	Golgi-matrix protein	BD Transduction Lab., USA	I
TGN38	Trans-Golgi network protein	Dr. G. Banting, UK/	I
*Ac*MNPV/BV	*Ac*MNPV	Dr. M. Summers	II
Luc	*Photinus pyralis* luciferase	Serotec, UK	II
Dynamin-2	dynamin-2	Dr. M McNiven	II
NTb (IL-2): Cy3-561	Ntb-domain of IL2-receptor	Dr. A. Dautry-Varsat	II
Flotillin-1	flotillin-1	BD Biosciences, USA	II
Arf6	ADP-ribosylation factor 6	Thermo Fisher Scientific, USA	II
RhoA	RhoA GTPase	SantaCruz Biotech., USA	II
Rac-1	Rac-1 GTPase	Millipore, USA	II
M2	FLAG epitope	Sigma Aldrich, USA	II
Myc 9E10	myc peptide	ATCC, USA	II, III
Actin	actin	Sigma Aldrich, USA	II, III
Tubulin	tubulin	Sigma Aldrich, USA	II, III
Rab5	Rab5 protein	BD Transduction Lab., USA	III
LAMP-2	LE/lysosomal protein	Biotech. Assoc. Inc., USA	III
Lamin A/C	nuclear lamin A/C	Novocastra Laboratories, UK	IV
PML	promyelotic leukaemia protein	Abcam, UK	IV
SC-35	nuclear speckle protein 35	Abcam, UK	IV

B. RABBIT POLYCLONAL ANTIBODIES

Antibody	Target	Source	Article
Streptavidin	Streptavidin	Dr. E. Bayer, Israel	III
IE-2	*Ac*MNPV immediate early virus protein 2	Dr. D. Knebel-Mörsdorf, Germany	IV
A-6455	green fluorescent protein	Molecular Probes, USA	IV
p80	coilin	Dr. A. Lamond, UK	IV

C. SECONDARY ANTIBODIES

Antibody	Source	Article
Goat Nanogold anti-mouse IgG	Nanoprobes, USA	I
Goat Nanogold anti-rabbit IgG	Nanoprobes, USA	I, III
Goat Alexa-546-conjugated anti-mouse IgG	Molecular Probes, USA	I-III
Goat Alexa-633-conjugated anti-mouse IgG	Molecular Probes, USA	II-IV
Goat Alexa-488- conjugated anti-rabbit IgG	Molecular Probes, USA	II-IV
Goat Alexa-633- conjugated anti-rabbit IgG	Molecular Probes, USA	II-IV
Goat anti-mouse IgG alkaline phosphatase	Promega, USA	II
Goat anti-rabbit IgG alkaline phosphatase	Promega, USA	III
Goat Alexa-488- conjugated anti-mouse IgG	Molecular Probes, USA	III, IV
Goat Alexa-555-conjugated anti-mouse IgG	Molecular Probes, USA	III, IV
Goat Alexa-555- conjugated anti-rabbit IgG	Molecular Probes, USA	III, IV
Goat anti-rabbit-HRP	Sigma Aldrich, USA	IV

For cointernalization studies (II, III), the cells were first transduced with virus or TAT-SA and then fed with TRITC-Dextran (Molecular Probes). Alexa-546- or TRITC-Transferrin or Alexa-488-labeled *E. coli* bioparticles (K-12 strain; Molecular Probes) were fed simultaneously or consecutively with virus or TAT-SA. After appropriate time points, the cells were PFA-fixed and immunolabeled. To separate the fluorescence of internalized and non-internalized *E. coli* particles, the extracellular fluorescence was quenched with trypan blue.

In inhibition studies (II-IV), the internalization of baculovirus or TAT-SA, as well as virus-mediated transgene expression (EGFP), was investigated in the presence of drugs that interfere with cholesterol content or its function on the plasma membrane, or inhibit dynamin GTPases or Na+/H+ exchanger. Additionally, actin disrupting, stabilizing or microtubule depolymerizing agents were used (Table 8). Positive and negative controls (transferrin internalization, *E. coli*/Echovirus-1 entry, Abs) were used to define the drug effects. The cell viability after drug treatments or TAT-SA/TAT-SA-PPAA internalization *per se* was determined by CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTT assay, Promega) according to manufacturer's protocol.

TABLE 8 Endocytic inhibitors used in the thesis.

Inhibitor	Function	Source	Article
Dynasore	dynamin1, dynamin2, and mitochondrial dynamin Drp1 inhibitor	Dr. T. Kirchhausen, USA	II
U73122	phospholipase C (PLC) inhibitor	Sigma Aldrich, USA	II
LY294002	phosphatidylinositol-3-kinase (PI3K) inhibitor	BioSource Int., USA	II
Nystatin	cholesterol clusteration	Sigma Aldrich, USA	II
Filipin	cholesterol clusteration	Sigma Aldrich, USA	II
MβCD	methyl-β-cyclodextrin, cholesterol depletion	Sigma Aldrich, USA	II, III
EIPA/Amiloride	Na+/H+ exchanger inhibitor	Sigma Aldrich, USA	II/III
Cytochalasin D	Actin depolymerization	Sigma Aldrich, USA	III
Nocodazole	Microtubule depolymerization	Sigma Aldrich, USA	III
Taxol	Microtubule stabilizer	Sigma Aldrich, USA	IV

4.5 Microinjection (III)

For TAT-SA and SA microinjection experiments (III), Hela cells were grown to subconfluency (50-70%) on microgrid coverslips. The injections were performed into the cytoplasm of living HeLa cells using a semiautomatic system comprising a Transjector 5246 and Micromanipulator 5171 (Eppendorf) attached to an inverted microscope. Capillaries for injections were prepared with a model P97 capillary puller from Sutter Instruments. After injections, the cells were observed by confocal microscopy (see 4.6 for further information).

4.6 Confocal Microscopy Studies in Mammalian Cells (I-IV)

Immunofluorescence Microscopy. With fixed samples, two laser scanning confocal microscopes were used (I-IV; Zeiss LSM 510, Carl Zeiss AG, Jena, Germany or Olympus Fluo-View 1000, Olympus Optical Co., Tokyo, Japan). In the imaging, appropriate excitation and emission settings were used for each dye (405-nm diode laser, 488/514-nm argon lasers, 543-nm and 633-nm HeNe-lasers). All 3D images were obtained by using 60x APO oil immersion objective (NA=1.35) or 63x Plan-Neofluor oil immersion objective (NA=1.25), in which the pixel resolution was adjusted to 100-110 nm/pixel (512x512 pixels/image) and stacks of 25-35 images were collected. In image processing, LSM510 and ImageJ softwares as well as Adobe Photoshop (CS2) were used.

In HepG2 cell synchronization experiments (IV), isoleucine deficient medium (Ham´s nutrient mixture F-10) and aphidicolin was used (Sigma Aldrich), to reach the G_1/S phase of the cell cycle. After virus transduction, PFA fix and/or immunofluorescence labeling (see 4.3 for further information) the fluorescence of viral capsids or transgene-mediated expression was monitored by confocal microscopy.

RNA interference (RNAi). To define the cellular regulators used in baculovirus entry (III) small interfering RNAs (siRNA) were used to knock down the cellular RhoA, Rac1 and dynamin2 in 293 cells (Dharmacon, Thermo Fisher Scientific, Fremont, CA, USA). Non-specific siRNA was used as a negative control, while siGLO act as a transfection marker. After transfection with Oligofectamine (Invitrogen), the cells were transduced with virus, PFA fixed, immunolabeled with appropriate Abs (see 4.3 for further information) and viewed by confocal microscopy.

