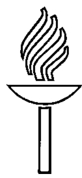

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ABSTRACT

Ojala, Kirsi
Development and applications of baculoviral display techniques
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Diss.

Baculoviruses, especially the *Autographa calicornica* multicapsid nucleopolyhedrovirus (AcMNPV), have become a popular choice for protein expression and have been used for the production of a large variety of heterologous proteins. In addition, display of foreign peptides and proteins on the surface of baculovirus particles has raised interest as an alternative to the prokaryotic phage display system, as insect cells are able to perform complex post-translational modifications of proteins of eukaryotic origin. Moreover, the ability of AcMNPV to transduce a wide range of mammalian cells has brought about the possibility to use these viruses as gene therapy vectors. The present study was aimed at the construction of efficient baculovirus display vectors that could bind to different mammalian cell types and transduce the corresponding target cells efficiently. Thus, the goal was to develop potential baculoviral tools for gene therapy applications. The foreign proteins studied were successfully incorporated on the surface of the baculovirus particles and shown to be functional regarding their ligand-binding activities. The recombinant baculoviruses were further specifically bound to the membrane of the desired cells. In addition, the baculovirus expression system was applied to shed more light on the synthesis and trafficking of rubella virus (RV) envelope proteins in mammalian cells. Together, these results showed that the tropism of AcMNPV could be modified, recombinant baculoviruses could be used as gene transfer vectors and expression of a marker gene in transduced mammalian target cells could be monitored. The baculovirus display and expression techniques are thus potential tools for various applications ranging from studies of protein interactions to gene therapy.

Key words: Baculovirus; display; gene therapy; gene transfer; rubella virus envelope proteins; targeting.

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I Mottershead, D.G., Alfthan, K., Ojala, K., Takkinen, K. & Oker-Blom, C. 2000. Baculoviral display of functional scFv and synthetic IgG binding domains. BBRC 275: 84-90

II Ojala, K., Mottershead, D.G., Suokko, A. & Oker-Blom, C. 2001. Specific binding of baculoviruses displaying gp64 fusion proteins to mammalian cells. BBRC 284: 777-784

III Toivola, J., Ojala, K., Michel, P.O., Vuento, M. & Oker-Blom, C. 2002. Properties of baculovirus particles displaying GFP analyzed by fluorescence correlation spectroscopy. Biol. Chem. 383: 1941-1946

IV Ojala, K., Koski, J., Ernst, W., Grabherr, R., Jones, I. & Oker-Blom, C. 2004. Improved display of synthetic IgG-binding domains on the baculovirus surface. Technol. Cancer Res. Treat. 3: 77-84

V Ojala, K., Tikka, P.J., Kautto, L., Käpylä, P., Marjomäki, V. & Oker-Blom, C. Expression and trafficking of fluorescent viral membrane proteins in baculovirus-transduced BHK cells. (submitted)

RESPONSIBILITIES OF KIRSI OJALA IN THE ARTICLES OF THIS THESIS

Article I: I carried out expression of the different display constructs and both of the ELISA experiments. I am responsible for writing the corresponding parts of the article.

Article II: I am responsible for the study and I also wrote the article.

Article III: I produced and concentrated the virus and wrote the corresponding part of the article. I also gave my suggestions on writing of other parts of the article.

Article IV: I am mainly responsible for this study and I wrote the article. The virus construction was started during my working period at the Institute of Applied Microbiology in Vienna, Austria under the supervision of Dr. Reingard Grabbherr and she also gave some suggestions during the writing. Johanna Koski is doing her master's thesis on this study under my supervision.

Article V: This project was initiated by Päivi J. Tikka for her master's thesis and she carried out a large part of the preliminary studies. After her graduation, I continued the work. Liisa Kautto is doing her master's thesis on some of these studies under my supervision. I am mainly responsible for the experiments and writing of the article with the help of Päivi J. Tikka.

All these studies were carried out under the supervision of Prof. Christian Oker-Blom.

ABBREVIATIONS

aa	amino acid
AAV	adeno-associated virus
AcMNPV	*Autographa californica* multicapsid nucleopolyhedrovirus
ADA	adenosine deaminase
AIDS	acquired immunodeficiency syndrome
BEVS	baculovirus expression vector system
BHK-21	baby hamster kidney cell line
BmNPV	*Bombyx mori* NPV
BSA	bovine serum albumin
BV	budded virion
C	complement
CBHI	cellobiohydrolase I of *Trichoderma reesei*
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CHO	Chinese hamster ovary cell line
CI-MPR	cation-independent mannose-6-phosphate receptor
CMV-IE	cytomegalovirus immediate early promoter
CRS	congenital rubella syndrome
CTD	cytoplasmic terminal domain
E.coli	*Escherichia coli*
E1	envelope protein 1
E2	envelope protein 2
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ER	endoplasmic reticulum
F	major envelope protein of group II BV
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FCS	fluorescence correlation spectroscopy
GFP	green fluorescent protein
gp64	major envelope glycoprotein 64 of group I BV
GV	granulovirus
HIV-1	human immunodeficiency virus type I
HSV	herpes simplex virus
Ig	immunoglobulin
kb	kilobase
kDa	kilodalton
LDL	low-density lipoprotein
LdMNPV	*Lymantria dispar* MNPV
LTR	long terminal repeat

mAb	monoclonal antibody
MHC	major histocompatibility complex
MLV	murine leukemia virus
MNPV	multicapsid nucleopolyhedrovirus
MOI	multiplicity of infection
NPV	nucleopolyhedrovirus
ODV	occlusion derived virion
OpMNPV	*Orgyia pseudotsugata* MNPV
ORF	open reading frame
Ox	2-phenyloxazolone
p.i.	post infection
p.t.	post transduction
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC-3	adenocarcinoma cell line
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PFA	paraformaldehyde
pfu	plaque forming unit
polh	polyhedrin promoter
RFP	red fluorescent protein
RNA	ribonucleic acid
RV	rubella virus
scFv	single-chain antibody
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulfate
SeMNPV	*Spodoptera exigua* MNPV
Sf	*Spodoptera frugiperda*
SFV	Semliki Forest virus
SNPV	single nucleopolyhedrovirus
TfR	transferrin receptor
TGN	*trans*-Golgi network
TM	transmembrane
VEGF	vascular endothelial growth factor
V_H	variable domain of a heavy chain
V_L	variable domain of a light chain
VSV	vesicular stomatitis virus
Z domain	synthetic IgG-binding domain of protein A

1 INTRODUCTION

Baculoviruses are large, double-stranded DNA viruses that infect insects, but do not replicate in mammalian cells and are non-pathogenic to humans. Starting in the 1940's, baculoviruses were first used as natural pesticides in the crop fields (Miller 1996). From 1983, baculoviruses have additionally been widely used for the production of complex, eukaryotic proteins in insect cell cultures (Kost & Condreay 1999). During the last decade, baculoviral display for the expression of peptides and proteins on the surface of this virus has become an increasingly studied system (Grabherr et al. 2001, Oker-Blom et al. 2003). In this way, baculoviruses have followed phages, which have been the choice for display applications for over a decade (Smith & Petrenko 1997). Both can be produced easily at high titers, although baculoviruses are somewhat more expensive to produce. Since bacteria are not able to perform complex post-translational modifications of proteins of eukaryotic origin, insect cells are a good choice for their display. The large insert capacity further favors the baculoviral system. Thus far, baculovirus display has been employed e.g. for library screening (Ernst et al. 1998), antigen presentation (Tami et al. 2000, Kaba et al. 2003) and antibody production (Lindley et al. 2000).

In addition, various studies have shown that baculoviruses are able to transduce a wide range of mammalian cells. This has expanded the baculoviral studies to involve gene transfer vehicles and subsequently, gene therapy vectors (Kost & Condreay 2002). This application is further supported by the non-pathogenic and replication-deficient nature of baculoviruses in vertebrate cells. However, development of targetable vectors is needed for successful gene therapy applications (Nabel 1999).

The main goal of this study was to develop efficient baculoviral display vectors that would be suitable for targeting and gene transfer to mammalian cells. This aim was approached by displaying single-chain antibodies (scFvs) or synthetic immunoglobulin G (IgG)-binding domains on baculovirus particles either as fusions to a second copy of the major envelope glycoprotein gp64 (I, II) or a heterologous membrane anchor derived from vesicular stomatitis virus (VSV) G protein (IV). Recombinant display viruses were then studied for their

binding specificity and transduction efficiency in mammalian cells. These studies are of importance when elucidating the possibilities of baculoviruses as one of the future choices for gene therapy applications.

In addition, baculovirus-mediated transduction of mammalian cells was utilized for the investigation of rubella virus (RV) envelope proteins (V). RV is a human pathogen, which can cause severe malfunctions if acquired during the first trimester of pregnancy. RV is not as well characterized as the other genus, *Alphaviruses*, within the family *Togaviridae* (Frey 1994). Here, the aim was to utilize baculovirus technology to reveal the intracellular localization of the RV envelope proteins during synthesis in RV susceptible BHK cells. This would possibly give insight into the biology of an RV infection and therefore help e.g. in the development of safe vaccines.

2 REVIEW OF THE LITERATURE

2.1 Baculoviruses

The *Baculoviridae* family are large, rod-shaped, enveloped viruses which contain circular double-stranded DNA genomes ranging from approximately 80 to 180 kbp. Baculoviruses include two genera; nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). The GVs have only one nucleocapsid per envelope and are generally small, whereas the NPVs contain either one nucleocapsid (single nucleopolyhedroviruses, SNPVs) or many nucleocapsids (multicapsid nucleopolyhedroviruses, MNPVs) per envelope and are much larger. The enveloped capsids are further embedded in an occluding protein matrix, composed of granulin in the case of GVs and polyhedrin for the NPVs. A single GV is embedded in each granular occlusion body, whereas the polyhedra contain many NPVs. Baculoviruses have a restricted host range, which is usually limited to a few closely related species within a single genus or family (Vialard et al. 1995, Blissard 1996, Miller 1996, Volkman 1997).

2.1.1 Baculovirus life cycle

During the infection cycle of *Baculoviridae*, two virion phenotypes are produced; occlusion derived virions (ODVs) and budded virions (BVs) (Fig. 1). The ODV type is responsible for primary infection of the lepidopteran host and remains viable in the environment due to the crystallized occluded protein matrix. In a natural infection, as the larvae ingest virion occlusion bodies, the alkaline pH of the insect midgut causes the dissolution of the occlusion bodies and the release of the ODVs (Blissard & Rohrmann 1990, Blissard 1996, Miller 1996, Volkman 1997). The ODVs infect the columnar epithelial cells of the midgut (Keddie et al. 1989) by fusion of the envelope of the ODVs with the membranes of the cellular microvilli. Nucleocapsids are released into the cytoplasm from where they are transported to the nucleus, probably by actin filament-mediated mechanism (Charlton & Volkman 1993), for viral transcription and replication. These

epithelial cells then produce the BV type, which buds from the basolateral side of the cells and is responsible for the systemic spread of the infection within the host and also for infection in cell cultures.

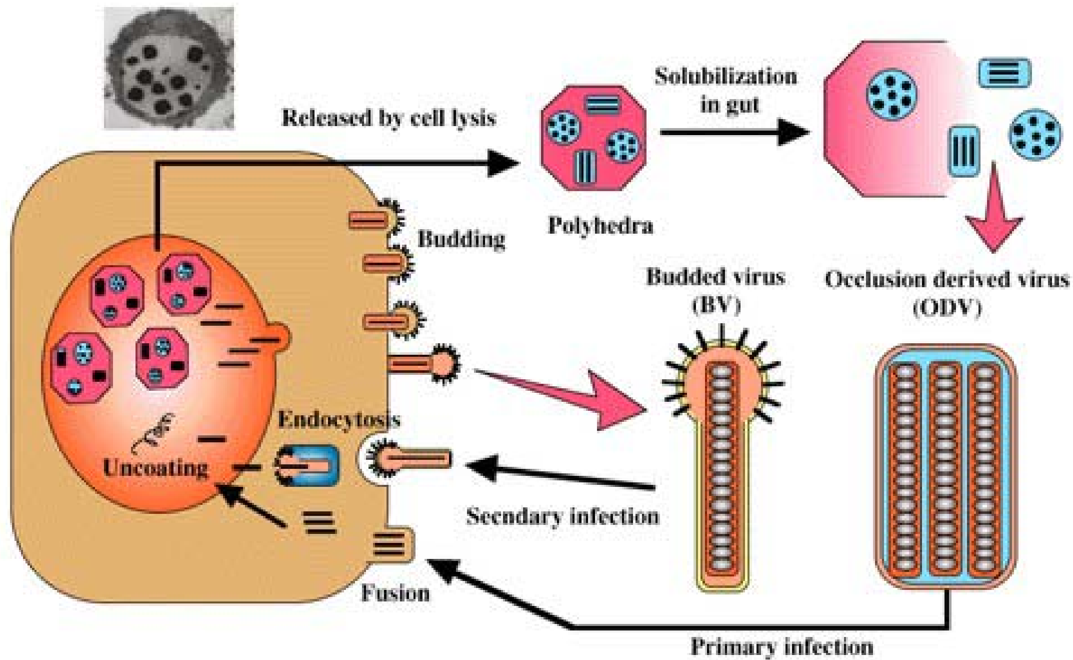

FIGURE 1 The infection cycle of the baculovirus. The figure is taken from the internet page www.biken.osaka-u.ac.jp/kenkyu/fuzoku/emajing_e. html

The infection cycle of baculoviruses can be divided into three phases: early (0-6 hours post infection), late (6-24 h p.i.) and very late phase (18-24 to 72 h p.i.). Budded virus is produced during the late phase of the infection and buds through the plasma membrane of the infected cells, acquiring a loosely fitting envelope. In contrast, ODVs are produced during the very late phase, enveloped within the nuclei and occluded within a crystalline matrix of the occlusion body protein. Occlusion bodies are released when cells are lysed (Blissard 1996, Miller 1996, Volkman 1997).

2.1.2 Virion structure

The most extensively studied baculovirus is *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), originally isolated from alfalfa looper (*Autographa californica*). Its genome is 134 kbp and contains 154 open reading frames (ORFs) (Ayres et al. 1994). The DNA and its associated protein, p6.9, comprise the nucleoprotein core, which is further enclosed by the capsid containing the major capsid protein p39 and other additional minor proteins (Fig. 2) (Blissard 1996). The nucleocapsids average 260 nm in length and 21 nm in diameter (Fraser 1986). The nucleocapsid and viral DNA of the ODV and BV

appear to be identical, differences however can be found in the envelopes and associated structures. For example, the major envelope protein, gp64, of the BV is not found in the ODV, whereas numerous other proteins are found only in the context of the ODVs (Braunagel et al. 1996a, Braunagel et al. 1996b, Belyavskyi et al. 1998, Beniya et al. 1998, Braunagel et al. 1999, Braunagel et al. 2001). The structural proteins of the ODV and BV are shown in Figure 2. In addition, the lipid composition of the envelopes of the BV and the ODV differ significantly, e.g. the major phospholipid of the BV is phosphatidylserine whereas those of the ODV are phosphatidylcholine and phosphatidyl-ethanolamine (Braunagel & Summers 1994).

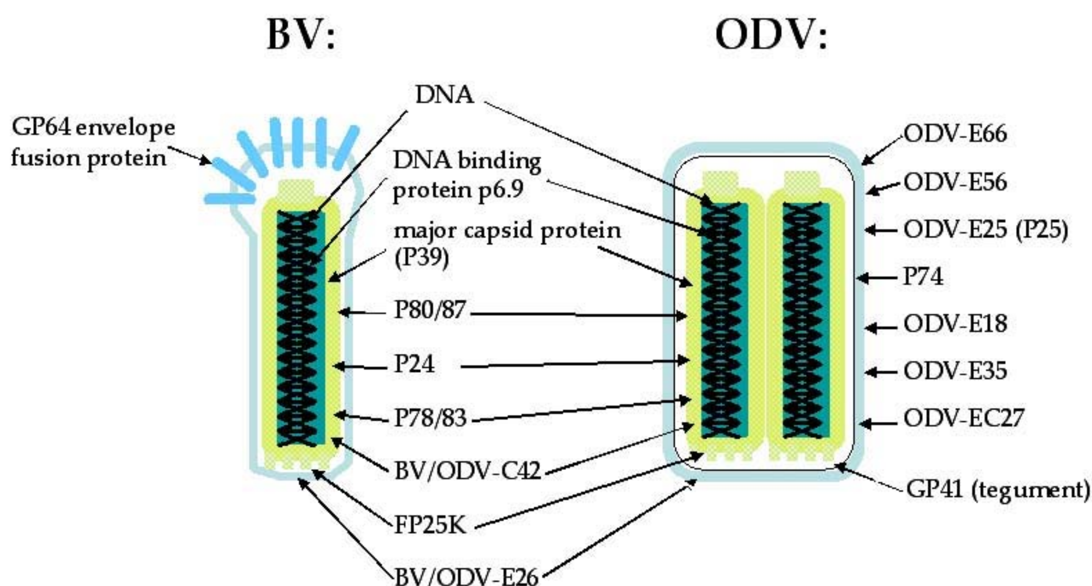

FIGURE 2 Schematic representations of the two forms of baculovirus and their structural proteins. The figure is modified from Blissard & Rohrmann (1990) and Blissard (1996) by Reetta Riikonen and Kirsi Ojala.

2.1.3 The major envelope glycoprotein gp64

The genera NPV of *Baculoviridae* can be subdivided into groups I and II, based on phylogenetic studies (Herniou et al. 2001). Budded viruses of group I NPVs, such as AcMNPV, *Bombyx mori* NPV (BmNPV) and *Orgyia pseudotsugata* MNPV (OpMNPV), contain the major envelope glycoprotein gp64. In contrast, other envelope proteins are characteristic to group II NPVs, including Ld130 for *Lymantria dispar* MNPV (LdMNPV) (Pearson et al. 2000) and Se8 for *Spodoptera exigua* MNPV (SeMNPV) (IJkel et al. 2000). These proteins have been named as F (fusion) proteins (Westenberg et al. 2002).

The gp64 protein is a homotrimeric type I integral membrane glyco-protein, which is present on the surface of the infected cell. From there it is incorporated to the envelope of budding viruses (Oomens et al. 1995) and is

concentrated at the ends of the virions. Gp64 is produced both at early and late phases of the infection cycle, the maximal rate of synthesis being observed at approximately 24-26 h p.i. (Blissard & Rohrmann 1989, Oomens et al. 1995). Trimers are phosphorylated, acylated and glycosylated. Acylation occurs by palmitoylation of the cysteine residue 503 within the transmembrane domain (Zhang et al. 2003). In addition, the trimers are associated by intermolecular disulfide bonds (Volkman & Goldsmith 1984). While gp64 is oligomerized rapidly, the efficiency is very low since only 33% of the synthesized gp64 reaches the trimeric state and is transported to the cell surface. In contrast, monomeric gp64 appears to be degraded within the cells (Oomens et al. 1995).

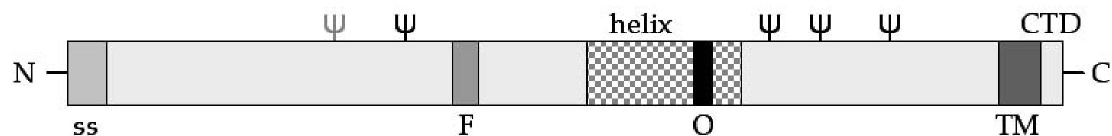

FIGURE 3 The schematic structure of AcMNPV gp64. CTD, cytoplasmic terminal domain; ss, signal sequence; F, fusion domain; O, oligomerization domain; TM, transmembrane domain; ψ, predicted N-linked glycosylation sites; helix, predicted alpha-helical region. The picture is modified from Monsma & Blissard (1995).

Gp64 of AcMNPV encodes a protein of 512 amino acids (aa), which is glycosylated at four of the five potential N-linked glycosylation sites, i.e. at asparagine residues 198, 355, 385 and 426, which are shown in Figure 3 as black (ψ) (Jarvis et al. 1998). The glycoprotein has an N-terminal signal sequence (20 aa) and a hydrophobic transmembrane domain (23 aa) near the C-terminus (7 aa) (Blissard & Rohrmann 1989, Oomens & Blissard 1999). It also contains two functional domains, i.e. the oligomerization domain and the fusion domain (Monsma & Blissard 1995) (Fig. 3). Gp64 is needed for efficient budding of the virion (Oomens & Blissard 1999) and cell-to-cell transmission during the infection cycle (Keddie & Volkman 1985, Monsma et al. 1996). In addition, gp64 is involved in attachment of the virion at the cell surface (Hefferon et al. 1999), although the receptor(s) has remained unknown. There are, however, reports indicating a role for glycoprotein(s) in virus binding (Wickham et al. 1992, Wang et al. 1997). Gp64 is further necessary and sufficient for low-pH-triggered membrane fusion (Blissard & Wenz 1992), which follows the uptake of BVs by receptor-mediated endocytosis (Blissard & Wenz 1992, Charlton & Volkman 1993, Chernomordik et al. 1995, Wang et al. 1997, Markovic et al. 1998, Kingsley et al. 1999). Some entry of BVs has also been observed by an alternate pathway, i.e. by fusion at the plasma membrane, when the gp64 is blocked using a specific antibody (Volkman & Goldsmith 1985, Volkman et al. 1986). Recent studies have demonstrated that a gp64-null baculovirus can be pseudotyped with the F proteins from LdMNPV and SeMNPV (Lung et al. 2002), as well as the G protein of vesicular stomatitis virus (VSV) (Mangor et al. 2001, Lung et al. 2002). These proteins can thus functionally substitute for gp64 as an envelope

protein. In contrast, pseudotyping is not possible with F protein homolog Ac23 from AcMNPV (Lung et al. 2003) or gp75 from Thogoto virus (Lung et al. 2002).

2.2 Baculovirus applications

2.2.1 Biological pesticides

In 1949, research to develop insect pathogens as biological pesticides was started and especially baculoviruses were considered as important candidates for this development. In 1975, the first insect virus was registered in the U.S. as a pesticide, and since then, several baculoviruses have followed as registered pesticides. Industrial interest in development of insect virus pesticides is increasing due to environmental concerns, resistance of insects to chemical pesticides and ability to genetically engineer the viruses to be more effective pesticides (Miller 1996).

2.2.2 Protein expression vectors

The potential of baculoviruses as heterologous protein expression vectors has raised interest for two decades, starting in 1983 when the first report of expression of a foreign protein, namely human beta interferon, in insect cells was published (Smith et al. 1983). Since then the baculovirus expression vector system (BEVS), primarily based on AcMNPV, has been widely used for the production of thousands of proteins (Jones & Morikawa 1996, Possee 1997, Grabherr et al. 2001). The advantages of AcMNPV as an expression vector include the capability to infect at least 32 lepidopteran species within 12 families. It can be easily studied in cell culture and thus has become a widely used expression vector (Volkman 1997). In addition, the cell lines supporting AcMNPV replication grow faster and have higher expression levels than the cell lines for another commonly used virus, BmNPV. Furthermore, the available transfer plasmids and viruses for AcMNPV exceed in number as compared to those for the BmNPV-based system (O'Reilly et al. 1994).

When compared to bacterial protein expression technology, the major advantage of BEVS is the capability to perform post-translational modifications, e.g. proteolytic processing, N-glycosylation, O-glycosylation, phosphorylation and acylation (Miller 1988, King & Possee 1992, O'Reilly et al. 1994). However, there are studies indicating limitations in modification of complex N-linked oligosaccharides, e.g. terminal sialic acid (Jarvis & Finn 1995, Marchal et al. 2001). This can be a significant problem especially with therapeutic recombinant proteins as the sugars often have an influence on glycoprotein function. Efforts have been recently devoted to create engineered baculovirus vectors or transgenic insect cell lines with extended N-glycan processing capabilities (Jarvis & Finn 1996, Jarvis et al. 2001, Seo et al. 2001, Jarvis 2003). In addition,

problems in phosphorylation have been encountered. Difficulties in N-glycosylation and phosphorylation may be due to the very high expression levels in the very late phase of infection which the cell's processing systems fail to keep pace with (O'Reilly et al. 1994).

The most commonly used cell lines for heterologous protein production with AcMNPV include Sf21AE and its clonal derivative Sf9 originally derived from the pupal ovarian tissue of fall armyworm *Spodoptera frugiperda*. Recently, the BTI-Tn-5BI-4 cell line, also known as High 5 cells, derived from the cabbage looper *Trichoplusia ni* egg cell homogenates, has become a popular choice (Luckow 1993, Kost & Condreay 2002). In contrast, when BmNPV is used as an expression vector, propagation of the virus is restricted to cell lines derived from the silkworm *Bombyx mori* (Luckow 1993).

Other advantages of the baculovirus-insect cell system are strong promoters, especially the polyhedrin (*polh*) and *p10* promoters. They are highly transcribed during the very late phase of infection, but are not essential for propagation of budded virus in cell culture. The use of these very late promoters usually leads to very strong expression of the heterologous proteins. It also interferes less with the BV production as compared to the use of late promoters such as *vp39* and *cor*, which controls the expression of the p6.9 protein. The employment of these late promoters might, however, improve the post-translational processing of the proteins, since the modification capacity of the cells appears to decline during the very late phase. Additional advantages of the BEVS include relatively easy construction and scale up of the viruses as compared to other viral vectors (King & Possee 1992, O'Reilly et al. 1994).

In addition to the expression of variety of recombinant proteins, the baculovirus expression system has been utilized in production and characterization of virus-like particles. This can be performed by using baculoviruses encoding multiple viral proteins or individual baculoviruses, each encoding a single protein. This method allows both the study of the virus assembly processes and the safe production of novel vaccines (Kost & Condreay 1999). The utilization of this system has been demonstrated in the production of the virus-like particles of the bluetongue virus (Belyaev et al. 1995, Roy 1996), papillomavirus (Benincasa et al. 1996), polyomavirus JC virus (Goldmann et al. 1999), herpes virus (Newcomb et al. 1999) and canine parvovirus (Gilbert et al. 2004).

2.2.3 Gene delivery vehicles in mammalian cells

The many advantages of baculovirus have recently increased the interest of several groups to study its use as a gene delivery vehicle for mammalian cells. Baculoviruses can be easily manipulated and produced to high titers (>10^9 plaque forming units (pfu) /ml) and they can accommodate large inserts (up to 47 kb have been tested) due to the flexibility of the nucleocapsid (Cheshenko et al. 2001). In addition, transient or stable transduction can be achieved, as well as simultaneous delivery of multiple genes. Furthermore, baculoviruses are unable

to replicate in mammalian cells, but are capable of transducing a broad range of cell types, if a promoter that is active in mammalian cells is utilized. Finally, little or no cytotoxicity is detected in the context of baculovirus transduction (Ghosh et al. 2002, Kost & Condreay 2002).

Due to the use of baculoviruses as biological pesticides, the first safety experiments on non-target cells were conducted in the 1960-70s. Further studies in the 1980s showed no replication capability of baculovirus in the mammalian cells tested and only low levels of reporter gene expression under mammalian promoters (Volkman & Goldsmith 1983, Kost & Condreay 2002). More extensive experiments with recombinant baculoviruses carrying mammalian cell-active promoters were started in the mid 1990s. In the study by Hofmann and colleagues, the virus was modified to contain the *Photinus pyralis* luciferase gene under the control of the cytomegalovirus (CMV) immediate early promoter and was used to transduce human, rabbit or mouse hepatocytes (Hofmann et al. 1995). A year later, in 1996, Boyce & Bucher equipped baculovirus with an expression cassette containing the Rous sarcoma virus promoter and lacZ reporter gene and used it to transduce a variety of cells lines, derived from both liver and other tissues (Boyce & Bucher 1996). Both studies showed high-level expression of the reporter gene in primary hepatocytes and hepatoma cells, but little or no activity in other cell lines. This led to an assumption that baculoviruses prefer cells of hepatic origin, which was supported by other studies (Sandig et al. 1996, Hofmann et al. 1998). However, these studies were followed by others in which a wide range cell types were shown to be efficiently transduced by baculoviral vectors. The vectors carried either the hybrid CAG promoter, composed of the CMV IE enhancer and chicken ß-actin promoter, and luciferase or *lacZ* gene (Shoji et al. 1997), or an expression cassette with the green fluorescent protein (GFP) under the regulation of a CMV promoter (Condreay et al. 1999). These reports described numerous mammalian cell lines, including a number of human, monkey and rodent cell lines that were successfully transduced. The list of cell lines was further expanded by experiments with a recombinant baculovirus pseudotyped with VSV G protein (Barsoum et al. 1997) and a hydrid baculovirus-T7 RNA polymerase system (Yap et al. 1997).

Later on, studies evaluating baculoviruses as gene transfer vectors for various cells have been conducted, showing susceptible cell lines such as osteogenic sarcoma cells Saos-2 (Song & Boyce 2001, Song et al. 2003), U-2OS and Saos-LM2 (Song et al. 2003) as well as prostate tumor cells like PC-3 (Song et al. 2003). Testing of baculovirus transduction efficiency has also widened to human and mouse pancreatic islet cells (Ma et al. 2000) and fish cells with good results (Leisy et al. 2003). As demonstrated by Condreay and co-workers, addition of sodium butyrate or trichostatin A to transduced cells enhanced significantly the level of reporter gene expression (Condreay et al. 1999). The effect of butyrate has been confirmed by many other reports thereafter (Airenne et al. 2000, Sarkis et al. 2000, Hu et al. 2003, Leisy et al. 2003). Butyrate is an agent that inhibits histone deacetylases and thereby induces hyperacetylation of

chromatin, affecting transcription regulation and this way enhancing gene expression in eukaryotic cells (Condreay et al. 1999, Sarkis et al. 2000).

The receptor(s) for baculovirus in mammalian cells is unclear as is the situation in insect cells also. However, electrostatic interactions, including negatively charged heparin sulfate, were shown to play an important role in the first step of transduction in 293 cells (Duisit et al. 1999). Another study by Tani and co-workers suggested that interaction of cell surface phospholipids and the baculovirus envelope protein gp64 was important for baculoviral entry into various mammalian cell lines as well as Sf9 cells (Tani et al. 2001). After binding, regardless of its mechanism, the entry is suggested to happen similarly through receptor-mediated endocytosis both in insect and mammalian cells (Hofmann et al. 1995, Boyce & Bucher 1996, van Loo et al. 2001). Following the escape from endosomes to the cytoplasm, the trafficking of baculovirus capsids in mammalian cells has been enlightened thus far only in two reports (van Loo et al. 2001, Kukkonen et al. 2003). The study by van Loo et al. indicates that actin filaments are involved in the transport of the capsids towards the nucleus (van Loo et al. 2001), similarly as suggested for the insect cells (Charlton & Volkman 1993, Lanier & Volkman 1998). Interestingly, van Loo's study suggests that the nucleocapsids, together with the viral genome, are transported through the nuclear pores for replication in the nucleus, as electron-dense nucleocapsids were observed inside the nuclei. In addition, the report clearly showed that baculoviruses can enter nondividing mammalian cells, as the expression of GFP under the control of CMV promoter was detected in cells arrested in the G_1/S phase. Moreover, viral nucleocapsids were observed in the nuclei of nonmitogenic cells (van Loo et al. 2001).

2.3 Display technology

The main advantage of molecular display technology is the physical linkage between the proteins or peptides displayed (phenotype) and the genes that encode them (genotype). This allows simultaneous selection of the genes with the proteins of desired affinity or function (Hoogenboom & Chames 2000). Many instances have documented the utility of the selection from a large library of variants for the isolation of novel peptides and proteins (Dunn 1996). Display has been performed using different bacterial and eukaryotic viruses, but also bacterial and yeast cells (Dunn 1996, Rodi & Makowski 1999). Additionally, several *in vitro* display technologies have emerged in recent years, including covalent display technology, polysome display and RNA-peptide fusions (FitzGerald 2000).

2.3.1 Phage display

Filamentous phages, M13, fd and f1, are bacterial viruses of about 1 µm in length and 6 nm in diameter. Their single-stranded, circular DNA genome is coated by the major coat protein pVIII, which is present in 2700 copies in wild type virions, constituting approximately 87% of the virion by mass. At one end of the viral particle there are five copies of the minor coat proteins pIII and pVI. The other end of the virion contains similarly five copies of the minor coat proteins pVII and pIX (Fig. 4). The phages infect strains of the F-pilus positive *Escherichia coli* by attachment of the N-terminus of pIII to the tip of the pilus and are further replicated in a non-lytic fashion (Smith & Petrenko 1997, Rodi & Makowski 1999).

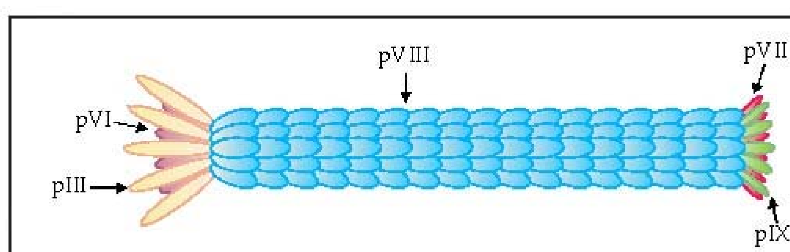

FIGURE 4 The structure of the filamentous phage M13. The coat proteins are indicated. The figure is modified from Smothers et al. (2002).