LIVE Confocal Microscopy. In live cell microscopy (Zeiss LSM 510), 293 (II, IV), HeLa (III) or HepG2 cells (IV) were maintained in Scotch chambers (Bananis et al. 2000) or plates and the 63x Plan-Neofluoar oil immersion objective (NA=1.25) and sample holder was heated to 37°C. In entry experiments (II, III) and nuclear studies (IV), the cells were transduced with p24mCherry (MOI 400) or luciferase or EGFP expressing baculoviruses (MOI 200-1000). In nuclear studies (IV), the host cell chromatin was stained with cell-permeable DNA probe Draq5™ (BioStatus Limited). In the imaging, appropriate excitation and emission settings were used. The 3D images were obtained from stacks of 30-35 images (512 x 512 pixels), in which the pixel resolution was adjusted to 100 nm/ pixel. The movie (IV) consisting of selected time frames after virus transduction was created with Image J software.

Quantitative Data Analysis. Quantification of fluorescence intensities in baculovirus transduced cells was performed by ImageJ software (Abramoff et al. 2004). In general, for internalization studies with *E. coli* bioparticles and drug-treatments (II), single images from middle plane of the PFA-fixed cells were obtained (pinhole ≥2) and the fluorescence intensity was determined by ImageJ. For nuclear studies (IV), 3D images from 30-40 live cells were collected (100 nm/pixel) and the data was further processed by ImageJ, in which the relative standard deviation of the nucleus and nucleolar fluorescence intensities were calculated. For colocalization analysis (III, IV), BioimageXD or ImageJ (Colocalization Plug-In) software was used from 20-30 cells (100 nm/ pixel).

4.7 Electron Microscopy Studies in Mammalian Cells (I-IV)

Nanogold Pre-embedding Immunoelectron Microscopy. To define the cellular compartments used in baculovirus or TAT-SA entry (I, III), HepG2 or HeLa cells were transduced (see 4.3 for further information) and fixed with PLP fixative. Next, the cells were washed with phosphate buffer, permeabilized with saponin and immunolabeled with appropriate primary Abs and nanogold-

conjugated secondary Abs (Table 2). After further washes with saponin and phosphate buffers, glutaraldehyde postfixation and NH_4Cl quenching were performed, followed by silver enhancement and gold toning. The cells were further postfixed with osmium tetroxide containing $K_4Fe(CN)$ and dehydrated with ethanol series, stained with uranylacetate, and embedded in LX-112 Epon (Ladd Research Industries). After polymerization, the samples were further stained with toluidine blue, cut with ultramicrotome (Reichert-Jung; Ultracut E), stained with uranylacetate and lead citrate and finally examined by A JEOL JEM-1200EX (JEOL Ltd. Tokyo, Japan).

Ruthemiun Red Staining. To examine the baculovirus entry route into HepG2 cells (I), carbohydrates of the outer leaflet of the plasma membrane were stained by ruthenium red. After transduction, the cells were first washed with cacodylate buffer, then simultaneously fixed and stained with glutaraldehyde containing ruthenium red in cacodylate buffer and finally postfixed with osmium tetroxide containing ruthenium red. Further dehydration, staining, polymerization and cutting were performed as described above.

Horse Radish Peroxidase (HRP) Labelling. To study the role of fluid-phase endocytosis in baculovirus entry (II), HRP labeling was used. 293 and HepG2 cells were first incubated at MEM containing HRP (type II, Sigma Aldrich). After virus transduction, the cells were fixed with PFA containing glutaraldehyde. HRP was detected with diaminobenzidine supplemented with hydrogen peroxide. The cells were then washed, post-fixed in osmium tetroxide, dehydrated, stained and processed for EM as described previously.

Pre-embedding Immunoelectron Microscopy. Baculovirus transduction was investigated in non-dividing, microtubule-stabilized, HepG2 cells (IV). The cells were grown to subconfluency and treated with taxol (Table 8). Prior to glutaraldehyde fixation, the cells were transduced in MEM containing taxol, postfixed and processed for electron microscopy as described previously.

4.8 Flow Cytometry (II, IV)

The transduction efficiency of recombinant baculoviruses was assessed by measuring the transgene-mediated expression of EGFP (I-II, IV) or luciferase (III) by flow cytometry or confocal microscopy. In flow cytometry (II, IV), after appropriate transductions (see 4.3 for further information) the cells were detached by scraping, concentrated by centrifugation and diluted in PBS. To detect the cellular DNA (IV), the cells were further fixed with ethanol and labelled with propidium iodide. The fluorescence of samples was analyzed with FACSCalibur and CellQuest software (Beckton Dickinson).

4.9 Quantitative RT-PCR (IV)

The transcription of baculoviral immediate early transregulator genes *ie-1* and *ie-2* was analyzed in transduced HepG2 and 293 cells with quantitative TaqMan RT-PCR (IV). After scraping and centrifugation of cells, total RNA was isolated using TRIZOL® reagent, treated with the deoxyribonuclease DNaseI and reverse-transcribed. SYBR® Green RT-PCR (ABI PRISM 7700 Sequence Detection System, Applied Biosystems) was used to quantify the levels of viral mRNA. To construct the standard curve, four serial dilutions of cDNA obtained from transduced cells were used.

4.10 SDS-PAGE, Immunoblotting and Chemiluminescence Detection (II, IV)

The functionality of siRNAs (II) was tested by SDS-PAGE and immunoblot analysis of dynamin-2, RhoA and Rac1 (see 4.5. for further information). Tubulin was detected as a loading control. The expression of the viral immediate early proteins IE-2 in HepG2 and *Sf*9 cells (IV) was detected by SDS-PAGE, western immunoblot analysis and chemiluminescence detection according to manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate, Pierce). After the transduction of HepG2 cells, the cells were collected on ice by scraping and centrifugating the pellet. *Sf*9 cells were infected at 27°C for set time periods and collected by centrifugation. Prior to detection, cells were further fractionated and collected according to Qproteome Cell Compartment Kit protocol (Qiagen).

5 REVIEW OF THE RESULTS

5.1 Baculovirus Uptake into Human Cells (I-II)

Baculovirus entry and transduction efficiency were defined in highly permissive human cell lines (HepG2, 293) by confocal and electron microscopy as well as with flow cytometry. After efficient binding, the internalization of the virus was detected by confocal microsopy in both cell lines (I, Figs. 2-3; II, Fig. 1). During early virus transduction (<30 min p.t.), ruffle formation on the cell surface was observed by confocal and electron microscopy (I, Fig. 3; II, Figs. 2, S2 and video S1). Additionally, by EM, baculoviruses were seen in fluid-phase marker HRP-labeled large endosomal vesicles at 5-15 min p.t., the co-internalization gradually increasing towards 15 min p.t. (II, Fig. 2).

Baculoviruses were further observed in EEs (eea-1 Ab) at 15 min-1 h p.t. by confocal microscopy (I, Fig. 4). The colocalization of baculovirus and late endosomal/lysosomal marker CI-MPR was detected at 45 min-1.5 h p.t. (I, Fig. 5). Additionally, large amounts of viruses were seen in lysosomes at 2-4 h p.t. by EM (CD-63 Ab; I, Fig. 5). The first viruses were seen in the nuclei of HepG2 cells at 3-4 h p.t. (I, Fig. 2). The expression profiles of the baculovirus-mediated gene transfer were shown to follow the previously published results in HepG2 cells (Salminen et al. 2005), showing the maximum EGFP-transgene expression under CMV promoter at 22-24 h p.t. (I, Fig. 1).

5.1.1 Role of Clathrin-mediated Endocytosis

The uptake of baculovirus into human cells has been suggested to be analogous to that of in insect cells, i.e. adsorptive endocytosis (Volkman & Goldsmith, 1985). By EM, we showed few viruses near clathrin coated pits at 30 min p.t. (I, Fig. 3). Notably, no clathrin coated vesicles containing viruses were seen and most of the viruses seemed to localize in larger non-coated plasma membrane invaginations and vesicles (I, Fig. 3; II, Fig. 2). Ruthenium red staining of the cell membrane was used to differentiate the invaginations and internalized vesicles (I, Fig. 3).