The first study concerning phage display was published in 1985 by George Smith, who used phages for expressing foreign protein fragments as a fusion to the coat protein pIII of M13 without destroying the infectivity of the particles (Smith 1985). Nowadays, fusion of peptides and proteins to the amino- or carboxy-termini of pIII protein is still the most common form of phage display (Rodi & Makowski 1999, Hoogenboom & Chames 2000). However, N-terminal and C-terminal fusions to pVIII (Clackson & Wells 1994, Rodi & Makowski 1999) and, to some extent, to pVI (Jespers et al. 1995) have also been employed for this purpose. The fused peptides can be incorporated to the native coat proteins or, in many cases, to a second copy of the proteins, which is included for preserving the essential functions of the native proteins. In addition, a phagemid vector can be used in which the fusion is constructed in the phagemid containing the sequence needed for packaging into virions and is transformed with a packaging-deficient helper phage. Especially in the case of pVIII, only short foreign peptides (6-10 amino acid residues) can be displayed on every copy of this protein, since it comprises a large fraction of the virion's mass and the fusions can alter its physical and biological properties drastically. However, larger peptides can be displayed if a wild type copy of pVIII is included to support phage production (Smith & Petrenko 1997, Rodi & Makowski 1999).

The selection of the phages displaying desired molecules is based on biopanning. The phages with specific binding properties are enriched from a

large pool of non-binding clones by multiple rounds of binding to the target. Unbound phages are washed away, bound phages eluted and amplified in host bacteria for the next round of panning (Hoogenboom et al. 1998). As the selection is repeated, the complexity of the pool is reduced and the phage displaying the desired ligand can be characterized by DNA sequencing (Larocca & Baird 2001). Many different selection methods can be used to separate the specific phages: the target can be attached to a solid support, to a column or be in a solution. It can be biotinylated or be a molecule on the surface of a cell or tissue, or even a living animal (Pasqualini & Ruoslahti 1996, Hoogenboom et al. 1998, Hoogenboom & Chames 2000, Larocca & Baird 2001). Biopanning has even be carried out in a patient by injecting phage libraries intravenously and collecting phages from tissue biopsies (Arap et al. 2002). In addition, elution can be carried out in different ways, e.g. using acidic or basic solutions, enzymatic cleavage or competition with excess target (Hoogenboom et al. 1998). Recently, selection for internalized ligands by recovering phage from cells (Barry et al. 1996) or recovering ligand genes from the transduced cells have been demonstrated (Larocca & Baird 2001).

The phage display technology has applications in numerous fields of research, varying from epitope mapping, receptor and ligand identification to antibody development and drug discovery (Smith & Petrenko 1997, Smothers et al. 2002). Proteins successfully displayed on phage include receptors (B domain of protein A, IgE receptor), ligands, enzymes (alkaline phosphatase, trypsin, glutathione transferase), hormones (human growth hormone, angiotensin), protease inhibitors (bovine pancreatic trypsin inhibitor, plasminogen activator inhibitor), DNA binding proteins (zinc fingers) and antibody fragments (different scFvs and Fab fragments) (Clackson & Wells 1994, Smith & Petrenko 1997). This method can also be used for altering protein functions (binding affinity or specificity), studying protein-protein interactions or identifying cell- or tissue-specific markers (Smothers et al. 2002). Other advantages of phage display are the cost-effectiveness and easy production of high titers of phages and their libraries, and that they can be amplified by simply infecting *E. coli* (Smith & Petrenko 1997).

2.3.2 Baculovirus display

Although the phage display technique has been widely used and complex display libraries can be generated, the limitations of this prokaryotic system for post-translational modifications and folding requirements of the displayed proteins has led researches to develop alternative eukaryotic display systems (Davies 1995).

During the last decade, several reports concerning baculoviruses as a potential eukaryotic display system have been published (Grabherr et al. 2001). The first proteins chosen to be displayed were glutathione-S-transferase and the external domain of gp120, a surface glycoprotein of human immunodeficiency virus type I (HIV-1). Both were successfully expressed on the baculovirus

surface as N-terminal fusions to the mature gp64 protein, and gp120 was shown to be functional in binding to its ligand, CD4 (Boublik et al. 1995). This report was followed by Grabherr and colleagues who demonstrated the presentation of the ectodomain of the HIV-1 gp41 envelope protein on the virus surface, either as a fusion to the entire gp64 or, alternatively, to its transmembrane domain (Grabherr et al. 1997). Further, the green fluorescent protein (GFP) and rubella virus (RV) envelope proteins E1 and E2 have been displayed on the baculovirus surface (Mottershead et al. 1997). All these studies employed a second copy of gp64 as a fusion partner, resulting in a mixture of recombinant fusion proteins and wild type gp64 on the virus surface. Recently, successful engineering of native gp64 for display purposes has been reported (Ernst et al. 2000, Spenger et al. 2002), when short peptides were inserted utilizing the naturally occurring restriction enzyme site, NotI, at the position 278 of gp64 (Ernst et al. 2000) or other positions of the gp64 open reading frame (Spenger et al. 2002).

Baculovirus surface display has been used for presentation of antigenic sites from the foot-and-mouth disease virus (Tami et al. 2000) and p67 antigen of *Theileria parva*, the causative agent of the cattle disease East Coast fever (Kaba et al. 2003). In addition, Lindley and co-workers have demonstrated the applicability of baculovirus display for the production of monoclonal antibodies against human nuclear receptors LXRβ and FXR by expressing these proteins on the N-terminus of gp64 (Lindley et al. 2000). Another study showed the generation of a monoclonal antibody against baculovirus-displayed SCAP, an ER protein that cleaves sterol element-binding protein-2. In this case, SCAP was not displayed as a fusion protein, but was expressed on budded virus in addition to the viral proteins, when insect cells were infected with a recombinant baculovirus encoding the SCAP gene. This demonstrates that membrane proteins can be functionally displayed on the baculovirus surface when they are expressed by using the BEVS (Urano et al. 2003).

In addition to the general employment of gp64, display on baculovirus surface has been established using a protein of foreign origin, the G protein of vesicular stomatitis virus (VSV). This protein, when expressed on the baculovirus surface, has been shown to enhance viral transduction of mammalian cells (Barsoum et al. 1997, Park et al. 2001, Tani et al. 2003) and is capable of replacing gp64 or fusions thereof in gp64-null baculoviruses (Mangor et al. 2001). In a previous study, the gp64 signal sequence and the membrane anchor from the VSV G protein were used for the display of GFP on the baculovirus surface (Chapple & Jones 2002). The level of display after fusion to gp64 is relatively low and is restricted to the poles of the virion. However, this novel display strategy led to more efficient display and non-polar distribution of GFP on the viral surface (Chapple & Jones 2002). Finally, Ernst et al. (1998) utilized the baculovirus display system for generation and screening of a eukaryotic expression library by inserting the HIV-1 gp41 epitope “ELDKWA” into the antigenic site B of influenza virus A hemagglutinin. The fusion proteins were expressed on the plasma membrane of baculovirus-infected insect cells

and affinity selected by fluorescence activated cell sorting (FACS), leading to selection of a single clone with increased binding capacity (Ernst et al. 1998).

Recently, an alternative display strategy was developed, in which the enhanced green fluorescent protein (EGFP) was fused with either the N- or C-terminus of AcMNPV major capsid protein vp39. This led to high level incorporation of the EGFP-vp39 fusion into the baculovirus capsid and additionally, seems to be compatible with oligomeric proteins. This novel display tool holds promise for baculovirus entry studies and intracellular targeting both in insect and mammalian cells (Kukkonen et al. 2003, Oker-Blom et al. 2003).

Together these different display strategies suggest applications for baculovirus display in the areas of drug screening, epitope mapping, cDNA library generation, protein-protein interaction studies and gene delivery (Grabherr & Ernst 2001, Grabherr et al. 2001, Oker-Blom et al. 2003).

2.3.3 Other viral display systems

The display of heterologous proteins has also been carried out by using mammalian viruses, including retro-, adeno- and adeno-associated viruses. Retroviruses contain a homotrimeric envelope protein, which has an extraviral domain called the SU subunit that is linked to a membrane-anchored TM subunit. The N-terminal domain of the SU subunit contains receptor-binding activity and critical residues which, if extensively mutated, can abolish binding. Short peptides have been inserted into the exposed loops of the receptor-binding domain of SU and larger proteins into the amino-terminus of SU or N-terminally truncated envelope proteins. The SU subunit also contains a hypervariable domain between N-terminal and C-terminal domains, which tolerates large insertions and deletions (Kayman et al. 1999, Russell & Cosset 1999). Many groups have modified the envelope protein of murine leukemia virus (MLV) to display different polypeptides, including some that are able to bind the Ram-1 phosphate transporter (Valsesia-Wittmann et al. 1996), epidermal growth factor receptor (Cosset et al. 1995) or erythropoietin receptor (Kasahara et al. 1994). The display of V1/V2 domain of HIV-1 gp120 enabled enrichment of the V1/V2-expressing viruses from mixtures with wild type viruses (Kayman et al. 1999). In addition, single-chain antibody fragments (scFvs) specific for the low density lipoprotein (Somia et al. 1995), human major histocompatibility complex (MHC) class I molecules (Marin et al. 1996), hapten (Russell et al. 1993) or carcinoembryonic antigen (CEA) (Konishi et al. 1998, Khare et al. 2001) are among proteins that are successfully displayed on the MLV surface. Another well-studied retrovirus is the spleen necrosis virus, which has also been used for the display of scFvs against a CEA-related protein (Chu & Dornburg 1995, Chu & Dornburg 1997), the transferrin receptor (TfR) (Jiang et al. 1998) or the human Her2neu protein, a member of tyrosine-kinase receptor family (Jiang et al. 1998, Jiang & Dornburg 1999). In addition, the feline leukemia virus tropism could be changed from subgroup A to subgroup C by

randomly substituting the amino acids in the receptor-determining region of the envelope protein and further selecting the library for functional protein (Bupp & Roth 2002). Moreover, pseudotyping with envelope proteins of foreign viruses has also been conducted. This has been demonstrated using the hemagglutinin of influenza virus (Hatziioannou et al. 1998), the G protein of VSV (Burns et al. 1993, Galipeau et al. 1999, Lee et al. 2001, Paquin et al. 2001) and envelope proteins of either HIV-1 (Schnierle et al. 1997) or Sindbis virus (Morizono et al. 2001).

In addition to enveloped viruses, non-enveloped viruses can be used for display. In this case the foreign peptide or protein is incorporated into the capsid of the virus. This has been demonstrated with adenoviruses whose HI loop of the fiber knob was used for the display of the TfR binding motif (Xia et al. 2000) or the FLAG epitope (Krasnykh et al. 1998). Additionally, the adenoviral capsid protein IX has been employed for the surface expression of heparin sulfate (Dmitriev et al. 2002). Furthermore, adeno-associated viruses have been modified to display the immunoglobulin G (IgG) binding domain of protein A (Ried et al. 2002) and tumor-targeting peptides (Grifman et al. 2001) as a part of their loop structures. The HI loop of the VP1 protein of polyoma virus has also been employed for display purposes (Gleiter & Lilie 2001).

Display of the protein moieties on the surface of these viruses is particularly aimed at the development of targeting vectors for gene therapy, which is reviewed below.

2.4 Gene therapy

The development of the methods for delivering genes into mammalian cells has inspired researchers to study the possibilities to treat human diseases by gene therapy (Mulligan 1993). Gene therapy can be defined as "the transfer of new genetic material to the cells of an individual with resulting therapeutic benefit to the individual" (Morgan & Anderson 1993). The basic idea of gene therapy is to treat or eliminate the cause of the disease instead of the treatment of the symptoms (Mountain 2000). At present, gene therapy is used only for the treatment of somatic cells rather than germ-line cells because of the ethical issues involved in the latter (Verma & Somia 1997, Mountain 2000). Different strategies have been employed to treat both inherited and acquired diseases with the help of gene therapy. These can be divided into two main types: viral (Table 1) and non-viral (or physical) vectors (Mountain 2000). Regardless of the type of the vector, there are several factors that are important for an ideal gene delivery vector. It should be suitable for efficient targeting and gene delivery, but lack the potential to self-replicate and produce infective virions in the host cells. In addition, reduced cytotoxicity and site-specific integration would be important. The vector should be cheap and easy to manufacture and, finally, the level and duration of transgene expression should respond to regulation, for

example via an inducible promoter (Verma & Somia 1997, Mountain 2000, Somia & Verma 2000, Lundstrom 2003). Although there is no such ideal vector available, and probably will not be, some of these properties are present in the currently used vectors. The choice of the vector would have to be decided separately for each trial, depending on the disease treated (Verma & Somia 1997, Lundstrom 2003).

TABLE 1 Properties of viral vectors.

Viral vector	Insert capacity	Advantages	Disadvantages
Retrovirus	8.8 kb	- genome integration - most extensively utilized - low immunogenicity	- infects only dividing cells - possibility of insertional mutagenesis - low titers
- lentivirus	9.6 kb	- infects both dividing and nondividing cells	
Adenovirus	7. 5 kb – 36 kb	- high titer - easy to scale up - high transfection efficiency	- no integration → short transgene expression - cytotoxicity - strong immune response - pre-existing antibodies
Adeno-associated virus (AAV)	4.5 kb – 10 kb	- genome integration (wild type) - non-pathogenic to humans - low immunogenicity	- helper virus needed - cytotoxicity - pre-existing antibodies - possibility of insertional mutagenesis
Herpes virus	30-50 kb (152 kb)	- broad host range - latent infection - infection of neurons	- no integration → short transgene expression - cytotoxicity - pre-existing antibodies - low titer
Alphavirus	7.5 kb	- broad host range - high titer - efficient transgene expression	- transient gene expression
Baculovirus	at least 50 kb	- high titer - non-pathogenic to humans - replication-deficient - easy to produce	- no integration → short transgene expression - immune response
Bacteriophage		- easy to manipulate - cheap to produce - replication-deficient	- low gene delivery efficiency - prokaryotic origin

2.4.1 Retroviruses

Retroviruses are single-stranded RNA viruses with (+) strand genomes of 3.5-9 kb, and are the most extensively studied viral vector systems for gene therapy applications. They comprise approximately half of the 300 clinical protocols approved in North America and Europe in the year 2000 (Morgan & Anderson 1993, Mountain 2000). A large portion of these vectors is based on the murine leukemia virus (MLV) (Robbins & Ghivizzani 1998). The replication-incompetent retroviral vectors can be produced in packaging cell lines which provide the viral protein encoding genes *gag*, *pol* and *env*. The production is carried out by transfecting these cells with the retroviral vector which has the insert in place of the virus-encoded proteins and contains a viral packaging signal psi (ψ) and long terminal repeats (LTRs) (Morgan & Anderson 1993, Wu & Ataai 2000). The expression achieved with retroviral vectors is prolonged due to the integration of the transgene into the cell's chromosome with the help of the LTRs and the virus-encoded integrase. However, the duration of the expression is usually shorter *in vivo* as compared to *ex vivo*. Moreover, the transfection efficiency is usually higher *ex vivo* than *in vivo*, partly because of the inactivation by complement encountered in *in vivo* methods (Verma & Somia 1997, Mountain 2000). For retroviral vectors, the limit of insert size is around 8 kb, which is usually enough to code for one large or several small proteins (Morgan & Anderson 1993, Mountain 2000). The major limitations are low titers and the ability to transfect only proliferating cells (Miller et al. 1990), but this can also be thought as an advantage in tumor therapy (Wu & Ataai 2000). In addition, the possibility of random integration of the vector-derived DNA into the chromosomes is of concern, since it can cause oncogene activation and/or inactivation of tumor-suppressor genes (Verma & Somia 1997, Marshall 2000). A significant concern in the use of retroviral vectors is the possibility of generation of replication-competent viruses, which has led to the development self-inactivating vectors. These vectors have deletions in the viral promoter and enhancer regions and are thus safe and transcriptionally regulatable (Somia & Verma 2000, Kootstra & Verma 2003).

Lentiviruses, such as HIV and simian immunodeficiency virus, have also recently been under investigation. They are basically like other retroviruses, but infect both dividing and nondividing cells (Lewis et al. 1992, Naldini et al. 1996, Verma & Somia 1997). Among the lentiviruses, HIV has been most often used as a gene delivery vector. However, clinical experience put limitations with respect to the HIV vectors, mainly because of the safety concerns due to the AIDS origin of the vector (Mountain 2000, Kootstra & Verma 2003, Lundstrom 2003). The recent development of lentiviral vectors, which do not replicate in human cells, has aimed at circumventing these safety problems (Lundstrom 2003). Previously described foamy viral vectors, also called spumaviruses, comprise one additional group of retroviruses, which have been studied for potential gene therapy applications. These viruses have a broad host range but are unable to replicate in nondividing cells. However, efficient transduction of

postmitotic cells has been demonstrated (Somia & Verma 2000, Koostra & Verma 2003).

2.4.2 Adenoviruses

Adenoviruses are linear double-stranded DNA viruses with a genome of 36 kb that cause mild upper respiratory and eye infections in humans (Robbins & Ghivizzani 1998). They can transfect a variety of dividing or nondividing cell types efficiently, but the gene expression is transient since the genome remains episomal in the transfected cells. The virus can be grown to high titers, up to $10^{11\text{-}12}$ pfu/ml. However, the maximum insert size of 7.5 kb sets limits to the use of these vectors (Kay et al. 1997, Mountain 2000). The serious disadvantages of the adenoviral vectors are the strong immune and inflammation responses, preventing repeated dosing as neutralizing antibodies develop against the administered viral particles. The therapy is further difficult due to the pre-existing antibodies from earlier exposures to wild type adenoviruses (Dai et al. 1995, Mountain 2000, Lundstrom 2003). However, a novel method of polymer coating of adenovirus vectors has been shown to shield the virus against antibody recognition (Lundstrom 2003).

The serotype 5 is the most studied adenovirus for gene therapy, followed by serotype 2 (Kay et al. 1997). There are four early genes (E1-E4), of which the E1 is deleted and replaced with the gene of interest. The viruses are further propagated in a cell line providing the E1 protein, usually the human cell line 293 (Morgan & Anderson 1993). Recently, adenoviral vectors, which lack most or all the viral early genes, have been developed. They exhibit reduced immune response and can accommodate larger inserts up to 36 kb. However, these so called "gutless" vectors are more difficult to construct and produce (Chen et al. 1997, Morsy et al. 1998, Mountain 2000, Wu & Ataai 2000, Kootstra & Verma 2003).

2.4.3 Adeno-associated viruses (AAVs)

A clear benefit of AAV is its capability to integrate into a chromosome, allowing long-term gene expression. The process is poorly characterized but wild type AAV integrates specifically into a single site of human chromosome 19. However, it seems that recombinant AAVs integrate less specifically (Mulligan 1993, Balague et al. 1997, Robbins & Ghivizzani 1998, Mountain 2000). This single-stranded DNA virus, containing either a (+) or (-) strand linear genome of approximately 4.7 kb, is a member of the *Parvoviridae* family and is non-pathogenic in humans (Robbins & Ghivizzani 1998). It can accommodate only 4.5 kb of foreign DNA, excluding larger cDNA and gene inserts. However, the packaging capacity has been extended by the observation that AAVs are able to form concatamers, i.e. a functional gene can be obtained in transduced cells by head-to-tail recombination of two genome sequences split over two AAV vectors. This method has increased the insert capacity up to 10 kb (Somia &

Verma 2000, Koostra & Verma 2003). Another disadvantage is the required use of helper virus, such as adenovirus or herpes simplex virus, in the manufacturing process. This can further cause contamination of the recombinant AAV with helper virus proteins and lead to costly purification (Verma & Somia 1997, Mountain 2000, Wu & Ataai 2000). However, recently helper-free production systems have been developed, which might lead to many AAV-based therapies in the future (Mountain 2000, Wu & Ataai 2000).

2.4.4 Baculoviruses

All the features mentioned earlier for the AcMNPV makes this virus an attractive tool also for gene therapy applications, i.e. large insert capacity, high titer, easy manipulation and scale-up, and lack of cytotoxicity and replication in mammalian cells (Pieroni & La Monica 2001, Ghosh et al. 2002, Kost & Condreay 2002). The encouraging results of mammalian cell experiments *in vitro* have led the researchers to test baculoviruses also *in vivo*. In previous studies, recombinant baculoviruses have been used for transduction of rabbit carotic arteries (Airenne et al. 2000) and brain (Lehtolainen et al. 2002), and mouse skeletal muscle (Pieroni et al. 2001) by using the CMV promoter and *lacZ* as a reporter gene. Moreover, *in vivo* gene transfer to neural cells of rodent brain (Sarkis et al. 2000) and to mouse eye (Haeseleer et al. 2001) was detected with an expression cassette constituting of GFP regulated by CMV promoter. In addition, luciferase activity was recently demonstrated in cerebral cortex and testis of mice by direct inoculation of baculoviruses *in vivo* (Tani et al. 2003).

Although baculovirus-mediated gene transfer usually leads to transient gene expression, there are studies demonstrating achievement of stable gene expression. This was shown by Condreay and colleagues when Chinese hamster ovary (CHO) cells were transduced with a baculovirus containing a dominant selective marker, allowing the isolation of a CHO cell line stably expressing GFP (Condreay et al. 1999). Later, it was demonstrated that small, discrete fragments of the recombinant baculovirus DNA, ranging from 5 to 18 kb in size, can randomly integrate into the host cell genome (Merrihew et al. 2001). Site-specific integration was achieved by using another approach, in which a hybrid baculovirus-AAV vector was constructed. This novel vector combined the transduction capacity of baculovirus with the elements of the AAV that are responsible for the integration into a specific site on chromosome 19, leading to prolonged transgene expression (Palombo et al. 1998).

Baculoviral vectors have also shown potential as a cytotoxic vector for tumor cells *in vitro*, when a baculovirus containing the p53 gene was used as a combination with chemotherapy (adriamycin) to kill osteogenic sarcoma cells (Song & Boyce 2001). Furthermore, the possibility to treat hepatic fibrosis was evaluated by efficient gene transfer to hepatic stellate cells, which are activated in response to liver injury (Gao et al. 2002). The differentiated hepatocytes form multicellular islands *in vitro* when grown in chemically defined medium, resembling *in vivo* liver. In these islands only peripheral cells were shown to be

efficiently transduced by baculoviruses. However, Bilello and co-workers demonstrated that the internal cells can be transduced if cells are subjected to transient calcium depletion, leading to transient lost of paracellular junctions (Bilello et al. 2001, Bilello et al. 2003). These studies further facilitate the development of advanced methods for liver gene therapy (Bilello et al. 2003).

The major drawback of baculovirus vector-based gene delivery *in vivo* is the inhibition by complement (C). This was demonstrated already in 1996 by Sandig et al., when *in vivo* gene transfer of a baculoviral vector into liver parenchyma was tested and showed negative results. This led the authors to study the influence of serum *in vitro* and to suggest the involvement of the classical complement cascade in baculovirus inactivation (Sandig et al. 1996). This assumption was later confirmed by Hofmann & Strauss from the same group, demonstrating that the assembly of very late C components was the major cause for baculovirus inactivation. Baculovirus infectivity was, however, maintained by utilizing either a functionally blocking antibody against C component 5, or a C inhibitor cobra venom factor were used as a pretreatment (Hofmann & Strauss 1998). Protection of the baculoviral vectors has been further achieved by soluble complement receptor type 1 (sCR1) (Hofmann et al. 1999) or the synthetic protease inhibitor FUT-175 (Tani et al. 2003), which block both the classical and alternative C pathways (Hofmann et al. 1999). Alternatively, the protection was obtained by displaying human decay-accelerating factor, a complement-regulatory protein, on the viral envelope as a fusion to the gp64 (Huser et al. 2001). The C system is also largely circumvented *in vivo* by direct injections into the brain of mice and rats (Sarkis et al. 2000). Furthermore, the ability to avoid the deleterious effects of serum components was demonstrated by collar-mediated gene delivery to rabbit carotid artery (Airenne et al. 2000). Pseudotyping of baculovirus with VSV G protein may also contribute protection against complement, as suggested by Barsoum et al. (1997) and Pieroni et al. (2001). Some recent studies show induction of interferons (Gronowski et al. 1999) and other cytokines (Beck et al. 2000) after uptake of baculovirus. In addition, evidence of limited inflammation has been observed (Airenne et al. 2000, Haeseleer et al. 2001).

2.4.5 Other viruses

There are a number of other viruses that are currently studied for their possibilities in gene therapy. These include herpes virus, alphaviruses such as Semliki Forest virus (SFV), Sindbis virus, and Venezuelan equine encephalitis virus, poxviruses (especially vaccinia virus), poliovirus and, more recently, phages. From these, poxviruses and herpes simplex virus (HSV) are the most advanced as gene therapy vectors (Mulligan 1993, Kay et al. 1997, Verma & Somia 1997, Mountain 2000). HSV is an enveloped double-stranded DNA virus with a 152 kb genome, encoding at least 80 genes (Koostra & Verma 2003). HSV vectors have an insert capacity of 30-50 kb, which gives HSV an advantage as compared to many other viral vectors (Robbins & Ghivizzani 1998, Wu & Ataai

2000). HSV infects a wide variety of cells types and remains episomal in the transduced cells, which minimizes the possibility of insertional mutagenesis. The virus can stay latent in the central nervous system, making it a potential vector for gene transfer to neurons (Mulligan 1993, Friedmann 1997, Friedmann 2000, Wu & Ataai 2000). However, the cytotoxicity and low titers obtained, in addition to pre-existing antibodies, limit the use of HSV as a gene transfer vehicle (Verma & Somia 1997). The development of HSV amplicons, which do not contain viral genes besides the *cis*-acting elements, have overcome these problems to a great extent. The amplicons have also large packaging capacity, theoretically up to 152 kb. However, long-term gene expression has not been demonstrated (Koostra & Verma 2003).

Alphaviruses, single-stranded RNA viruses, have also gained attention as possible gene delivery vectors due to their broad host range, easy high-titer production and efficient transgene expression. Recombinant vectors are generated by co-transfecting helper vector RNA with an alphavirus replicon, leading to only one round of RNA replication, but no further production of virus progeny. The major limitation of alphaviruses is transient transgene expression in every susceptible cell type, although this feature can be useful in certain cancer gene therapies or vaccination (Wahlfors et al. 2000, Lundstrom 2003).

Lately, phages have also raised interest as a vector for gene therapy. They share many of the advantages of baculoviruses, i.e. lack of replication in mammalian cells, easy manipulation and cost effective production of high titer stocks. However, the efficiency of gene delivery is low compared to other viral vectors (Larocca & Baird 2001).

2.4.6 Non-viral vectors

As a whole, non-viral and physical gene transfer methods are less efficient than viral vectors. In addition, the gene expression is transient, but there is no limit to the size of the genes they can deliver. In addition, non-viral vectors are not immunogenic and are easy to prepare (Kay et al. 1997, Mountain 2000). The most important non-viral methods include naked DNA, condensed DNA particles composed of DNA and cationic lipids or polycationic carriers and needle-free injections (Friedmann 1997, Mountain 2000, Ogris & Wagner 2002).

Naked DNA, usually plasmid DNA, is locally injected into the tissues where the cells take up the DNA by an unknown mechanism. The most promising application is naked DNA vaccines, as even small amounts of antigen encoded by the plasmid can elicit humoral and cellular immune responses, leading to immunity (Felgner 1997, Mountain 2000). Manufacturing of naked DNA is simple and cheap, but the gene-delivery is inefficient and in many cases only transient expression is observed. Furthermore, this transfer method is not suitable for targeting (Mountain 2000).

The complex of DNA and cationic lipids is formed by electrostatic interaction, when the cationic headgroup binds the negatively charged DNA.

The complex also includes a lipid tail, which enables the hydrophobic collapse of the lipid-DNA (Ogris & Wagner 2002). These complexes are somewhat efficient *ex vivo*, but the overall gene transfer efficiency into the nucleus is low, probably due to the poor uptake and inefficient trafficking within the cells (Kay et al. 1997, Mountain 2000, Ogris & Wagner 2002). The polycationic carrier/DNA complexes contain DNA within nonlipid, cationic polymer coat, which consists of naturally occurring histones or protamines, or synthetic compounds, e.g. poly-amino-acids, polylysine, polyarginine or polyhistidine (Ogris & Wagner 2002). Both types of condensation protect the genes from degradation by cellular enzymes and enhance targeting and uptake (Friedmann 1997, Mountain 2000). For example, the polycationic carriers usually contain primary amino groups, which can be used for chemical attachment of additional components to enable further protection and targeting (Ogris & Wagner 2002).

The gene delivery by needle-free injection can be achieved by using a gene gun, a device that uses high-pressure helium stream to "shoot" gold particles coated with DNA straightly to the cytoplasm. A variety of cell types can be transfected *ex vivo*, but *in vivo* this method is applicable mainly to skin cells. The "gene gun" technology may have promising applications in the preventative vaccination similar to naked DNA (Mountain 2000).

2.4.7 Targeted gene delivery

Only a few publications concerning *in vivo* targeting of viral vectors have been published, but there have been many studies describing different strategies for modification of the binding characteristics of viral vectors (Peng & Russell 1999). Targeted gene delivery can be achieved by modifying the viral vector surface either by displaying heterologous peptides and proteins, or by pseudotyping the vectors with foreign proteins as mentioned above. Targeting can also be accomplished by using bispecific antibodies. These strategies have been demonstrated to lead to more efficient targeting by many of the viral vectors used in gene therapy to date. Targeting can also be used to inhibit gene delivery to a specific target cell, to change or narrow the tropism, or to modulate the trafficking and localization of the gene delivery vehicles (Peng & Russell 1999, Russell & Cosset 1999).

In addition to the various examples of viral display of different peptides and proteins listed above, successful retargeting has been demonstrated in several other studies. For example, display of epidermal growth factor (EGF) and single-chain antibodies against melanoma antigen or human MHC class I molecule as a part of influenza hemagglutinin on a pseudotyped retrovirus has led to specific binding to cells expressing the targeted cell surface molecules (Hatziioannou et al. 1999). Furthermore, redirected binding of adenoviral vectors has been achieved by modifying the virus particle to express for example an α_v integrin-binding RGD motif (Wickham et al. 1997b), a peptide specific for the heparin sulfate receptor (Wickham et al. 1997b) or peptides

which selectively bind to human umbilical vein endothelial cells (Nicklin et al. 2000). Also, a recombinant HSV-1 has been engineered to display the erythropoietin hormone and was thus targeted to erythropoietin receptor-bearing cells (Laquerre et al. 1998).

Bispecific antibodies can be used as a linker, which binds both to the vector surface and a receptor on the cell membrane. This provides a molecular bridge between these two and may be used to redirect vector attachment (Peng & Russell 1999, Russell & Cosset 1999). In many cases, the antibody complex serves two functions: it blocks native receptor binding and simultaneously redirects the virus to a novel receptor (Wickham 2000). This has been demonstrated with antibody linkers such as those that contained in one end anti-FLAG antibody, which bound to an adenovirus displaying the FLAG epitope, and in the other end either an anti-α_v integrin (Wickham et al. 1996), human CD3 antibody (Wickham et al. 1997a) or anti-E-selectin antibody (Harari et al. 1999) which bound to a respective cellular receptor. Also, an antibody against the adenoviral fiber protein conjugated to folate was shown to target an adenoviral vector to folate receptor expressing cells, simultaneously preventing the binding to the native receptor (Douglas et al. 1996). The same was shown for a pseudotyped retrovirus bearing an avian leukosis virus envelope protein, which was redirected by an antibody bridge to cells expressing the epidermal growth factor receptor (Boerger et al. 1999). The bispecific antibody can also be used solely to redirect the vector, preserving the native receptor binding (Wickham 2000).

Inverse targeting, i.e. inhibition of infection of a specific cell line, has been demonstrated by using retroviral vectors displaying either EGF or stem cell factor, which bind to the EGF and Kit receptors, respectively. This display selectively destroys the ability to infect cells expressing these receptors and hence the viral vectors specifically transduce receptor-negative target cells in a mixed cell population (Cosset et al. 1995, Fielding et al. 1998). Another strategy is to mutate or delete the domain required for binding to the natural receptor, as shown for retrovirus (MacKrell et al. 1996) and adenovirus (Von Seggern et al. 1999), and add novel binding domains to retarget the vectors. Moreover, display of a protease-cleavable receptor-blocking domain on the virus surface can be used for restriction of the host range. In this case, exposure of the virus particle to a certain protease at the site of high target protease activity cleaves the blocking domain and restores the virus infectivity (Peng & Russell 1999, Russell & Cosset 1999). The examples include retroviral vectors displaying domains cleaved and activated by proteases such as factor Xa (Morling et al. 1997), matrix metalloproteinases, plasmin and furin (Russell & Cosset 1999).