Clathrin-mediated entry was further excluded by showing discrete localization of expressed clathrin, transferrin and recycling marker rab11 with baculoviruses at 5-60 min p.t. (I, Fig. 4; II, Figs. 1, S1). In all tested cell lines transferrin was shown to internalize the cells at faster rate than baculovirus. Additionally, dynamin, required for vesicle scission in CME, was not required for uptake or transduction of the virus. This was observed by experiments with dynamin inhibitors, such as dynasore (Macia et al. 2006), affecting dynamin-1/-2 and mitochondrial dynamin Drp1 or siRNA against dynamin-2 (II, Fig. 1). Effectiveness of the inhibitors was determined by their ability to block the uptake of transferrin. Additionally, clathrin-independent, but dynamin-dependent IL-2 receptor (II, S4), dynamin-independent GPI-AP (data not shown) or flotillin-1 (II, Fig. 3) did not localize in the same cellular regions as with baculovirus.

5.1.2 Role of Phagocytosis-like Mechanism

Due to extensive ruffle formation, presence of viruses in large vesicles (I, Fig. 3; II, Fig. 2) and involvement of actin in efficient transduction (Salminen et al. 2005), the roles of macropinocytosis and phagocytosis were studied in baculovirus entry. Baculovirus transgene expression but not the early entry was interfered by amiloride analog EIPA (II; Figs. 3, S3), an inhibitor of macropinocytosis (West et al. 1989). Furthermore, CA, DN mutants or siRNA of macropinocytosis mediators p21-kinase Pak1, Rab34 or RhoGTPase Rac1 had no effect on baculovirus uptake or transduction efficiency (II, Figs. 3, 5). The data thus suggest that macropinocytosis does not regulate baculovirus uptake in human cells.

The involvement of phagocytosis-like entry in the baculovirus transduction was first observed with phagocytic marker *E. coli* bioparticles in HepG2 cells. In the presence of virus, the cellular uptake of *E. coli* particles increased significantly (II, Fig. 6). Bioparticles were also seen to colocalize with the virus at 5-10 min p.t. (II, Fig. 6). In transfection experiments, baculovirus transduction was demonstrated to be dependent on underlying regulation of RhoA- and Arf6GTPases (II, Figs. 4, 5), previously identified as actors in phagocytosis (Caron et al. 1998, Zhang et al. 1998). Interestingly, both CA and DN mutant forms of Arf6GTPases were shown to decrease the virus-mediated transgene expression (II, Fig. 4), CA-mediated inhibition likely occuring through inactivation of RhoA (Boshans et al. 2000). The virus expression levels also declined in cells treated with phospholipase C and phosphatidylinositol-3-kinase inhibitors, required in phagocytosis (II, Fig. 3).

In contrast to phagocytosis mechanism characterized in macrophages, baculovirus was able to express its transgenes in cholesterol-depleted cells (II, Fig. 3). Interestingly, the co-internalization of virus and *E. coli* particles in cholesterol-depleted cells did not decrease the uptake of *E. coli* into cells (unpublished data). Cholesterol-clustering agent filipin was also shown to enhance the baculovirus-mediated transgene expression (II, Fig. 3), suggesting the upregulation of a raft-independent pathway (II). Altogether, these results

suggested that baculovirus uptake is devoid of lipid rafts and induces RhoA and Arf6-regulated phagocytosis-like mechanism for efficient transduction in non-phagocytic human cells. Two other RhoGTPases Cdc42 and Rac1, known to associate with phagocytosis in macrophages (Caron et al. 1998), were not required in baculovirus-mediated transgene expression (II, Fig. 6). Table 9 presents the timeline of the baculovirus early entry observed in studies I-II.

TABLE 9 The timeline of baculovirus uptake and the involved cellular mediators.

p.t.	Cellular marker/inhibitor	Colocalization/Effect on Transduction	Notes
5-15 min	HRP	*yes*	EM; endocytic vesicles
5-60 min	transferrin	no	CME marker
15 min-1.5 h	rab11	no	RE marker
5-60 min	clathrin	no	light chain
5 min-1 h	*E. coli*	*yes*	enhances uptake, colocalizes with virus at 5-10 min p.t.
0-60 min	dynasore	no	inhibits dynamin-1,-2
0-6 h	dynamin-2 siRNA	no	inhibits dynamin-2
15 min-1.5 h	eea-1	*yes*	EE marker
30 min	CCP	yes?	EM; few viruses
5-60 min	IL-2R	no	IL-2 route marker
5-60 min	flotillin-1	no	flotillin route
5-60 min	GPI-AP	no	GPI-AP route
0-24 h	EIPA	*yes*	Na^+/H^+ inhibitor
0-6 h	EIPA	no	
0-24 h	U73122	*yes*	PLC inhibitor
0-24 h	LY294002	*yes*	PI-3 kinase inhibitor
0-24 h	methyl-β-cyclodextrin	no	cholesterol depletion
0-24 h	nystatin	no	cholesterol clustering
0-24 h	filipin	*yes*	cholesterol clustering enhances uptake
0-24 h	Arf6	*yes*	Arf6GTPase
0-24 h	RhoA	*yes*	RhoGTPase
0-6 h	RhoA siRNA	*yes*	RhoGTPase
0-24 h	Cdc42	no	RhoGTPase
0-24 h	Rac1	no	RhoGTPase
0-6 h	Rac1 siRNA	no	RhoGTPase
0-24 h	Pak1	no	p21-kinase
0-24 h	Rab34	no	RabGTPase
45 min-2 h	CI-MPR	*yes*	LE marker
30 min-4 h	TGN-38	no	trans-Golgi marker
30 min-4 h	GM130	no	Golgi-matrix marker
2 h-4 h	CD-63	*yes*	lysosomal marker

Abbreviations: HRP, horse radish peroxidaase; RE, recycling endosome; eea-1, early endosomal antigen-1; EE, early endosome; CCP, clathrin coated pit; IL-2, interleukin-2; GPI-AP, glycosyl phosphatidylinositol-anchored proteins; PLC, phospholipase C; PI-3, phosphatidylinositol-3-kinase; Arf6, ADP-ribosylation factor 6; LE, late endosomes.

5.2 TAT-Streptavidin in Human Cells (III)

The internalization and delivery properties of the TAT-streptavidin vector (Albarran et al. 2005) were further evaluated in this thesis in various mammalian cells by confocal and electron microscopy. The internalization and functionability of TAT-SA was primarily followed in HeLa cells, although the efficient uptake was also shown in A549 and non-cancer cell line MRC-5 (III, Fig. 1). First, the high stability of TAT-SA and TAT-SA bound biotin was demonstrated with SDS-PAGE and immunoblotting experiments. TAT-SA was shown to retain its tetrameric conformation as apoform or when bound to biotin in spite of the heat treatments (37-68°C) and reducing agents (III, S1).

In live confocal microscopy, the internalization of Alexa488-labeled TAT-SA or TAT-SA complexed biotin was shown to be very rapid starting at 5 min p.t. However, after 4 h p.t. majority of the constructs were still located in the cytoplasm in vesicle-like structures. In addition, only minority were seen in the nucleus by confocal (III, Fig. 1) or immunoelectron microscopy techniques (III, Fig. 2). To identify this cellular barrier, cytoplasmic microinjection of TAT-SA was performed. The nuclear localization signal of TAT peptide was shown to be functional as the complex rapidly localized into the nucleus. Injected Alexa-488-labeled streptavidin, on the other hand, remained at the cytoplasm and did not enter the nucleus (III, Fig. 1).

5.2.1 Internalization of TAT-Streptavidin

The uptake of streptavidin has been suggested to occur via CME (Wilbur et al. 1998, Alon et al. 1993). Here, TAT-SA was detected in live HeLa cells with CME-marker transferrin and fluid-phase endocytosis marker dextran. Partial colocalization was detected with transferrin at 5-30 min p.t., whereas dextran seemed to colocalize significantly with TAT-SA during 15 min-4 h p.t. (III, Figs. 3, 4, 5; video S2). Additionally, expression experiments with dominant negative mutant form of AP180-C, required for the efficient assembly of clathrin coated pits, decreased but did not inhibit the cellular internalization of TAT-SA (III, Fig. 3). As a control, transferrin was shown to be inhibited in AP180-C expressing cells.

The different inhibitors of cellular uptake were also applied, such as amiloride and cytochalasin D. In comparison with untreated control cells, the internalization of TAT-SA was markedly reduced with both drugs affecting actin-dependent uptake (III, Fig. 5). Treatment with nocodazole, which disrupts the microtubules, had only minor effect on vector internalization. The inhibitory effects on the cytoskeleton were assured by immunolabeling actin filaments or microtubules from the drug-treated cells. Additionally, treatment with methyl-β-cyclodextrin, a cholesterol depletion agent known to affect the caveolae route (Nabi and Le 2003), did not prevent the entry of TAT-SA into living cells (III, Fig. 5). Furthermore, TAT-SA internalized efficiently into caveolin-deficient HepG2 cells (data not shown).

5.2.2 Delivery of Biotinylated Molecules via TAT-Streptavidin

The delivery of biotinylated molecules via TAT-SA vector has been described previously (Albarran et al. 2005). However, the cellular destination of the vectors is unknown. For imaging the functionality of TAT-SA with biotinylated cargos, we used biotin-633 dye as a marker. Confocal microscopy experiments together with fluid-phase marker dextran showed that the vector was efficiently delivered into cells. However, the majority of the constructs accumulated in the cytoplasm into vesicle-like structures instead of the nuclei. These structures were defined by immunolabeling as late endosomal/lysosomal compartments (LAMP-2 Ab; III, Figs. 3, 7 and video S3).

To circumvent the degradation in these compartments, biotinylated-PPAA, known to enhance endosomal release, was complexed with TAT-SA. Quantitative analysis demonstrated that nuclear localization of TAT-SA and TAT-SA-biotin-633 dye increased in the presence of PPAA (III, Figs. 6, 7 and video S4). Importantly, no significant cytotoxicity was seen in transduced cells at 4-72 h p.t. As a proof-of-principle, biotinylated linear DNA fragments expressing EGFP under CMV promoter were complexed with TAT-SA and transduced into HeLa cells. No expression was, however, detected (unpublished data).

5.3 Nuclear Accumulation of Baculoviruses in Human Cells (IV)

Baculoviruses were shown to accumulate into nuclei of HepG2 and 293 cells starting at 6h p.t. by confocal and electron microsopy. Maximum fluorescence intensity of the immunolabeled viral capsids was determined in lamin A/C Ab lined nuclei at 8 h p.t. (IV, Fig. 1). In electron microscopy, baculoviruses were seen near heterochromatin regions lining the nuclei (IV, Fig. 1). As in previous studies of van Loo *et al. (*2001), baculovirus was able to internalize into aphidicoline- (G_1/S phase), as well as taxol-treated, microtubule-stabilized, non-dividing cells (IV, Fig. 1).

5.3.1 Virus-induced Alterations in Nuclear Compartments

Various subnuclear structures were studied relative to baculovirus location in HepG2 cells by confocal microscopy. Baculovirus transduction was shown to alter the localization and size of PML NB structures in HepG2 cells particularly at 6 h p.t., in which the size of the structures were shown to increase and amount to decrease (IV, Fig. 4). Since PML NBs have proposed to be sensitive for cellular stress, the localization of interferon-inducible protein nucleophosmin was detected in the same cell line. Baculovirus did not relocalize the protein from the nucleolus to the nucleoplasm as a result of virus-induced cellular stress but remained at the nucleolus during transduction (data

not shown). As a control, canine parvovirus was shown to mediate nucleophosmin relocalization in its highly infective cell line (unpublished data).

Other nuclear substructures, such as nuclear speckles, nucleolus and Cajal bodies were shown to be absent of viruses in confocal microscopy studies. In HepG2 cells, baculoviruses were seen to localize in the vicinity of sp100 and PML proteins at PML NB foci (IV, Fig. 5). In quantitative image analysis, however, no colocalization correlation was found between virus capsid and PML protein or sp100 (data not shown). Intriguingly, at later time points (>24 h p.t.) baculovirus transduction was shown to disperse the host chromatin (DRAQ5, H2B-EYFP) in HepG2 cells. The alterations were especially observed in heterochromatin lining the nucleolus and nuclei. By quantitative image analysis, the change in chromatin structure was observed to increase gradually with higher viral dose (IV, Figs. 6, 7). Flow cytometry studies of propidium iodide labelled cells were performed to compare the total DNA fluorescence of untransduced cells with virus transduced cells. The results indicated that the potentially labeled virus genome was not responsible for the detected chromatin alterations, since the viral dose had no significant effect on the detected DNA fluorescence intensities. Additionally in 293 cells, in the presence of high baculovirus-mediated EGFP-expression, the chromatin alterations were not detected. This implicates that the observed changes in transduced HepG2 cells did not result from EGFP overexpression (data not shown).

5.4 Transcription and Translation of Baculovirus Proteins in Human Cells (IV)

The viral immediate early protein transcription was detected with quantitative RT-PCR in HepG2, 293 and *Sf*9 insect cells. In HepG2, mRNAs of *ie-1* and *ie-2* genes were detected already at 4 h p.t., gradually increasing towards 48 h p.t. Transcription was also observed in 293 cells, however, at substantially lower amounts (IV, Fig. 2). At protein level, immediate early virus proteins (IE-2 Ab) were detected for the first time in human cells in cytosol and nuclear fractions of HepG2 cells at 24 h p.t. by SDS-PAGE and immunoblot analysis (IV, Fig. 3). *Sf*9 cells were used as a control to demonstrate the viral protein expression in infected insect cells.

6 DISCUSSION

To successfully deliver biomedically relevant cargos into the target cells and acquire the desired therapeutic response, active cellular transport systems are required. Gene therapy, which is an emerging new treatment for various inherited and acquired diseases, utilizes virus-derived and non-viral vectors to carry transgenes across tissues into cells (Verma & Weitzman 2005, Thomas, and Ehrhardt & Kay 2003). The lack of knowledge of cellular entry pathways and intracellular factors mediating the transgene delivery has, however, hindered the refinement of transductional targeting, endosomal release and nuclear delivery of the applied vectors. This thesis focuses on defining intracellular mechanisms of two potential gene therapy vectors, insect infecting *Ac*MNPV-baculovirus (I, II, IV) and a novel streptavidin-based non-viral vector TAT-SA (III).

6.1 Uptake Mechanisms of Baculovirus and Streptavidin-based Vectors in Mammalian Cells

6.1.1 Baculovirus Vectors

*Ac*MNPV-baculovirus represents a potential gene therapy vector for its easy genetic engineering, production of high titer stocks, non-toxicity and the inability to replicate in human cells (Hu 2006, Kost & Condreay 2002). Several studies have focused on the improvement of baculovirus-mediated gene transfer by gp64, VSV-G or CD46-display strategies or by optimizing the gene delivery efficiency in serum free environments (Airenne et al. 2008, Hu 2006, Mäkelä & Oker-Blom 2006). To enable the development of improved delivery strategies and to obtain more efficient and stable baculovirus-mediated transgene expression in mammalian cells, the elucidation of the exact entry route and intracellular mechanisms are essential. In this study, functional transduction and gene transfer were detected in highly permissive human HepG2 hepatocarcinoma and 293 epithelial kidney cell lines (I, II).

Uptake of particles by mammalian cells can occur either via pinocytotic or phagocytotic mechanisms. Many viruses employ clathrin-mediated endocytosis (CME) to enter their host cells. In baculovirus infected insect cells, virus-containing endosomes have previously been observed after 10 min p.i. (Blissard 1996). Although the involvement of clathrin coated vesicles in baculovirus uptake has been proposed, neither the receptor nor exact entry mechanisms are known in insect or human cells.