2.4.8 Diseases treated by gene transfer

Almost two-thirds of the clinical gene therapy trials have been for cancer (Ogris & Wagner 2002). There are several forms of gene therapy that can be used for cancer treatment; in many cases the genes used for cancer gene therapy are

aimed towards killing the tumor cells either directly (toxin genes) or indirectly (suicide genes such as HSV thymidine kinase). Also approaches to block the cell cycle, induce apoptosis or correct gene defects, e.g. by introducing tumor suppressor genes, have been used. Alternatively, genes for tumor angiogenesis blocking or cell metastasis inhibition have been tested (Mountain 2000, Ogris & Wagner 2002). The enhancement of immune responses against tumors is further widely employed for cancer treatment. In these methods the cancer cells, which are often capable of hiding themselves from the immune system, are made more visible e.g. by introducing genes encoding cytokines or antigens predominantly found on cancer cells. This should then activate lymphocytes and lead to an attack towards the cancer (Blaese 1997).

Various vascular diseases, such as atherosclerosis, postangioplasty restenosis and peripheral atherosclerotic vascular disease are also attractive targets for gene therapy due to the easy access of blood vessels and, in most disorders, requirement of only a temporary expression of transgenes (Ylä-Herttuala 1996, Ylä-Herttuala & Martin 2000). The therapeutic techniques often aim towards the inhibition of smooth muscle cell proliferation (Ohno et al. 1994). The inhibition of connective tissue formation and growth factor activities are also important (Ylä-Herttuala 1996). In addition, stimulation of angiogenesis by introducing angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factors, has been used in the treatment of peripheral vascular disease, coronary heart disease and ischaemic myocardium (Ylä-Herttuala & Martin 2000, Ylä-Herttuala & Alitalo 2003). People suffering from familial hypercholesterolemia have an elevated cholesterol level due to a defect in their low density lipoprotein (LDL) receptor, leading to atherosclerosis and coronary heart disease. The receptor protein is synthesized mainly in the liver and thus gene therapy is focused on transduction of hepatocytes, mainly *ex vivo*, with a vector introducing a functional LDL receptor gene (Morgan & Anderson 1993, Ylä-Herttuala 1996).

To date, many clinical trials on gene therapy, in addition to cancer, are focused on gene transfer into hematopoietic stem cells. They are the ultimate progenitors of all blood cells and would provide a continuous supply of genetically modified hematopoietic cells (Mulligan 1993, Kay et al. 1997). Therefore, gene therapy of these cells would be attractive for a treatment of a large number of congenital and acquired blood cell diseases. The gene therapy is carried out by harvesting the cells from a patient, transferring the desired gene *ex vivo*, usually by retroviral vectors, and infusing transduced cells back to the patient (Kay et al. 1997). The first hematopoietic gene therapy trials focused on adenosine deaminase (ADA) deficiency, an inherited fatal disease in which a T cell defect leads to severe combined immune deficiency (SCID). A normal copy of the ADA gene was introduced into the T lymphocytes using a retroviral vector (Morgan & Anderson 1993). Recently, successful treatment of SCID-X1 was attained by using a retrovirus-derived vector to infect $CD34^+$ cells of patients. SCID-X1 is an X-linked inherited disorder of lymphocyte differentiation, which is caused by mutations in the gene encoding a γ_c cytokine

receptor subunit of various interleukin receptors. The therapy with a retrovirus carrying this gene was shown to result in sustained correction of the SCID-X1 phenotype (Cavazzana-Calvo et al. 2000).

Numerous other diseases and disorders have also been targets of gene therapy, including cystic fibrosis, hemophilia A and B, and neurological diseases such as Parkinson's and Alzheimer's (Verma & Somia 1997). Furthermore, many studies have focused on gene therapy of infectious diseases, particularly acquired immunodeficiency syndrome (AIDS). The therapies for AIDS aim primarily to stop HIV from infecting or replicating inside the cells. This can be achieved for example by generation of inactive forms of the HIV proteins or antibody fragments within cells, preventing the assembly of virus particles. In addition, the enhancement of the immune response against HIV by intracellular vaccination can be used (Mulligan 1993, Blaese 1997).

2.5 Rubella virus

Rubella virus (RV) belongs to the family *Togaviridae* as the only member of the genus *Rubivirus*, whereas the other genus *Alphavirus* consists of 27 viruses. RV is a significant human pathogen, causing a mild childhood disease known as German measles, but if acquired during the first trimester of pregnancy, the fetus may suffer from severe malformations. This condition is known as congenital rubella syndrome (CRS) and commonly involves birth defects such as deafness, mental retardation and heart defects (Frey 1994).

2.5.1. The RV genome

The rubella virus genome contains a positive, single-stranded 40S RNA genome, which is 9756 nucleotides in length excluding the 5′ cap structure and 3′ terminal poly(A) tail. The poly(A) length is heterogeneous, the mean length being 53 nucleotides (Wang et al. 1994). The genome contains two long open reading frames (ORFs). The 5′ proximal ORF is 6345 in length and encodes the nonstructural proteins. The 3′ proximal ORF, 3189 in length, encodes the structural proteins. The nonstructural proteins are involved in RNA replication while the structural proteins, i.e. the capsid protein and the two envelope proteins E1 and E2, form the virion, which is approximately 60 nm in diameter. The capsid protein is associated with the viral genome, whereas the E1 and E2 proteins are anchored to the viral envelope (Frey 1994).

2.5.2 The envelope proteins of RV

The RV structural proteins are processed from a single polypeptide, p110, which is encoded during the viral infection from a subgenomic 24S RNA (Oker-Blom 1984). The proteins are organized in the order NH_2-C-E2-E1-COOH

(Oker-Blom 1984) so that the signal sequence of the E2 protein is located at the C-terminus of the capsid protein and similarly, the signal sequence of E1 can be found at the C-terminus of E2 (Frey 1994) (Fig. 5). The post-translationally cleaved p110 gives rise to these proteins of sizes 33 kDa (capsid protein), 42-47 kDa (E2) and 58 kDa (E1) (Oker-Blom et al. 1983, Pettersson et al. 1985). The envelope proteins E1 and E2 are class I transmembrane proteins, i.e. they contain a single transmembrane sequence. They are also type I glycoproteins with four or five potential N-linked glycosylation sites, respectively. In addition, E2 is O-glycosylated (Frey 1994).

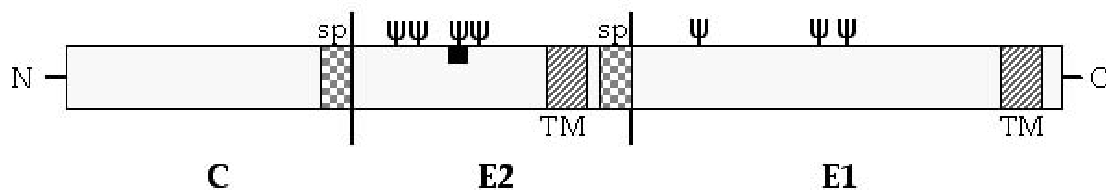

FIGURE 5 The rubella virus polyprotein p110, precursor of the structural proteins. C, capsid protein; E1 and E2, the envelope proteins; sp, signal peptide; TM, transmembrane domain; Ψ, glycosylation sites; black box, O-linked glycosylation site. The figure is modified from Frey (1994).

There appear to be six different major antigenic epitopes within E1 recognized by antibodies that exhibit neutralization and/or hemagglutination inhibition activity. E2 seems to contain three epitopes, one possibly being a neutralization domain (Frey 1994).

The E1 and E2 glycoproteins are translocated to the endoplasmic reticulum (ER) by the corresponding signal peptides. There they form a heterodimer, which is needed for efficient transport of both proteins to the Golgi complex (Hobman et al. 1993). The proteins reside in the Golgi where the RV nucleocapsid assembly and intracellular budding of RV particles occur in most cell lines, e.g. in BHK cells (Frey 1994). Both proteins have been shown to contain intracellular retention signals: the E1 retention signal arrests the protein in the ER in the absence of E2 (Hobman et al. 1992, Hobman et al. 1997), while E2 contains a Golgi retention signal which is enough to retain the E1-E2 heterodimer in this organelle (Hobman et al. 1995). The studies also indicate that the transmembrane and cytoplasmic domains of the E2 and E1 proteins are required for early and late steps, respectively, in the virus assembly (Garbutt et al. 1999). This is supported by a study demonstrating that when the budded viral particles are released from the cells possibly via the secretory pathway, the E1 envelope protein, and especially its transmembrane and cytoplasmic domains, is required for virus release (Yao & Gillam 1999, Yao & Gillam 2000).

2.5.3 Rubella virus entry

The natural host for the rubella virus is the human, but the virus replicates in a wide variety of vertebrate cell lines, although usually at low titers. Higher titers

can be obtained by using BHK-21 cells and Vero cells and thus these cell lines have mainly been utilized for RV studies (Frey 1994). The attachment and entry route of the RV in susceptible cells is only poorly characterized. However, studies by Mastromarino and colleagues using erythrocytes (Mastromarino et al. 1989) and Vero cells (Mastromarino et al. 1990) indicate that membrane lipid molecules, especially phospholipids and glycolipids, are involved in the cellular binding sites for RV. These studies also suggest that different lipid components participate in the attachment and the fusion step (Mastromarino et al. 1989), whereas proteins did not appear to have a role as an RV receptor (Mastromarino et al. 1990).

After the rapidly occurring initial binding, viruses enter the cells via the endocytic pathway, which has been demonstrated by using lysosomotropic compounds (Petruzziello et al. 1996). The internalization is slow as a period of 7 hours is required for all adsorbed virus to enter the cells (Petruzziello et al. 1996). This is different from alphaviruses, which are rapidly internalized (Marsh & Helenius 1989). The viruses are presumably released from the endosomes by a low pH-induced conformational change of the RV envelope proteins. This was shown by treating the glycoproteins with acid, which led to an irreversible change of the proteins, making the E1 polypeptide very resistant and E2 very susceptible to trypsin. These results suggest that E1 plays a predominant role in membrane fusion (Katow & Sugiura 1988). In addition, it has been shown that a region of E1 between amino acids 81 and 109, the internal hydrophobic domain, is involved in the membrane fusion (Yang et al. 1998, Qiu et al. 2000).

2.5.4 Expression of recombinant RV structural proteins

In general, authentic RV is used in vaccination and diagnostic tests for the detection of RV-specific antigens. However, the production of native RV particles in mammalian cells is laborious and the isolation of the protein subunits in native form is difficult as the antigenicity is easily lost (Seto & Gillam 1994). For this reason, strategies to produce individual RV structural proteins by heterologous expression systems have been developed. Especially, the baculovirus expression system has been employed to produce the capsid (Schmidt et al. 1998), E2 (Seto et al. 1995) and E1 proteins (Lindqvist et al. 1994, Seto & Gillam 1994, Johansson et al. 1996, Schmidt et al. 1996). Antibodies against E1 are predominant after most RV infections, but in the case of CRS, antibodies against E2 are usually more abundant (Frey 1994). This supports the need to use individual RV proteins instead of native RV for diagnosis of rubella infections (Seto & Gillam 1994, Seto et al. 1995). Hence these purified recombinant viral proteins may have applications especially in diagnostics, vaccine development and immunization, but also in structural studies (Seto & Gillam 1994, Johansson et al. 1996).

3 AIMS OF THE STUDY

The display of foreign peptides and proteins on the surface of bacteriophages has been an important tool for over a decade for library engineering, e.g. antibody and antigen selection (Hoogenboom & Chames 2000) and recently, also for gene delivery (Larocca & Baird 2001). However, the inability of bacteria to process complex eukaryotic proteins has raised an interest to develop a eukaryotic display system, which would be a) easily scaled up, b) able to accommodate large inserts and c) be safe for use in various applications, including gene therapy. The baculovirus display system has recently shown potential where these aspects are concerned (Grabherr et al. 2001). Thus the specific aims of this study were:

1. To create baculoviral vectors that display functional protein moieties on their surface.

2. To engineer the display vectors for monitoring gene expression in both insect and mammalian cells.

3. To improve the efficiency of foreign peptide or protein display on the baculovirus surface.

4. To evaluate the engineered baculovirus vectors as targetable gene delivery vehicles in mammalian cells, the final goal being gene therapy applications.

5. To apply the efficient baculoviral transduction of mammalian cells for studying the synthesis and trafficking of a viral membrane protein in these cells.

4 SUMMARY OF THE MATERIALS AND METHODS

The materials and methods are described in more detail in the original publications I-V.

4.1 Cell lines

For cloning of the plasmids, *E. coli* JM109 cells (II, IV, V) were used. The recombinant baculoviral genomes were produced in *E. coli* DH10Bac cells (V). For baculovirus propagation and purification, *Spodoptera frugiperda* 9 (Sf9) or 21 (Sf21AE) cells, were used (I-V). In cellular binding and transduction studies, either baby hamster kidney (BHK-21) cells (II, IV) or an adenocarcinoma cell line, PC-3 (II), were employed.

The bacterial cells were grown in suspension cultures in Lucia-Bertani medium at 37 °C in the presence of appropriate antibiotics, when selecting the desired plasmid clones (I, II, IV, V). The insect cells were grown in monolayer or suspension cultures at 28 °C in serum free medium; either HyQ SFX-Insect medium (HyClone Inc., Logan, UT, USA) or Insect-Xpress (Cambrex, Walkersville, MD, USA) without antibiotics (I-V). BHK cells were maintained in a monolayer in BHK-21 medium (Gibco-BRL, MD, USA) containing 5% fetal bovine serum (FBS), 10% tryptose phosphate broth, 1% penicillin/streptomycin, and 2 mM L-glutamine (II, IV, V). PC-3 cells were maintained in DMEM (Gibco-BRL) supplemented with 4500 mg/l glucose, 10 mM Hepes, 10% FBS, 2 mM L-glutamine and 1% nonessential amino acids (II).

4.2 Isolation and specificity of CEA-binding scFv

A human single chain antibody (scFv) specific for carcinoembryonic antigen (CEA) was isolated from a mixture of scFv/kappa and scFv/lambda

phage libraries by five rounds of panning using CEA (Calbiochem, Darmstadt, Germany). The DNA encoding the scFv was isolated and cloned into the pKK2223-3 tac vector (Takkinen et al. 1991). The specificity of the CEA binding scFv was determined by competitive ELISA, in which increasing amounts of soluble CEA was added to the culture supernatant of the transformed *E. coli* strain RV 308 and incubated on a microtiter plate coated with CEA. Binding was detected with an anti-myc peptide antibody and alkaline phosphatase-conjugated anti-mouse IgG (H+L) (Promega, Madison, WI, USA) (I).

4.3 Plasmids

For construction of the viral clones displaying various polypeptides as a fusion to the second copy of the viral envelope protein gp64 (I), the plasmid pFLAGgp64 was used (Mottershead et al. 1997). The plasmid contains the FLAG epitope and the signal sequence of the GluR-D glutamate receptor. The recombinant viruses were equipped with a fluorescent expression cassette introduced by a plasmid p10CEG, based on pAcUW42 (BD PharMingen San Diego, CA, USA) (II). This plasmid contains GFP under the transcriptional regulation of the polyhedrin promoter and EGFP under the CMV-IE promoter. The plasmid for constructing the display virus, in which the membrane anchor of VSV G protein was used (IV), is described elsewhere (Chapple & Jones 2002). In addition to the C-terminal 70 amino acids of VSV G protein, this plasmid contains the signal sequence of AcMNPV gp64. Construction of plasmids for the fluorescent rubella virus envelope proteins was performed by using the pFASTBAC1 vector (Bac-to-bac®, Baculovirus Expression Systems, Invitrogen Incorporation, Carlsbad, CA, USA) (V).

4.4 Construction of recombinant baculoviral vectors

The ability of baculoviruses to display various peptides and proteins was studied by fusions either to the N-terminus of a second copy of the baculoviral major envelope protein gp64 (I-III) or to a heterologous membrane anchor of vesicular stomatitis virus (VSV) G protein (IV) (Table 2). The displayed proteins included single-chain antibodies (scFvs) specific for 2-phenyloxazolone and CEA (I, II), and the synthetic IgG-binding (ZZ) domains of protein A (I, II, IV). The construction of hapten binding scFvs specific for 2-phenyloxazolone (Ox scFv) has been described previously (Alfthan et al. 1995). Briefly, the Ox scFv variable domains V_H and V_L were connected with two different linkers; either a 28 aa linker peptide from the fungal cellulose CBHI (L1) or a ($GGGGS_3$) linker peptide (L4) (Alfthan et al. 1995). The various scFvs (I) and the ZZ domains (I,

IV) were amplified by polymerase chain reaction (PCR) and cloned into the respective plasmids for transfections.

To study viral infection and transduction in insect and mammalian cells, respectively, the recombinant display viruses AcCEAscFvgp64, AcZZgp64 (II) and AcZZVSVgTM (IV) were further equipped with a fluorescent expression cassette, which is labeled EGFP at the end of each construct. In addition, a viral vector called Ac-EGFP without any displayed protein, i.e. expressing only wild type gp64, but containing the fluorescent expression cassette, was constructed for control purposes (II).