Baculoviruses cannot replicate in mammalian cells, although virus internalizes and transduces efficiently various cell types (Volkman et al. 1985). In this study, baculoviruses were shown to bind and internalize into human carcinoma cells concentrating on specific cell surface areas at 5-15 min p.t. (I). EM and confocal microscopy experiments of HepG2 and 293 cells showed high frequencies of non-branched ruffles after 15 min of exposure to the virus (II, video S1). Only few viruses were seen near clathrin coated pits by EM and none in clathrin coated vesicles or caveolae (I, II). Viruses seemed to bind and internalize the cells efficiently within first 5-15 min, during which large, fluid-phase marker positive, virus-containing endosomes were observed (II). CME markers did not associate with viruses at any detected time point (I, II). Additionally, the inhibition of dynamin GTPase, required in vesicle scission in CME, had no effect on baculovirus transduction (II). The possible recruitment of clathrin (Veiga et al. 2005, 2007) in virus transduction was also exluded in living cells (II). Altogether, the data suggested that CME was not required for functional baculovirus entry into human cells. The size of the viral particle (~0.26 μm) relative to clathrin coated vesicles (0.1-0.15 μm, ≤.0.2 μm; Bishop 1997, Rejman et al. 2004) or caveolae (0.05-0.09 μm; Bishop 1997) also implicated an alternative cellular entry mechanism.

Uptake of fluid from the extracellular environment i.e. fluid-phase endocytosis can occur in mammalian cells within either micropinocytotic (≤0.1 μm) or macropinocytotic vesicles (0.5-5.0 μm; Amyere et al. 2002, Swanson et al. 1995). In baculovirus transduction, high cellular ruffling (I), requirement of actin (Fujita et al. 2006, Lanier & Volkman 1998, Salminen et al. 2005) and the presence of multiple baculoviral particles (~0.26 μm/virus) per endocytic vesicle (I, II) indicated the role of macropinocytosis or phagocytosis in baculovirus transduction. Macropinocytosis is accompanied with frequent cell surface ruffles and is regulated by actin, RhoGTPase Rac1, p21-kinase Pak1, phosphoinositide 3-kinase, phospholipase C, and Rab34 GTPase (Jones, 2007). Viruses, including Vaccinia, HIV-1 and adenovirus have been shown to utilize macropinocytosis for their entry (Maréchal et al. 2001, Meier et al. 2002, Mercer et al. 2008). In present study, the uptake of baculovirus was shown to be independent of macropinocytic regulators Rac1, Pak1 or Rab34. The virus-mediated transgene expression was, however, found to be decreased in the presence of phosphoinositide 3-kinase and phospholipase C inhibitors (II), which are required for both macropinocytosis and phagocytosis (Araki et al. 1996).

Phagocytosis is similar to macropinocytosis in many respects. Both processes require actin, RhoGTPases and phosphoinositide 3-kinase for the

engulfment of large cellular membrane segments (>0.5 μm diameter; Caron et al. 1998). In addition to professional phagocytes, most cell types have some phagocytic ability. RhoAGTPase mediated phagocytosis-like uptake of HSV-1 was recently demonstrated in primary human corneal fibroblasts and nectin-expressing Chinese hamster ovary cells (Clement et al. 2006). Although phagocytosis has previously proposed to be induced only by particles larger than ~0.5 μm, HSV-1 particles (~0.2 μm) were shown to activate the route by an undefined signaling mechanism. In this study, baculovirus entry enhanced cellular ruffle formation and the uptake of phagocytic tracer, *E. coli* bioparticles in non-phagocytotic, highly permissive, human cancer cells (II). Efficient baculovirus-mediated expression was further shown to be Rac1 and Cdc42-independent but RhoA-dependent (II), similar to functional entry of HSV-1. Baculovirus transduction also required the actin regulating Arf6 GTPase for its entry (II). Arf6 has been associated with phagocytic uptake in macrophages and with the entry of *Chlamydia* (Balana et al. 2005, Zhang et al. 1998). To conclude, baculovirus seems to employ actin-dependent, RhoA and Arf6-regulated phagocytosis-like mechanism to enter human cells. Although the uptake seemed to be dynamin-independent, further studies are required to define its role in functional entry of baculovirus. Table 10 summarizes the various entry routes studied as well as the cellular factors affecting baculovirus uptake.

TABLE 10 The endocytic routes and their utilized markers relative to observed functional baculovirus entry in human cells.

Cellular factor/marker	CME	CCI	Macropinocytosis	Phagocytosis	Baculovirus Uptake
Actin	√	√	√	√	√
PLC *	-	-	√	√	√
PI3K *	-	√	√	√	√
RhoA	-	√	-	√	√
Arf6	-	√	√	√	√
E.coli	-	-	-	√	√
HRP	-	-	√	√	√
Cdc42	-	√	√	√	-
Rac1	-	√	√	√	-
Pak1	-	√	√	-	-
Rab34	-	√	√	-	-
IL-2	-	√	-	-	-
GPI-AP	-	√	-	-	-
Flotillin	-	√	-	-	-
Cholesterol	√	√	√	√	-
Dynamin	√	√	√/-	√	-
Transferrin	√	-	-	-	-

Abbreviations: CME, clathrin-mediated endocytosis; CCI; clathrin and caveolin-independent pathways. *only inhibitors utilized, activation/overexpression have shown to affect CME.

In immunolabeling studies by EM and confocal microscopy, baculovirus was shown to localize in structures positive for the eea-1, CI-MPR and CD-63 markers (I). This is consistent with previous qualitative confocal studies by Kukkonen *et al.* (2003). Apart from contributing to early/late endosomes and lysosomes, all of the above mentioned markers have been linked to maturating phagosomes (Fratti et al. 2001, Ellson et al. 2001, Huynh et al. 2007, Kitano et al. 2008). Additionally, known baculovirus transduction inhibitors ammonium chloride, chloroquine and bafilomycin A all neutralize acidic compartments or interfere with the maturation of the phagosomes, thereby affecting the transduction efficiency (Pieroni et. al 2001, Schneider et al. 2000, Schnupf et al. 2007, van Loo et al., 2001, Via et al. 1998). These results implicate that instead of previously presented early endosomes, baculovirus may be released to cytoplasm from maturing phagosomes. To elucidate the virus-associated endosomal compartment further studies with baculovirus membrane glycoprotein gp64 and phagosomal/early endosomal markers are required. In the future, induction of phagocytosis may be utilized to improve baculovirus-mediated gene transfer *in vitro*.

6.1.2 TAT-Streptavidin

Cell penetrating peptides, such as the TAT peptide derived from HIV-1, are able to across the cell membrane and deliver cargo up to 100-fold larger than their own size (Lundberg & Langel 2003, Trehin et al. 2004). In recent years, cell penetrating peptides have been utilized to deliver an array of pharmacologically relevant cargos into cells, such as plasmids, liposomes and nanometer-sized particles (Trehin et al. 2004). In this study, the internalization and delivery capability of a TAT-streptavidin vector was characterized in human cells (TAT-SA; III).

Streptavidin-biotin system has previously been widely used in radioimmunotherapy, as well as in various biotechnological applications (Boerman et al. 2003, Wilbur et al. 1999). In TAT-SA vector, streptavidin acted as a carrier for biotinylated molecules into cells and HIV-derived TAT-peptides enhanced the entry and nuclear import of the vector (III). Previous structural and preliminary functional characterization of the construct was performed in the laboratory of Professor Patrick Stayton (University of Washington, Seattle, USA), including mass spectrometry, SDS–PAGE and biotin off-rate determination (Albarran et al. 2006). The internalization of the vector was also assessed in NIH3T3 mouse fibroblasts and Jurkat T cells by flow cytometry. We extended the characterization of this vector, showing that TAT-SA is non-cytotoxic to human cells and retains its tetrameric structure (60 kDa) at high temperatures and under reducing conditions. TAT-SA was also shown to be efficiently internalized into all tested cell lines, having no preference for any cell type (III).

The uptake characteristics of the TAT-conjugated cargos has been previously defined to be dependent on the properties of the cargo molecule, TAT concentration, and the cell line (Jarver & Langel 2004, Lindsay et al. 2002).

As a larger partner of the TAT_{47-57}-SA fusion construct, streptavidin (140 aa SA vs. 11 aa of TAT-peptide/monomer), was expected to mediate the uptake and intracellular trafficking of the vector by receptor-mediated endocytosis (Alon et al. 1993, Wilbur et al. 1998). Consistent with studies of Albarran et al. (2006), we observed that streptavidin (theoretical pI 6.04) entered HeLa cells more slowly than the positively charged TAT-SA (III; theoretical pI 9.92). Notably, no nuclear localization of the streptavidin was found even after cytoplasmic microinjection (III), indicating that the TAT-peptides significantly enhanced the entry process.

In subsequent cellular studies, the majority of TAT-SA was observed to colocalize with the fluid-phase marker dextran and with LAMP-2 labelled lysosomes, possibly indicating degradation of the construct. Cellular entry into HeLa cells was shown to be disrupted by the macropinocytosis inhibitor amiloride and the actin depolymerising agent cytochalasin D, suggestive that uptake was occurring via macropinocytosis. A small portion of internalized TAT-SA was also shown to colocalize with CME markers (III). No further characterization of the entry process was performed in this study due to high localization of the vectors to lysosomes, indicating that the construct *per se* was not suitable for therapeutic applications and required additional endosomal releasing agents.

6.2 Nuclear Delivery and Localization of Baculovirus and Streptavidin-based Vectors in Mammalian Cells

6.2.1 Baculovirus Vectors

The nuclear envelope of mammalian cells is a double membrane contiguous with the endoplasmic reticulum, having ~3000-4000 nuclear pore complexes per cell (Macara 2001, Whittaker 2003). Previously, nuclear transport of gold particles at least 39-nm-diameter in size has been shown to occur through nuclear pores (Pante et al. 2002). The cigar-shaped baculovirus nucleocapsid is thought to be transported intact through the nuclear pore (Salminen et al. 2005, van Loo et al. 2001). Nuclear localization of vp39-capsid protein labelled viruses has been demonstrated at 6 h p.t. (Salminen et al. 2005). In present study (I, IV), unenveloped baculovirus capsids were observed docking on the nuclear membrane already at 3-4 h p.t., suggesting a faster entry rate for the virus. Moreover, successfull transduction of non-dividing human cells implied that the nuclear entry of baculovirus did not depend on mitotic disintegration of the nuclear membrane (IV), being consistent with previous studies (van Loo et al. 2001).

In the nucleus, baculovirus accumulated time-dependently into specific foci and produced immediate early proteins in highly permissive HepG2 cells. However, only a minority of internalized viral capsids entered the nucleus (IV). Viral immediate early gene transcription was demonstrated to occur as early as

4 h p.t., corresponding to the time point at which virus capsids were first detected in the nuclei. We showed for the first time, that translation of the baculovirus *ie-2* mRNA occurs during human cell transduction (IV).

Transcription of all immediate early baculovirus transregulators, including *ie-0, ie-1, ie-2* and *pe-38*, has now been demonstrated in human cells (IV; Fujita et al. 2006, Kenoutis et al. 2006). The transcription process is presumably mediated by host RNA polymerase II and its associated transcription factors being analogous to transcription machinery utilized in the infection of insect cells (Friesen 1997). Interestingly, microarray analysis with IE-1 and IE-2 expressing recombinant baculoviruses was recently used to characterize the function of viral transcriptosome in transduced green monkey kidney Vero E6 cells (Liu et al. 2007). The results showed that IE-1 overexpression resulted in activation of *gp64* and *pe38* viral genes and upregulation of *ie-2*, *he65*, *pcna*, *orf16*, *orf17* and *orf25*. The overexpression of IE-2, on the other hand, activated only two viral genes *pe38* and *orf17*. Furthermore, simulatenous overexpression of IE-1 and IE-2 resulted in activation of 38% of *Ac*MNPV genes showing their synergistic effect on genome activation in mammalian cells. More studies are, however, required to clarify the role of endogenous IEs in *Ac*MNPV gene transcription in human cells.

Nucleus consists of highly organized and dynamic substructures. Various DNA viruses have been shown to alter the distribution of nuclear substructures during infection by inducing degradation of structures or by altering their morphology (Everett 2006; Hivin et al. 2005, Maul 1998, Rodrigues et al. 1996). In baculovirus infected insect cells, transfected viral *ie-2* has been reported to express and localize near PML NBs adjacent to the viral DNA replication centers (Mainz et al. 2002, Murges et al. 2001). In the present study (IV), baculovirus capsids were found to accumulate in the vicinity of PML NBs at 6 to 24 h p.t. visualized with antibodies against PML and sp100 proteins. Earlier, PML NBs have been reported to be associated with viral transcription sites (Everett 2001, 2006; Maul et al. 1998). In order to demonstrate baculovirus function in the nuclei and develop its nuclear targeting, the localization of the virus genome needs to be defined in the context of the nuclear substructures.

Previously, Rabies virus and the lymphocytic choriomeningitis RNA viruses have been demonstrated to cause accumulation of PML proteins into larger bodies (Blondel et al. 2002, Borden et al. 1998). Here, PML NB structures were shown to concomitantly increase in size and decrease in amount during transduction, thus implicating a baculovirus-induced effect starting at 6 h p.t. (IV). Apart from virus infection, the size and amount of PML NBs has been shown to alter during cell cycle, stress and interferon responses (Borden et al. 1998, Dellaire et al. 2006). As an indicator that a non-interferon mediated PML NB response was occurring during baculovirus transduction, the interferon-inducible protein, nucleophosmin was shown to remain in the nucleolus during viral transduction (IV) and not to traverse into the nucleoplasm as a result of cellular stress (Kurki et al. 2004, Yang et al. 2002). Since baculovirus has been shown to induce interferon response in human cells at 18-24 h p.t. (Gronowski et al. 1999), the observed upregulation of PML proteins (IV) due to a stimulated

cellular response cannot be excluded, until further studies are performed. Additionally, the role of the cell cycle was not defined relative to baculovirus-induced PML NB growth.

PML NBs are interesting targets for cancer therapy purposes, due to their regulative role in apoptosis, p53 function, tumor suppressor, DNA repair, and chromatin dynamics (Borden 2002, Everett 2006). In this study, in addition to the alterations in PML NBs, baculovirus transduction was demonstrated to induce chromatin dispersion in HepG2 but not in 293 cells (IV). The differences were likely to reflect the lower viral transcription efficiency observed in 293 cells. In insect cells, baculovirus early proteins have been suggested to induce the marginalization of the host cell chromatin, due to the expansion of the virogenic stroma for nucleocapsid assembly (Granados et al. 1981, Nagamine et al. 2008, O´Reilly et al. 1994). In human cells, baculovirus altered chromatin composition possibly by post-translational modification of histones or by earlier reported chromosomal integration of baculovirus DNA fragments (Merrihew et al. 2001). For future applications the durability and stability of this gene expression and its possible long-term effects on immunity need to be further characterized.

Altogether, the significance of the virus-induced cellular changes and their intranuclear targeting remain to be elucidated. In general, poor decondensation of the DNA, nuclear disposition, and/or lack of virus-specific transcription activators have been suggested to affect the transduction efficiency of gene therapy vectors (Hama et al. 2007). Although subnuclear targeting of reporter plasmids to PML NBs via PML IV and the sp100 domains was recently reported (Block et al. 2006), more information of the nuclear structures themselves and their mechanisms are required for successful targeting *in vivo*.