The recombinant display baculoviruses were produced in Sf9 cells by homologous recombination, i.e. transfecting the cells with viral DNA and plasmid and further purifying the viruses by subsequent rounds of plaque purification (I, II, IV). The viruses encoding the RV envelope fusion proteins, cloned into the pFASTBAC1 vector, were produced in DH10Bac cells by transposition according to the manufacturer's instructions (Invirogen Incorporation) (V). The purified viruses were propagated in Sf9 cells infected at multiplicity of infections (MOIs) of 1-10 plaque forming units (pfus)/cell and titered by end-point dilution (O'Reilly et al. 1994). For storage, viral stocks were supplemented with 2.5% FBS and stored at 4°C. Viral stocks concentrated either by ultracentrifugation (III) or by sucrose gradient and ultracentrifugation (IV) (Mottershead1997) were resuspended in TE (III) or PBS (IV) and titers were determinated as described above (IV).

TABLE 2 Baculoviral display constructs.

Signal sequence	Displayed protein	Fusion partner	Publication
GluR-D	CEA scFv, ($GGGGS_3$)-linker	AcMNPV gp64	I, II
GluR-D	1-2x Z domains of protein A	"	I, II
GluR-D	Ox scFv, ($GGGGS_3$)-linker (L1)	"	I
GluR-D	Ox scFv, CBHI-linker (L4)	"	I
GluR-D	GFP	"	III
gp64	ZZ domains of protein A	VSV G TM+CTD	IV

4.5 Characterization of the display viruses

4.5.1 Western blotting

Protein samples derived either from infected cells or concentrated viruses were analyzed by SDS-PAGE by standard procedures (Laemmli 1970) and transferred to nitrocellulose membrane (Towbin et al. 1979). Immunostaining was carried out by using different primary antibodies (Table 3) and alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG (H+L) (Promega) secondary antibodies. The immunoblots were developed by adding the substrate solution containing NBT and BCIP (Bio-Rad Laboratories, CA, USA)

(I, II, IV, V). In the case of the recombinant viruses described in the paper IV, the amount of the concentrated viruses applied to the wells was set to an equal level according to the obtained pfus.

TABLE 3 Primary antibodies used in the studies.

Antibody	Target	Source	Provider
B12D5	gp64 envelope protein	mouse	Dr. Loy Volkman
M1	FLAG epitope	mouse	Sigma, MO, USA
anti-CEA	CEA	mouse	Calbiochem, Germany
IgG	-	bovine	Sigma, MO, USA
IgG	-	rabbit	Sigma, MO, USA
anti-TfR	human TfR	rabbit	Cymbus, UK
R322	αvβ5 integrin	rabbit	Dr. Jyrki Heino
anti-avidin	avidin	rabbit	Dr. Markku Kulomaa
GM130	*cis*-Golgi	mouse	BD Biosciences, CA, USA
PDI	ER	mouse	Stressgen, Canada
CD63	lysosomes	mouse	Zymed, CA, USA
CI-MPR	late endosomes, *trans*-Golgi	rabbit	Dr. Varpu Marjomäki

4.5.2 Enzyme-linked immunosorbent assay (ELISA)

To analyze the binding properties of the fusion proteins, ELISA assays were utilized. Microtiter plates of 96-wells were coated with different antibodies and antigens specific for the displayed proteins diluted in 0.1 M bicarbonate buffer (pH 9.6). These included bovine IgG, CEA (I, II), Ox_{19}BSA (I) and rabbit IgG (IV). After overnight incubation at 4 °C, wells were washed and blocked with 1% BSA/PBS. Samples of either insect cell lysates (I) or recombinant viruses (II, IV) were added to the wells. After washes, bound viruses were detected with anti-gp64 mAb and alkaline phosphatase-conjugated anti-mouse IgG (H+L) (Promega). After addition of the substrate solution, the absorbance was measured at 405 nm at different time points. For the competition ELISA, viral samples were incubated with increasing concentrations of competing ligand prior to addition to the microtiter plates (I).

4.5.3 Fluorescence Correlation Spectroscopy (FCS)

To study the diffusion properties of the viral construct displaying GFP as a fusion to the AcMNPV gp64 (Mottershead et al. 1997), as well as the number of fluorescent molecules on the viral surface, the recombinant virus Ac-EGFP (Mottershead et al. 1997) was analyzed by FCS. The virus was concentrated and resuspended in TE buffer (1mM Tris, 0.1 mM EDTA, pH 7.4). Virus samples were diluted 1:200 in citrate-phosphate buffer (pH 7.0) and exposed to different concentrations of n-octyl-β-D-glucopyranoside (0.1-50 mM), Triton X-100 (0.01-3 mM) or SDS (0.01-5 mM) (all detergents were provided by Sigma-Aldrich) at room temperature for 10 min. The effect of the detergents on the characteristics of the recombinant viral particle was compared to those of soluble GFP (Oker-

Blom et al. 1996) or untreated virus by using a ConfoCor 2 instrument (Carl Zeiss, Jena, Germany) and Ar^+ ion laser with an excitation wavelength of 488 nm. Emitted photons were collected by using a 530-600 nm bandpass filter. The instrument was operated in an autocorrelation mode and the software was used to calculate the autocorrelation function, diffusion time and number of particles per observation volume according to the manufacturer's instructions (Carl Zeiss) (III). FCS measures the diffusion properties of fluorescent molecules in a small subvolume of 0.25 fl defined by a strictly focused laser beam, enabling fast measurements of dilute samples. The fluctuation arising from the collected fluorescence is averaged and an autocorrelation curve is formed**.** The curve gives information about the size and concentration of the molecules under investigation (Rigler & Elson 2001).

4.5.4 Electron microscopy (EM)

The characterization of fusion protein distribution on the virus surface was carried out by incubation of concentrated, recombinant display baculoviruses on grids for 1-2 h at room temperature. Bound viruses were labeled with rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA), washed and further labeled with protein A-conjugated gold particles (10 nm) (Dr. G. Posthuma and Dr. J Slot, Utrecht, The Netherlands). After washes, viruses were negatively stained with uranyl oxalate. The grids were further coated with methylcellulose and viruses detected at 60 kV with a JEOL JEM-1200 EX transmission electron microscope (Jeol Ltd., Tokyo, Japan (IV).

4.6 Detection of protein expression in insect cells

To show that the fluorescent marker gene, GFP (II, IV) or the fluorescent fusion proteins E1-EGFP, E2-EGFP or E2-RFP (V) (under the transcriptional regulation of polyhedrin promoter) were correctly expressed and functional in Sf9 cells, infected cells were fixed 72 h p.i. with 4% paraformaldehyde (PFA) in PBS at room temperature for 20 min. Fixed samples were mounted in Mowiol (Calbiochem) supplemented with 25 mg/ml of antifading agent DABCO (Sigma-Aldrich). Samples of the cells were applied on microscope slides, covered with cover slips and examined with a confocal microscope (Carl Zeiss Laser Scanning Microscope, Axiovert 100M, LSM510, Jena, Germany) by using a 63x objective and immersion oil.

4.7 Mammalian cell experiments

4.7.1 Binding studies

BHK (II, IV) or PC-3 (II) cells were grown on cover slips for 24 h, placed on ice and washed with ice-cold PBS. Cells were incubated with the recombinant viruses with equal pfu's (II) or at MOI 50-100 (IV) for 1 h on ice. After three washes with PBS (5 mg BSA/ml), bound viruses were detected on ice by using anti-gp64 mAb and Alexa Fluor 546 (IV) or 594 (II) labeled goat anti-mouse IgG (H+L) (Molecular Probes, Inc., Eugene, OR, USA). Samples were fixed with 4% PFA/PBS and mounted in Mowiol/DABCO. When the binding was performed with an intermediate antibody (II, IV), BHK cells were incubated on ice with polyclonal anti-integrin antibody against α5β1, PC-3 cells with monoclonal anti-CEA antibody and both cell lines with irrelevant anti-avidin antibody (Table 3) or in the absence of antibody for 1 h prior addition of the viruses. Samples were analyzed either with a DMR fluorescence microscope (Leica, Wetzlar, Germany) (II) or the confocal microscope (IV).

To demonstrate that the cells express the desired proteins on their surface, i.e. CEA and α5β1 integrin on the PC-3 and BHK cell membrane, respectively, the cells were treated with either anti-CEA, anti-integrin or anti-avidin antibodies on ice for 1 h, and further incubated with Alexa Fluor 546 (IV) or 594 (II) labeled goat anti-rabbit IgG (H+L) (Molecular Probes) as above. As a control, cells were treated with secondary antibodies only. Samples were fixed and analyzed as described above.

4.7.2 Transduction efficiency studies

For demonstration of EGFP activity in mammalian cells, BHK or PC-3 cells were transduced with the recombinant baculoviruses at 37 °C in the presence of 5-10 mM sodium butyrate (Condreay et al. 1999). At 24 h post transduction, cells were fixed with 4% PFA/PBS and gene expression was analyzed by fluorescence or confocal microscopy (II, IV).

In order to study the transduction efficiency of various recombinant display viruses, they were first allowed to bind to the cell membrane for 1 h in the presence or absence of an intermediate antibody. After washes, cells were incubated for 24 h at 37 °C in order to gain EGFP expression in transduced cells. Cells were washed, harvested with trypsin, fixed with 2% PFA in PBS and resuspended in PBS. The samples were run on a Becton Dickinson FACSCalibur flow cytometer, which counted 10 000 cells per sample, and data was analyzed by using CELLQUEST software (II, IV).

4.7.3 Localization of RV fusion proteins in BHK cells

The BHK cells, grown on cover slips, were transduced with the recombinant baculoviruses, AcCE1-EGFP and AcCE2-RFP, containing the genes for the fluorescent RV proteins under the CMV promoter. The viruses were diluted 1:2 in BHK-21 medium either separately or in combination. Sodium butyric acid was added to a final concentration of 5 mM (Condreay et al. 1999). Cells were transduced for 24, 36 or 48 h at 37 °C, washed with PBS and fixed with 100% methanol for 6 min at -20 °C. After washes with PBS, cell organelles were labeled with ER, *cis*-Golgi, *trans*-Golgi network (TGN), late endosomes or lysosome specific antibodies (Table 3) and Alexa Fluor conjugated anti-rabbit or anti-mouse secondary antibodies (Molecular Probes). Localization of the fluorescent fusion proteins was analyzed with the confocal microscope (V).

5 REVIEW OF THE RESULTS

5.1 Binding activity of CEA scFv

Isolation and production of the scFv specific for CEA (CEA scFv) was performed by five rounds of CEA panning and producing the selected clone in bacteria. The expression of the scFv was confirmed by immunoblotting (I, data not shown). The binding specificity was tested by competitive ELISA from culture supernatant, showing that the binding of the scFv to CEA was inhibited in a concentration dependent manner when increasing amounts of the competing antigen were added to the supernatant (I, Fig. 2).

5.2 Characterization of the display viruses

5.2.1 Western blot analysis

To demonstrate that the generated recombinant baculoviruses were properly produced in infected insect cells, the cells (I, II) and/or purified viruses (I, II, IV) were analyzed by SDS-PAGE and immunoblotting. The results showed that the correct fusion proteins, composed of gp64 and either scFvs or the Z domains, were produced in insect cells (I, Fig. 3) as the size of the bands corresponded to the calculated molecular masses of the fusion proteins (Table 4). Similar results could be observed from the immunoblots of the budded viruses displaying the scFvs and ZZ domains (I, Fig. 4; II, Fig. 3). In addition, the ZZ domains fused to the VSV G membrane anchor were successfully displayed on the virus surface (IV, Fig. 2).

The level of incorporation of the fusion proteins into the virus particles was lower compared to that of wild type gp64 (I, Fig. 4; II, Fig. 3 B; IV, Fig. 2 A). This was especially observed with the baculovirus displaying the Ox scFv (L1),

in which the variable domains were connected by the CBHI linker, showing a significantly lower level of the fusion protein on the virus surface (I, Fig. 4).

TABLE 4 Molecular masses of the displayed fusion proteins.

Fusion protein	Molecular mass in kDa
CEA scFv –gp64	92.5
Ox scFv (L1) –gp64	91.5
Ox scFv (L4) –gp64	90
Z domain –gp64	72
ZZ domains –gp64	79
GFP –gp64	98
ZZ domains –VSV G TM+CTD	22

5.2.2 Ligand-binding activity

Functional binding of the displayed proteins to their respective ligands was tested in ELISA assays by using cell lysates from infected insect cells (I, Fig. 5), viral samples from culture supernatants (II, data not shown) or viruses purified by sucrose gradient (IV, Fig. 4). The only fusion protein incapable to give a signal above the background was the gp64 -fused Ox scFv with CBHI-linker (I, Fig. 5 and 6), which is consistent with the results obtained from western blotting. The results show that the baculovirus displaying two Z domains of protein A, instead of only one, has higher binding efficiency to its ligand (I, Fig. 5). The binding efficiency of the recombinant virus was further clearly improved by using a fusion of the ZZ domains and VSV G membrane anchor for display as shown in paper IV (Fig. 4). The data from competitive ELISA experiments used in paper I additionally show the specificity of binding of the recombinant baculoviruses displaying CEA scFv, Ox (L4) scFv or the ZZ domains (I, Fig. 6).

5.2.3 Analysis by fluorescence correlation spectroscopy

To study the properties and efficiency of fusion protein incorporation of the display baculoviruses, the recombinant virus AcGFPgp64 displaying GFP as a fusion to the gp64 (Mottershead et al. 1997) was concentrated, exposed to various detergents and analyzed by FCS. Untreated virus was shown to have a diffusion time of 3,300 µs, corresponding to a diffusion coefficient of (2.9±0.7) $x10^{-8}$ cm^2s^{-1} and an apparent hydrodynamic radius of 83 ±21 nm (III, Table 1). In addition, the concentration of the fluorescent virus particles was shown to be approximately $7x10^{10}$/ml. Treatment of the recombinant virus with Triton X-100, SDS or β-octylglucoside at concentrations of 0.4 mM, 5 mM and 25 mM, respectively, resulted in a decrease of the diffusion time to a value of as low as 150 µs (III, Table 1 and Fig. 3). Similarly, the calculated diffusion coefficient

increased to approximately $6x10^{-7}$ cm^2s^{-1} and the hydrodynamic radius decreased to approximately 4 nm in the presence of the detergents. This demonstrates that fluorescent molecules were liberated from the dissolved viral lipid membrane as a consequence of the treatments (III, Table 1). The detergents were also shown to increase the calculated particle number approximately 3-fold (III, Fig. 4), suggesting that a minimum of three trimers containing the fluorescent fusion protein were released from the viral membrane as complexes with the detergent. This was further supported by data from the SDS treatment, which led to a decrease of counts per molecule, i.e. brightness of measured particles by a factor of three (III, Fig. 5). As a control, soluble GFP was identically analyzed in the presence of SDS, but no significant changes in diffusion time or counts per molecule could be detected (III, Fig. 5).

5.2.4 Distribution of the ZZ domains on the virus particle

To compare the distribution and number of the fusion proteins between ZZ domains displayed as a fusion to either gp64 or the VSV G membrane anchor, the recombinant baculoviruses AcZZgp64-EGFP and AcZZVSVgTM-EGFP were subjected to electron microscopic studies. The displayed proteins were detected with rabbit IgG and protein A-conjugated gold particles and the viruses were further stained with uranyl oxalate. The EM analysis showed a heterologous distribution of the ZZ-VSVgTM fusion protein on the viral membrane, i.e. proteins were found both in the head domains and stalk of the viruses (IV, Fig. 5 A). In contrast, the ZZ-gp64 fusion protein was detected only in the head domain (IV, Fig. 5 B). The number of attached gold particles varied mainly between 2 to 7 per virus for the AcZZVSVgTM-EGFP whereas only one or two gold particles per virus were observed in the case of the AcZZgp64-EGFP virus. The negative control, Ac-EGFP, showed no binding of the gold particles (IV, Fig. 5 C). The fragile nature of the baculovirus particles (Mottershead et al. 1997) and the harsh staining procedure led to degradation of the lipid envelope of many virus particles, which made the overall detection somewhat difficult. However, enough intact viruses were present in the samples for reliable analysis.

5.2.5 Expression of GFP in insect cells

The fluorescent marker gene, GFP, cloned under the transcriptional regulation of the polyhedrin promoter was shown to be functional in infected Sf9 cells. Cells infected with AcZZgp64-EGFP or AcCEAscFvgp64-EGFP expressed high amounts of GFP, as fluorescence was detected both in the cytoplasm and nuclei of the cells (II, Fig. 2 A & B). Similar results were obtained with other recombinant baculoviruses containing the fluorescent expression cassette, i.e. Ac-EGFP and AcZZVSVgTM-EGFP (data not shown). No fluorescence was detected when uninfected cells were analyzed (II, Fig. 2 D). Further, the expression of GFP fused to the gp64 was detected on the plasma membrane of

infected cells (II, Fig. 2 C), demonstrating the transport of functional fusion proteins to the surface of the cells.