6.2.2 TAT-Streptavidin

Mechanisms of endosomal release of non-viral vectors are not well known. Targeting ligands and endosomolytic moieties for endosomal escape have improved the gene transfer efficiency of many non-viral vector types, thus avoiding degradation of the construct by lysosomes and enabling their nuclear delivery (Medina-Kauwe & Hamm-Alwarex 2005). In our previous cellular localization studies with TAT-SA, only a small proportion of the vector was detected in the nuclei, and the delivery of biotin dye (III) or biotinylated linear EGFP-expressing DNA was not successful (unpublished data).

To test the hypothesis that poor endosomal release of the TAT-SA vector was responsible for the inability to localize to the nucleus, the biotinylated pH-responsive polymer PPAA was added to TAT-SA (III). PPAA has been shown to disrupt endosomes at a pH of 6.5 or below, cause the cytosolic release of cargo molecules, and enhance the delivery of antibody-targeted conjugates *in vitro* and lipoplexes *in vivo* (Jones et al. 2003, Lackey et al. 1999, 2002; Kyriakides et al. 2002). The relocalization of the TAT-SA vector in the presence of PPAA was first presented qualitatively by Albarran *et al.* (2005) with fluorescence microscopy. To further define the effect of PPAA on the endosomal release of

TAT-SA, we performed quantitative confocal microscopy experiments in living HeLa cells. PPAA was found to significantly improve nuclear delivery of TAT-SA, without significant cytotoxicity up to 72 h p.t. (III, video S4). Additionally, TAT-SA was able to mediate delivery of biotin dye to the nucleus in the presence of PPAA. However, the setup was not suitable for further applications *in vivo* as the presence of two biotinylated cargos and four biotin sites created reduced efficiency for correct binding. As a result, experiments with a second generation of TAT-SA vectors, having covalently attached endosomal releasing peptides, are currently underway (Laakkonen et al. unpublished).

6.3 Towards Baculovirus- and Streptavidin Vector-mediated Therapeutic Gene Transfer

The goal of this thesis was to define the intracellular mechanisms of two potential gene therapy vectors, baculovirus and non-viral TAT-streptavidin and to develop their use for gene therapeutic applications. Non-replicative baculovirus vectors have previously been shown to efficiently transduce various mammalian cell types (reviewed in Hu 2006). So far baculoviral vectors have been used for *in vivo* applications mainly in rabbits, mice, and rats (reviewed in Airenne et al. 2008). Since the serum complement system has been shown to inactivate baculovirus mediated transgene expression *in vivo* (Hofmann et al. 1998), transduction and the delivery methods have been optimized in serum free environments, with collar devices or targeting to immunopriviledged areas (Airenne et al. 2000, Hoffman et al. 1998b). This approach has led to successful transductions of neural cells, carotid arteries and ocular tissue (Airenne et al. 2000, Haeseleer et al. 2001, Li et al. 2005, Wang et al. 2006). Successful systemic delivery of tropism modified baculovirus vectors into immunocompromised mice bearing breast carcinoma xenografts was recently reported (Mäkelä et al. 2008).

Due to the need for vector targeting strategies, it is important to define the mechanisms of viral entry into cells and its intracellular pathways affecting the function of the vectors. Here, the large size of the baculovirus, aggregated viral particles, or unknown signaling mechanism, caused induction of phagocytosis-like uptake of baculovirus by non-phagocytic mammalian cells. The role of heparan sulfates in this process, identified as contributing factors to baculovirus binding to mammalian cells (Duisit et al. 1999, Wang et al. 1997), is so far unknown. The functional virus uptake likely requires actin rearrangement and signalling pathways involving RhoA- and Arf6GTPases but not dynamin GTPases or lipid rafts. Additionally, the activation of phosphoinositide-3-kinase and phospholipase C may be involved in actin reorganization. Intriguingly as such, baculovirus altered the internalization of *E. coli*s, a process known to require cholesterol in their entry (II). The utilization of baculoviruses to disturb the natural endocytic route of pathogens can therefore be speculated. In fact,

baculovirus administration *in vivo* has shown to protect mice against lethal encephalomyocarditis virus (Gronowski et al. 1999) and influenza virus infections (Abe et al. 2003) by unknown mechanisms. Enhanced entry via phagocytosis may also be the reason for exclusive baculovirus-mediated transgene expression of retinal pigment epithelial (RPE) cells *in vivo* after subretinal injection (Haeseleer *et al.* 2001), since RPEs are known to have high phagocytic ability (Mayerson et al. 1986, Willermain et al. 2002). The enhancement of phagocytosis in mammalian cells might therefore improve the baculovirus-mediated gene transfer in future.

After entry, baculovirus is released from early endosomal/phagosomal vesicles via a viral envelope protein gp64-mediated mechanism and transported to the nucleus as an intact capsid. The findings that baculovirus alters the nuclear localization of PML NBs, and disperses the host cell chromatin and produces immediate early proteins (IV), raises new safety concerns for the use of this vector type in gene therapy applications (Fig. 6). Baculoviruses have usually been considered to be safe, non-transcribing and non-replicating viruses (Hu et al. 2006). The extent of the endogenous viral transcription in mammalian cells is undefined; although it is known not to reach the replication stage (Liu et al. 2007, Volkman and Goldsmith 1983). In insect cells, six essential and three stimulatory genes have been identified in baculovirus replication: DNA polymerase, DNA helicase, *lef-3, ie-1, lef-1* and *lef-2, ie-2, pe-38* and *p55*, which blocks the apoptosis (Friesen 1997). Of these, detectable viral mRNA levels have been observed with four genes *ie-1, ie-2, pe-38* and recently *lef-3* (Kaikkonen, Laakkonen et al., unpublished data), which is required for transient viral replication and binds single-stranded DNA in insect cells (Evans et al. 1997). Therefore, for safety reasons, modification of the baculovirus vector by deleting some of the viral genes needs to be considered depending on the desired therapeutic effect. Construction of *ie-1* deficient baculovirus vectors and insect host cell lines producing rescuing IE-1 are already in progress (Kenoutis et al. 2006).

With TAT-SA vector, proof-of-principle experiments showed the ability of this non-viral vector to carry biotinylated cargo into human cells. Further work, however, needs to be performed to enhance the endosomal release of the construct and to define the suitable therapeutic applications. Smaller cargo, such as siRNA or cytotoxic drugs might be preferable to deliver instead of negatively charged DNA, which has affinity for the positively charged TAT-SA. Subsequent to delivery studies of the second generation of TAT-SA vectors at the cellular level, we are planning to follow their biodistribution *in vivo* and assess their ability to carry suitable cargo for therapeutic purposes.

To conclude, in different cell types the available entry mechanisms, nuclear transport machinery, and the cell/tissue specific regulation of the expression vary. The present advantages and disadvantages of the vectors studied in this thesis project are summarized in Table 11. Further insight into the cellular mechanisms of the gene therapy vectors not only provide information about the underlying molecular machinery but also may bear fascinating novel concepts for biomedical applications.