5.3 Binding of the display viruses to mammalian cells

The recombinant baculoviruses displaying either the CEA scFv or the ZZ domains of protein A were investigated in mammalian cells in order to study whether the viruses could be specifically bound to certain cell lines. The ZZ domains bind to any IgG molecule (Jendeberg et al. 1996) and thus the binding of a virus displaying these domains can be studied with any cell type, if a cell surface –specific antibody is available. Here, the α5β1 integrin was chosen as it is known to be expressed on the surface of various cell types and was thus suitable for BHK cell experiments (Hynes 1992, Koyama & Hughes 1992).

First, the PC-3 and BHK cells were shown to abundantly express the target proteins CEA (II, Fig. 4 C) and α5β1 integrin (II, Fig. 5 D), respectively, on their surface by incubating the cells with anti-CEA or anti-integrin antibodies. PC-3 is an adenocarcinoma cell line, previously shown to express CEA on its surface (Inoue et al. 1988), which was confirmed by these results. The recombinant virus AcCEAscFvgp64-EGFP was allowed to bind to PC-3 cells by incubating the virus with these cells on ice to prevent viral inoculation. The virus was found to bind efficiently to the PC-3 cells (II, Fig. 4 A) whereas only low levels of binding was detected with the control virus Ac-EGFP (II, Fig. 4 B). In addition, high binding levels of AcZZgp64-EGFP to PC-3 cells were detected when the cells were pretreated with anti-CEA (Fig. 6 A). The binding was shown to be specific as the binding level of this virus in the absence of an intermediate antibody was low (Fig. 6 B) and comparable to that of Ac-EGFP. Similar results were obtained with BHK cells when the cells were pretreated with anti-integrin antibody and the AcZZgp64-EGFP virus was shown to bind abundantly to the surface of the cells (II, Fig. 5 A). The specificity of binding via this antibody was demonstrated when the virus was exposed to cells without any antibody pretreatment. In that case, the amount of bound virus was low (II, Fig. 5 C) and comparable to the binding of Ac-EGFP in anti-integrin treated BHK cells (II, Fig. 5 B).

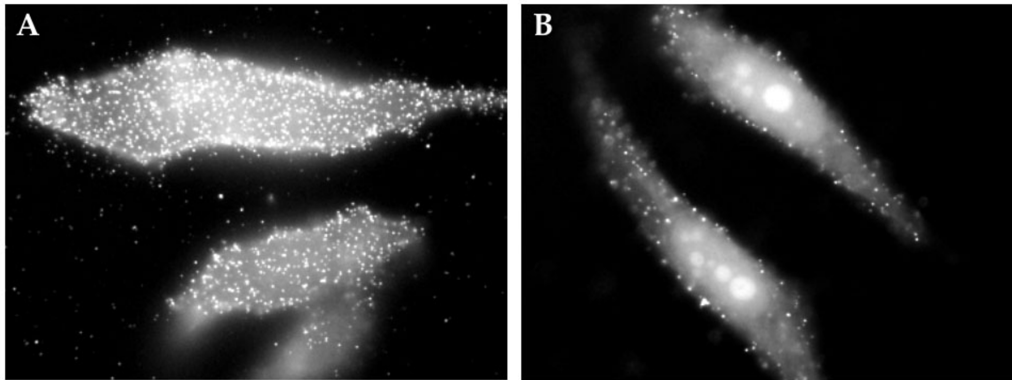

FIGURE 6 Binding of recombinant baculovirus AcZZgp64-EGFP to the surface of PC-3 cells A) via anti-CEA antibody or B) without an intermediate antibody.

Binding of the recombinant baculovirus AcZZVSVgTM-EGFP was similarly studied in BHK cells pre-incubated with anti-integrin antibody, irrelevant anti-avidin antibody or without any intermediate antibody. As a comparison, binding of AcZZgp64-EGFP and Ac-EGFP was investigated. Extensive binding of the display virus AcZZVSVgTM-EGFP to cells via the specific antibody, anti-integrin, was clearly observable (IV, Fig. 6 A). However, this virus was also shown to bind to cells pretreated with anti-avidin (IV, Fig. 6 D) and to untreated cells (data not shown) at a higher level than detected with the control viruses. While only low levels of binding of the negative control virus Ac-EGFP was detected in all cells (IV, Fig. 6 C & F), binding of the positive control AcZZgp64-EGFP to cells via anti-integrin was clear (IV, Fig. 6 B). This virus bound to anti-avidin treated cells at a level comparable to that observed with Ac-EGFP (IV, Fig. 6 E).

5.4 Transduction efficiency of mammalian cells

The specific binding of the recombinant display viruses observed in the experiments described above was exploited for studies in which the transduction efficiency of the different viruses was compared. The viruses were bound to the cell membrane either directly or via a specific antibody, allowed to be internalized and to express the fluorescent marker gene, EGFP, cloned under the CMV-IE promoter, which is active in mammalian cells. At 24 h post transduction, cells were fixed and analyzed either by fluorescence microscope or flow cytometry. In each experiment, cells transduced by the control virus Ac-EGFP were used as a basis to which other samples were compared.

Each recombinant baculovirus carrying the fluorescent expression cassette was shown to successfully transduce the mammalian target cells, which was detected as bright fluorescence in PC-3 (II Fig. 6 B & D) and BHK (II, Fig. 6 A &

C, IV Fig. 3) cells. Nevertheless, no improvement of the transduction could be seen with any of the specifically bound viruses by using GFP for monitoring. The transduction efficiency of AcCEAscFvgp64-EGFP in PC-3 cells was equal to that of Ac-EGFP. Similar results were obtained with AcZZgp64-EGFP and AcZZVSVgTM-EGFP when introduced to the BHK cells via anti-integrin or irrelevant antibodies. The percentage of fluorescent living cells reached 20 % at the highest for any given sample at the MOI of 100 in the prescence of butyric acid, while the percentage varied between 0.5 and 5 % in the absence of butyric acid.

5.5 Characterization of the rubella virus envelope fusion proteins

5.5.1 Expression in Sf9 and BHK cells

The full-length RV envelope protein E1 was fused to EGFP and similarly the E2 protein either to EGFP or the red fluorescent protein (RFP) (V, Fig. 1) for monitoring the expression in either infected insect cells or transduced mammalian cells. The fluorescent fusion proteins under the polyhedrin promoter were produced by using the baculovirus expression system. Expression of the fusion proteins in infected insect cells was detected by immunoblot analysis and bands migrating with molecular masses corresponding to the apparent sizes of the fusion proteins were detected (V, Fig. 2 A). In the cells infected with E2-EGFP or E2-RFP expressing viruses, lower molecular mass bands were also detected, which most likely represent either degradation products or unglycosylated forms of the apoprotein. In addition, cells were analyzed by confocal fluorescence microscopy (V, Fig. 2 B). Protein expression was clearly detected, whereas no fluorescence could be seen in uninfected cells. Analysis by confocal microscopy of fluorescent fusion proteins under the CMV-IE promoter, i.e. CE1-EGFP and CE2-RFP, was carried out similarly for BHK cells with similar results (V, Fig. 2 C). In addition, western blot analysis showed identical bands for CMV-expressed proteins in BHK cells as for proteins expressed in insect cells by the polyhedrin promoter, although at a lower level (data not shown).

5.5.2 Trafficking of the fluorescent RV proteins in BHK cells

In order to study the synthesis and intracellular localization of the RV envelope proteins, they were expressed in BHK cells under the CMV promoter as fluorescent fusion proteins: E1 with C-terminally fused EGFP (CE1-EGFP) and similarly E2 with RFP (CE2-RFP). Cells were transduced with the recombinant baculoviruses and the expression and trafficking of the proteins was analyzed at 24, 36 or 48 h post transduction (p.t.) (V, Fig. 3).

First, at all time points some of the proteins were detected in the ER (Tables 4-6). At 24 h p.t., individually expressed CE1-EGFP was mainly seen in the *cis*-Golgi compartment, whereas CE2-RFP was mostly detected in the ER, but partially also in *cis*-Golgi or the TGN. When expressed together, CE1-EGFP and CE2-RFP co-localized in the ER, but CE1-EGFP seemed to largely locate also in the *cis*-Golgi (Table 5 and V, Fig. 3).

TABLE 5 Co-localization at 24 h post transduction.

24 h	**ER**	***cis*-Golgi**	**TGN**	**lysosomes**
E1	+	++	+	-
E2	++	+	+	+/-
E1+E2	++	-, E1 ++	-, E1 +	+/-

++, distinct co-localization; +, slight co-localization; +/-, scarce co-localization; -, no co-localization

After 36 h incubation, CE1-EGFP was still mainly in the *cis*-Golgi, while CE2-RFP had moved to the TGN. Some overlap with the lysosomal marker could also be detected. Co-expression of the proteins seemed to enable translocation of them both into the TGN and lysosomes. Again, high amounts of CE1-EGFP were observed in the *cis*-Golgi (Table 6 and V, Fig. 4).

TABLE 6 Co-localization at 36 h post transduction.

36 h	**ER**	***cis*-Golgi**	**TGN**	**lysosomes**
E1	+	+	+/-	-
E2	++	+/-	++	+/-
E1+E2	++	-, E1 ++	+	+

++, distinct co-localization; +, slight co-localization; +/-, scarce co-localization; -, no co-localization

At 48 h p.t., some portion of CE1-EGFP had increased co-localization with the TGN and lysosomal markers although the *cis*-Golgi retention prevailed. CE2-RFP was now mainly seen in the lysosomes, as were both proteins when expressed together. However, part of the co-expressed CE1-EGFP could not proceed beyond the *cis*-Golgi (Table 7 and V, Fig. 5).

TABLE 7 Co-localization at 48 h post transduction.

48 h	**ER**	***cis*-Golgi**	**TGN**	**lysosomes**
E1	+	++	+	+/-
E2	+	-	+/-	+
E1+E2	++	-, E1 ++	+/-	++

++, distinct co-localization; +, slight co-localization; +/-, scarce co-localization; -, no co-localization

6 DISCUSSION

Development of the baculovirus display system during the recent years has followed the route of phage display, which has proven to be highly useful e.g. in library screening (Hoogenboom et al. 1998, Hoogenboom & Chames 2000), protein-protein or protein-peptide interaction studies (Smothers et al. 2002) and lately, also in preliminary gene transfer applications (Poul & Marks 1999, Larocca & Baird 2001). The major advantage of the baculovirus system over phage display is the capability to introduce complex post-translational modifications to proteins of eukaryotic origin and hence express them in a functional form. Further, the nonpathogenic nature of baculoviruses to vertebrates and the capacity to tolerate large DNA inserts add value to the development of this technology (Grabherr et al. 2001). Thus, these two pro- and eukaryotic display techniques complement each other depending on the application under study. For example, when creating libraries of unmodified peptides, phage display is more suitable. Similarly, baculoviral display should be advantageous when post-translational modifications of complex proteins are a prerequisite.

Due to the several beneficial features of the baculovirus display system, the main goal of the study presented in this thesis was to develop *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) based display vectors that would efficiently express various proteins on their surface, either as fusions to the major envelope protein gp64 or, alternatively, to a heterologous membrane anchor, i.e. that of the vesicular stomatitis virus (VSV) G protein (Fig. 7). Further, the recombinant display viruses were examined for their potential as targetable gene delivery vehicles for mammalian cells. Moreover, the baculovirus expression system was exploited for studies on the synthesis and trafficking of fluorescent fusions of foreign viral envelope proteins in transduced mammalian cells.

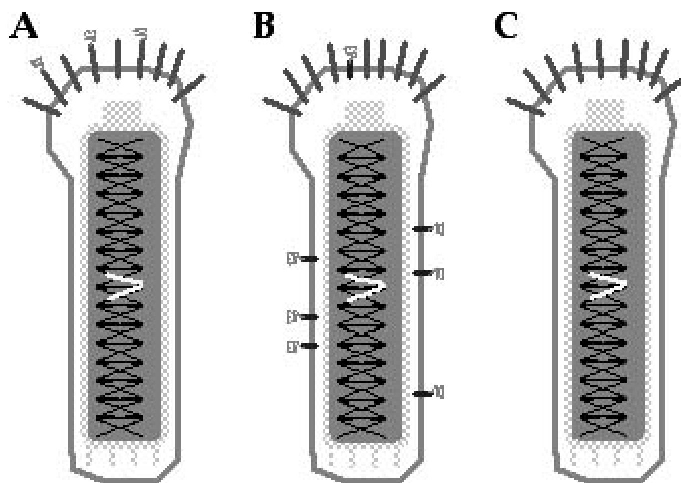

FIGURE 7 The recombinant baculoviral display constructs developed in this study. A) display as a fusion to a second copy of AcMNPV gp64, B) display by using a heterologous membrane anchor of VSV G protein and C) Ac-EGFP, control virus without any foreign fusion protein. All constructs were equipped with a fluorescent expression cassette, containing EGFP and GFP under CMV and polyhedrin promoters, respectively (marked as white).

6.1 Functional display of foreign proteins on the baculovirus surface

The antibody fragments such as scFv and Fab, used instead of full-length antibodies, have been shown to be effective for display purposes (Hoogenboom et al. 1998). ScFvs are composed of variable, i.e. antigen binding, domains of antibodies linked by a flexible linker peptide (Bird et al. 1988). In this study, the murine scFv specific for 2-phenyloxazolone and a human scFv specific for CEA were chosen for display on the baculovirus surface as fusions to the N-terminus of the AcMNPV major envelope protein gp64. By analyzing purified virus in western blotting, successful presentation of the antibody-gp64 fusions on the baculoviral surface was confirmed (I & II). The incorporation of the Ox scFv linked by cellobiohydrolase I (CBHI) into the baculovirus particles was, however, weak. A possible explanation for this could be that in its original host, the filamentous fungus *Trichoderma reesei*, the CBHI peptide is known to be O-glycosylated (Tomme et al. 1988). Therefore O-glycosylation of the linker in insect cells might obstruct proper folding and subsequent display of the antigen binding domain.

Protein A is a cell-wall bound pathogenicity factor from *Staphylococcal aureus*. It contains five different domains, one of them being the B domain. The synthetic IgG-binding domain (Z) is an engineered, more stable analogue of the B domain. The Z domain contains two amino acid substitutions relative to the B domain, i.e. Ala1 → Val and Gly29 → Ala, and it binds tightly to the Fc -portion of many IgG subclasses (Braisted & Wells 1996, Jendeberg et al. 1996, Tashiro et

al. 1997). Here, two baculovirus constructs were engineered to display either one or two Z domains. Both fusion constructs were clearly detected by immunoblot analysis and appeared to be displayed on the viral surface at similar levels (I & II). A molecule composed of two Z domains fused to the VSV G protein TM and CTD domains was further shown to be expressed on the baculovirus particle and could be detected with both monoclonal and polyclonal antibodies (IV). In addition to the band of about 22 kDa, the estimated size of the monomeric ZZ-VSVgTM fusion protein, multiple higher molecular mass bands could be detected. A previous study by Robison et al. demonstrated that a VSV G protein consisting of a 42 aa ectodomain, 20 aa TM domain and 29 aa CTD domain was expressed as a trimer (Robison & Whitt 2000). Our VSV G protein contains a 21 aa ectodomain in addition to the TM and CTD domains. Although shorter than the ectodomain studied by Robison et al., the fusion protein is most likely expressed also as a trimer. It has also been shown that only trimers of the VSV G protein are transported to the Golgi complex and hence to the cell membrane (Doms et al. 1987). As our fusion protein was quite efficiently expressed on the surface of the baculovirus particles, it is likely to be oligomerized during assembly. Some of the bands in our immunoblots were in accordance with the dimeric and trimeric forms of the fusion protein. It is possible that the fusion protein is partially resistant to SDS-treatment, since similar SDS-resistant forms of the VSV G protein have also been reported by others (Doms et al. 1987, Weisz et al. 1993). However, some bands could not be explained by oligomerization and could therefore possibly be a result of different glycosylation patterns of the fusion proteins.

Detection of significantly higher amounts of wild type gp64 on the baculovirus particles as compared to any of the gp64 fusion proteins (I, IV) could possibly be explained by a competition between the fusion protein and wild type gp64 for access to the virus surface (Boublik et al. 1995, Chapple & Jones 2002). Moreover, larger fusion proteins are more likely to interfere with gp64 oligomerization and thus viral incorporation of the fusion protein (Mottershead et al. 1997). On the other hand, the fusion to gp64 may also have an influence on properties of the fusion protein, e.g. its folding or binding affinity.