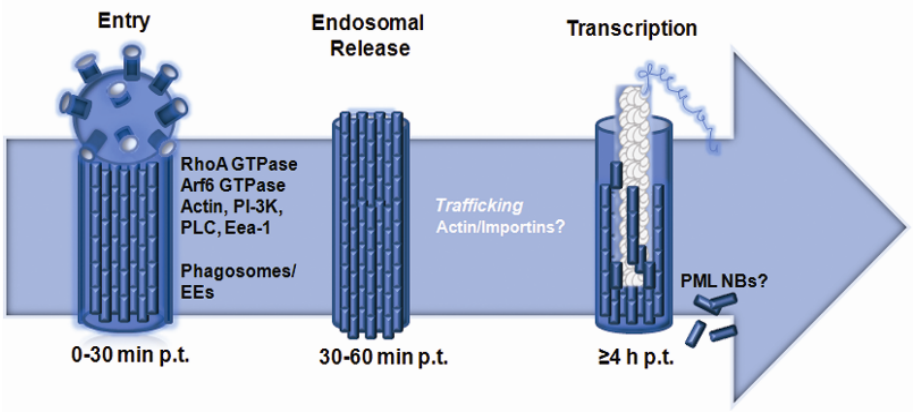

FIGURE 6 Schematic image of the *Ac*MNPV-baculovirus route in human cells leading to host cell alterations (Laakkonen 2008). Entry of virus occurs at 0-30 min p.t. via phagocytosis-like mechanism, having multiple factors involved. Uncoating and endosomal release from the phagosomes/early endosomes (EEs) takes place at 30-60 min p.t. Following the intracellular trafficking presumably via actin filaments, viruses are delivered into the nucleus through nuclear pores. Viral immediate early gene transcription starts at 4 h p.t. The role of PML NB structures in transcription is not known. Host cell changes were first detected at 6 h p.t. as an accumulation of PML NB structures and later as an alteration of host cell chromatin composition.

TABLE 11 Advantages and disadvantages in baculovirus and streptavidin-mediated gene transfer

A. Baculovirus Vectors
+ Easy genetic engineering and capacity for large DNA insert(s) (>100kb)
+ Easy and fast production in insect cells (>10^{10} pfu/ml, FDA approved)
+ Stable in storage (≤6 months)
+/- Efficient internalization into a variety of human cells
+ Low cytotoxicity even at high viral doses
+ Non-pathogenic, replication restricted in human cells
- Hampered by inactivation of the complement system *in vivo*
*The longest transgene expression *in vivo* lasted for ~178 days
*Systemic delivery demonstrated in immunocompromised mice
+ Suitable for gene therapy of immunopriviledged regions
B. Streptavidin-based Vectors
+ Easy production in *E. coli* or in insect cells (in *E.coli* ~230 mg/l achieved)
+ High affinity for biotin
+ Stable in storage for long periods and *in vivo*
+ Non-cytotoxic, non-pathogenic
- Immunogenic
+/- Efficient internalization into human cells
+ Efficient *in vivo* targeting e.g. via biotinylated antibodies
+ Phase I and II clinical trials in radioimmunotherapy
+ Systemic delivery demonstrated in immunocompetent mice
- Few gene transfer studies performed to date

Abbreviations: pfu, plaque forming unit; FDA, Food and Drug Administrtion (US)

7 CONCLUSIONS

The main conclusions of this thesis are:

1. Baculovirus vectors (*Ac*MNPV) utilize a phagocytosis-like uptake mechanism, regulated by RhoA and Arf6 mediators, to gain efficient transduction into non-phagocytic human cells. The induction of this route may be used to improve the baculovirus-mediated gene transfer efficiency *in vitro*.

2. Non-viral vector TAT-streptavidin is internalized into various human cells. The endosomal releasing agent PPAA enhances nuclear entry of the vector and is non-cytotoxic to cells. Proof-of-principle delivery of biotinylated molecules was demonstrated with biotin-dye. Due to its positive charge, the vector is suggested to be most suitable for drug delivery.

3. Baculovirus accumulates into nuclei near chromatin regions in human cells. Transcription and translation of viral immediate genes was demonstrated to occur concurrently with alterations in the localization and size of the subnuclear structure PML NBs and in the structure of the host cell chromatin. Due to the safety concern, modification of the vector needs to be considered depending on the utilized gene therapy application.

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In Jyväskylä, August 2008

Johanna Laakkonen

YHTEENVETO (RÉSUMÉ IN FINNISH)

Bakulovirus ja streptavidiini geeninsiirtovektoreina ihmisen soluissa

Geeniterapia on kokeellinen lääketieteellinen hoitomuoto, jonka pyrkimyksenä on korvata solujen perintöaineksen puuttuva tai vaurioitunut geeni ja sen tuote. Geeninsiirtoon käytetään sekä viruksiin perustuvia että synteettisiä vektoreita eli kuljettimia. Toistaiseksi kliiniset kokeet ovat kohdistuneet pääasiassa syövän, sydän- ja verisuonisairauksien ja yksittäisten geenivirheiden aiheuttamien sairauksien hoitoon. Tässä väitöskirjassa on tutkittu hyönteisvirukseen sekä bakteerin streptavidiini-proteiiniin perustuvia geeninsiirtokuljettimia solutasolla.

Bakulovirukset (*Baculoviridae*) ovat suuri ryhmä vaipallisia DNA-viruksia, jotka monistuvat vain niveljalkaisissa. Geeninsiirto bakuloviruksella (*Ac*MNPV, ~260 x 68 nm) on osoitettu nisäkässoluissa *in vitro* ja *in vivo*. Bakuloviruksen helppo tuotto, muokkaus ja turvallisuus ovat tehneet viruksesta potentiaalisen kuljettimen geeniterapiaan. Vaikka viime vuosina bakuloviruskuljettimia on onnistuttu kohdentamaan tiettyihin solutyyppeihin, on viruksen karakterisointi solutasolla jäänyt suurelta osin tuntemattomaksi.

Tämän väitöskirjan osatöissä seurattiin bakuloviruksen sisäänmenoa ja lokalisaatiota ihmisen maksa- ja munuaissyöpäsolulinjoissa. Tutkimuksessa osoitettiin viruksia solujen sisällä isoissa vesikkeleissä. Viruksen todettiin käyttävän soluun sisäänmenossaan Arf6- ja RhoAGTPaasien säätelemää fagosytoosinkaltaista mekanismia. Solujen fagosytoosireittiä indusoimalla voidaan mahdollisesti parantaa jatkossa bakuloviruksen sisäänmenoa ja sitä kautta geeninsiirron tehokkuutta nisäkässoluissa.

Tumassa bakulovirusten havaittiin keräytyvän yhteen. Viruksen läsnäolo kasvatti myös promyelosyyttileukemia-tumarakenteita ja muokkasi isäntäsolun kromatiinia. Vaikka bakulovirukset eivät pystykään monistumaan ihmisen soluissa, niiden havaittiin tuottavan virus infektion varhaisen vaiheen proteiinia. Bakuloviruskuljettimien kehityksen kannalta on tärkeää määrittää syntyneet soluvasteet ja muokata kuljetinta siten, että se sopii kunkin geeniterapian sovelluksen tarkoitukseen.

Väitöskirjan kolmannessa osatyössä tutkittiin synteettistä TAT-streptavidiini kuljetinta. Streptavidiinin affiniteettia biotiini-vitamiiniin on aiemmin käytetty hyväksi monissa bioteknologisissa ja diagnostisissa sovelluksissa. Tässä osatyössä streptavidiini kiinnitti biotinyloidut makromolekyylit TAT-streptavidiini kuljettimeen ja solukalvon läpäisevä TAT-peptidi kuljetti ne edelleen solun sisään ja tumaan.

Tutkimuksessa TAT-streptavidiinin osoitettiin menevän tehokkaasti ihmisen soluihin. Suurin osa kuljettimista päätyi kuitenkin hajotusreiteille lysosomeihin. Kalvorakenteista vapautumista ja sitä kautta TAT-peptidien välittämää tumakuljetusta edistettiin biotinyloidun polypropyyliakryylihapon avulla. Fluoresoivan biotiinin tehokas kuljetus soluihin ja edelleen tumaan osoitti TAT-streptavidiini kuljettimen toiminnallisuuden ihmisen soluissa.

Tämä väitöskirja edistää osaltaan synteettisten ja virusperäisten kuljettimien kehittämistä geeniterapian sovelluksiin. Se myös osoittaa TAT-streptavidiini kuljettimen laajan potentiaalisuuden biotinyloitujen molekyylien kuljetuksessa soluihin. Kuljettimien solunsisäisien mekanismien karakterisoiminen on olennaisen tärkeää luotaessa tehokkaita, pysyviä ja turvallisia sovelluksia lääketieteellisiksi hoitomuodoiksi.

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