The binding ability of the display viruses or fusion proteins was tested in ELISA assays (I), which further supports the results obtained by western blotting: Ox scFv and CEA scFv joined by $(GGGGS)_3$ linker peptide and Z domain(s) showed high binding activity to their ligands, i.e. $Ox_{16}BSA$, CEA and bovine IgG, respectively. In contrast, the Ox scFv with the CBHI linker exhibited very low binding, which is in agreement with the poor incorporation of this fusion protein into the viral membrane. The IgG-binding specificity of the virus displaying two Z domains was significantly higher than that of a single Z domain in spite of similar incorporation of these proteins into virus particles. This is consistent with earlier data showing that a molecule composed of two Z domains has 20 times tighter binding than one Z domain (Jendeberg et al. 1996). Additional experiments with viral supernatants by using competitive

ELISA provided information regarding the binding specificity, which is in agreement with the results of traditional ELISA, i.e. binding was specific for all viruses tested except for the one displaying Ox scFv with the CBHI linker (I).

When the recombinant virus presenting the ZZ-VSVgTM fusion protein on its envelope was tested in ELISA assays, further improvement of the binding was observed as compared to the binding of the recombinant virus displaying the ZZ domains as a gp64 fusion (IV). Since the virus particle amount in each sample was set to be equivalent according to the infectivity of Sf9 cells (pfus), the improved binding cannot be explained by higher concentration of the AcZZVSVgTM-EGFP virus sample applied to the wells. However, the total virus particle number may not be the same as the number of infectious virus particles (Volkman & Goldsmith 1985) and current methods for calculating the virus titers are not completely reliable. On the other hand, the similar intensity of the gp64 protein bands in the immunoblots (IV) supports the equivalency of the virus particle amounts used in the ELISA assays. Thus, improved binding is likely to be a consequence of high expression levels of the protein chimera on the viral envelope, possibly leading to easier and faster binding of the virus. In addition, if the fusion protein is embedded all over the viral envelope as expected (Chapple & Jones 2002), the angle in which the virus encounters the ligand at the bottom of the wells is not as critical as it is when the ZZ domains are fused to gp64 and are thus found only at one end of the virus. Hence, better access of the ZZ-VSVgTM protein to its ligand during incubation would lead to a higher binding ability.

6.2 Characterization of fluorescent baculovirus by FCS

By analyzing the baculovirus displaying GFP protein as a fusion to gp64, various properties of this recombinant virus could be characterized (III). An approximate particle size of 166 ±42 nm for the virus was calculated, which is roughly consistent with the EM studies by M. Fraser who demonstrated that the mean length of a baculovirus particle is approximately 260 nm (Fraser 1986). Although the average size measured by using the FCS was smaller than the value from the EM studies by Fraser, the differences most likely resulted from the different methods and can thus be compared only as approximate values. As the baculovirus particle is large and rod-shaped, the recombinant fluorescent virus was expected to have a longer diffusion time than the GFP-gp64 fusion protein alone. This was shown to be correct, as the diffusion time of the intact virus was approximately 3,300 μs, whereas detergent-treatment decreased the diffusion time of fluorescent molecules to a value of 150 μs. These data suggest that the detergents dissolved the viral membrane, releasing membrane-bound fusion proteins probably as detergent-fusion protein complexes. In addition, treatments of the recombinant virus with the different detergents resulted in decrease of counts per molecule to one third and

simultaneous three-fold increase in particle number as compared to untreated viruses (III). The three-fold changes illustrate that a minimum of three GFP-gp64 fusion protein trimers exist on the surface of one recombinant baculovirus. The concentrations of detergents used in the experiments are not able to reduce the disulfide-linked gp64 trimers into monomers, which further supports this estimation. Since the SDS treatment of soluble GFP did not show significant changes in the counts per molecule or the diffusion time, the effect of this detergent on the properties of the recombinant display virus was not related to photochemical effects, i.e. changes in the properties of GFP. The result regarding the amount of fluorescent fusion proteins on the viral surface should be considered more as an estimation rather than an exact value, since FCS characterizes mainly globular particles and in addition, lateral diffusion of the fusion protein on the viral envelope may have an impact on the results. Further, the number of GFP molecules per each gp64 trimer could not be estimated by the methods used in this study. Additionally, since GFP is a relatively big protein to be used as a chimeric fusion partner, i.e. approximately 240 aa (Tsien 1998), it is possible that its size might interfere with efficient incorporation of the fusion protein into the viral surface. Proteins smaller than GFP might be incorporated to the virus particle more easily and thus would give higher average fusion protein number. This was in fact shown when the FLAG epitope was displayed on the baculovirus surface at higher levels than bigger fusion partners (Mottershead et al. 1997). These results give additional proof to the low copy number of gp64-fusion proteins on the baculovirus envelope.

6.3 Electron microscopic detection of fusion protein display

To reveal the location of the fusion proteins on the viral envelope, the viruses displaying the ZZ domains were examined by using EM techniques by labeling the ZZ domains with rabbit IgG and protein A gold (IV). The polar display of the ZZ-gp64 fusion proteins in either end of the virus particles was in line with previous reports (Blissard 1996, Chapple & Jones 2002) as was the low number of gold particles per virus (Boublik et al. 1995, Mottershead et al. 1997). In contrast to the ZZ-gp64 fusion, the ZZ-VSVgTM fusion protein was detected at various sites of the viral envelope and the number of gold particles was higher, indicating better incorporation of this fusion protein into the virus particles. These results are consistent with those of Chapple et al. (2002) and suggest that the CTD of VSV G protein can somehow facilitate the fusion protein incorporation into the budding baculoviruses. As suggested by Chapple et al., this may be due to the interaction of the CTD with the underlying baculovirus nucleocapsid or to the small size of the CTD, leaving it unnoticed by the budding virus (Chapple & Jones 2002). The specificity of the gold particle labeling was confirmed since no gold particles could be detected on the membrane of the control virus Ac-EGFP.

6.4 Specific binding of recombinant display baculoviruses

The increasing interest towards viruses as gene therapy vectors has resulted in a number of reports in which various viral vectors have been studied for their gene delivery properties. As a common feature for these studies, the need to develop targetable vectors has become evident (Russell & Cosset 1999, Lundstrom 2003). In most occasions, it would be desirable to transduce all cells of a certain type, but viruses usually prefer to transduce only a few cells of various types (Dobbelstein 2003). Targeting would facilitate the systemic administration of the vectors *in vivo* as the viruses would find their way to the target cells or organs by the vascular circulation (Peng & Russell 1999). In addition, improved localization of targeted vectors would allow lower vector doses and is likely to increase safety and lower the immune response (Wickham et al. 1997b, Wickham 2000). Targeting could mainly be achieved by two different ways: either by genetic modifications of the viral coat or by covalently/noncovalently linking the targeting moiety to the virus particle (Wickham 1997, Wickham 2000).

The carcinoembryonic antigen (CEA) is a highly glycosylated protein, which is expressed on the cellular membrane of various types of human cancer cells, but especially on those of gastrointestinal cancer of the colon and stomach (Hammarstrom 1999). Successful targeting of retrovirus vectors displaying a CEA scFv to various CEA-expressing cells, such as RCM-1 (human colorectal adenocarcinoma), MKN-45 and KATO-III (gastric cancer cell lines) has been described previously (Konishi et al. 1998, Khare et al. 2001). Our results points out that also a baculovirus displaying the CEA scFv can be bound to a CEA-expressing adenocarcinoma cell line, PC-3, while only a low signal from the control virus Ac-EGFP was detected (II). Targetable vectors specifically recognizing these various CEA-expressing cancer cells would thus be advantageous for the treatment of numerous carcinomas and demonstrates the potential of baculoviruses as a targetable gene therapy vector.

Recently, numerous viral vectors, such as those based on Sindbis virus (Ohno et al. 1997, Wickham 1997), retrovirus (Hatziioannou et al. 1999, Morizono et al. 2001), polyoma virus-like particle (Gleiter & Lilie 2001) and AAV (Ried et al. 2002), all displaying the Fc-binding domain of protein A have been constructed. All these studies have demonstrated that efficient targeting can be achieved by using an antibody specific for the target cell between the virus and the cell. In the studies reported here in, similar encouraging results were obtained when the recombinant baculovirus containing the ZZ-gp64 fusion protein on its surface was shown to efficiently bind to PC-3 or BHK cells previously coated with a monoclonal anti-CEA antibody or a polyclonal anti-integrin antibody, respectively. The specificity of binding was shown as the binding of AcZZgp64-EGFP without an antibody bridge was comparable to the non-specific binding of Ac-EGFP both in PC-3 cells and BHK cells (II). The beauty of the employment of the IgG-binding domain is in its wide

applicability: there is no need to generate a battery of targetable vectors for each cell type, but instead, by simply choosing an antibody specific for the desired target cell, the tropism of a common vector can be modified.

These results demonstrate that there are two different ways to target the recombinant display baculoviruses *in vitro* to specific cell lines. These are either direct targeting by displaying a targeting motif, such as a scFv (Fig. 8, pathway A), or by using an intermediate antibody (Fig. 8, pathway B).

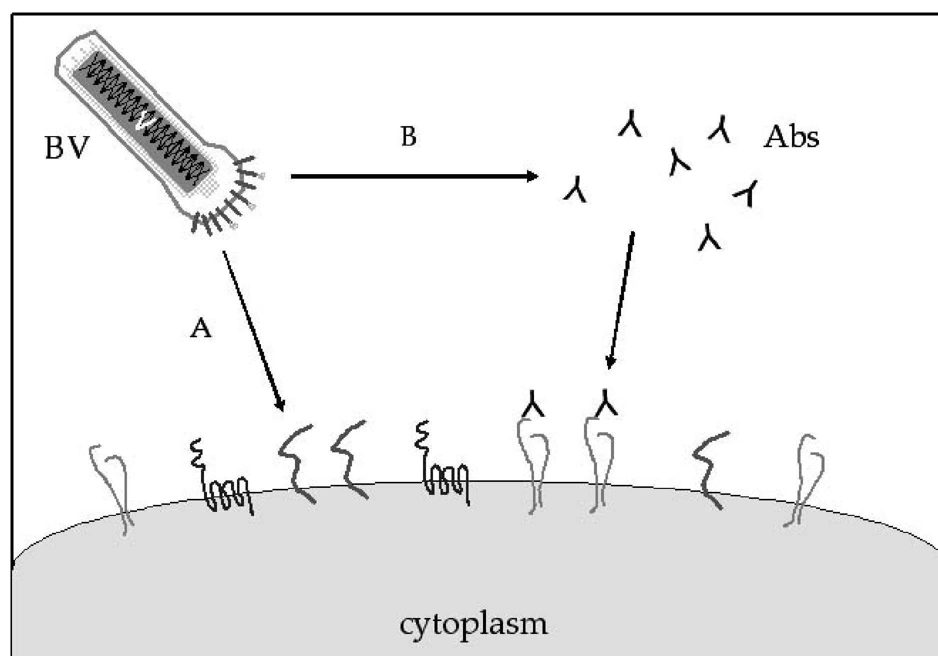

FIGURE 8 Schematic representations of the targeting routes of the recombinant display baculoviruses constructed in this study. A) direct binding to the cell surface protein by displaying a targeting motif and B) antibody-mediated targeting. BV, budded virus; Abs, specific antibodies against cell surface protein.

Based on the encouraging ELISA results, in which improved IgG-binding of the recombinant virus displaying the ZZ-VSVgTM fusion protein was obtained, increased binding of this virus to the cell surface was anticipated as compared to AcZZgp64-EGFP. The difference in the amount of bound virus between AcZZVSVgTM-EGFP, AcZZgp64-EGFP and Ac-EGFP was very clear and in line with the expectations (IV). However, the enhanced binding of the AcZZVSVgTM-EGFP vector to the BHK cell membrane via an irrelevant anti-avidin antibody was unexpected and similar binding was not detected with the AcZZgp64-EGFP. The 21 aa long ectodomain of our VSV G protein anchor could possibly explain the non-specific binding, since it has been shown that a G protein ectodomain of 42 aa residues, together with the transmembrane and cytoplasmic domains, is sufficient to cause the binding of VSV to BHK cells (Jeetendra et al. 2002). Although the ectodomain in our display construct is half of the length, an earlier study by the same group demonstrated that only 12-16 aa residues of the ectodomain along with the TM and CTD is needed for efficient virus budding (Robison & Whitt 2000). Since the shorter ectodomain

was not examined for its binding ability in that study, it is possible that it is sufficient also for binding of the virus. In addition, it has been shown that the short, 14 aa residues long ectodomain can promote both pH-dependent and independent fusion potential of some foreign viral fusion proteins (Jeetendra et al. 2002). Therefore, although unlikely, it cannot be excluded that the ectodomain of 21 aa residues used in our display vector facilitates the fusion activity of the AcMNPV gp64 and in this way strengthens viral binding to the cell membrane even under conditions (i.e. at 4 °C) where no endocytosis and/or membrane fusion should occur.

In these microscopical studies, no quantification of virus binding was carried out. This would of course give more accuracy to the results and may reveal even subtle differences regarding the levels of viral binding. The estimation of the amount of bound virus in this study was nevertheless based on the comparison of multiple experiments and samples. However, to further investigate the possibilities of the AcZZVSVgTM-EGFP for targeted gene transfer in more detail, quantification of the binding efficiency is needed.

6.5 Efficiency of gene transfer to mammalian cells by display baculoviruses

Numerous studies have shown that baculoviruses are able to efficiently transduce a wide range of mammalian cells, including those of human, porcine, rodent or non-human primate origin (Ghosh et al. 2002). Therefore a targetable baculoviral vector is needed to attain restricted transduction of a specific cell type. Here, the transduction by previously bound baculoviruses was analyzed in order to find out whether the specific binding would also lead to increased transduction efficiency. By using an EGFP gene under a mammalian promoter (CMV), the percentage of fluorescent cells at 24 h post transduction was determined. Surprisingly, the results showed similar amounts of fluorescent cells in each sample, irrespective whether the virus was more efficiently bound to the target cells or not (II, IV). It seemed that the low binding levels of the controls led to an equal transduction percentage than what could be observed with specific display vectors.

Although in many occasions the modified viral vector enhances transduction of the target cells, as demonstrated with some retroviral (Kasahara et al. 1994, Somia et al. 1995, Boerger et al. 1999) and adenoviral (Wickham 1997, Wickham et al. 1997b, Harari et al. 1999) gene delivery vehicles, there are also studies showing that this is not always the case (Peng & Russell 1999). For example, problems in efficient gene delivery with some targetable retrovirus vectors have been common (Russell et al. 1993, Cosset et al. 1995, Marin et al. 1996, Zhao et al. 1999). According to Russell & Cosset (1999), the problems are probably due to sequestration of the targeted receptor-bound particles or the failure to activate the fusion machinery upon binding to the targeted cells.

Similar observations have been made by Zhao and colleagues, who indicate that inability of the chimeric protein to undergo conformational change leads to gene transfer block (Zhao et al. 1999). In addition, different cell lines or receptors have been shown to be differentially susceptible to targeted retroviruses (Roux et al. 1989). The poor transduction has been improved e.g. by optimizing the space between the displayed domain and the fusion partner (Valsesia-Wittmann et al. 1996).

Also in this study, the displayed proteins were thought to interfere with the fusion activity of gp64 and thus reduce the possibilities of the specifically bound baculovirus vectors to efficiently transduce the target cells. With this is mind, the novel baculovirus vector AcZZVSVgTM-EGFP was constructed with expectations of higher transduction levels since the gp64 was left intact in this recombinant virus. Thus, it should be able to act normally in the endosomal release of the virus. Although the exact mechanism of baculovirus entry in mammalian cells has not been extensively studied, there are reports showing that the virus is internalized by the endocytic pathway also in vertebrate cells, followed by an acid-induced fusion event (van Loo et al. 2001, Kukkonen et al. 2003). This is consistent with studies on insect cells (Volkman & Goldsmith 1985, Blissard & Wenz 1992, Wang et al. 1997, Hefferon et al. 1999) and suggests strongly that gp64 also plays an important role during mammalian cell entry. However, leaving the gp64 intact did not increase the percentage of transduced cells (IV), indicating that the barrier is somewhere else than in the endosomal release. The blocking step can in theory be located to various sites: (i) the recombinant virus might be targeted to a cell membrane protein, which does not allow efficient endocytosis or membrane fusion, as suggested for retroviruses (Russell & Cosset 1999), (ii) although the display virus is internalized, it takes place by a route that is not natural for baculoviruses and thus the internalized virus may end up in lysosomes or virus particles are recycled back to the cell surface, (iii) the internalized virus is possibly released normally to the cytoplasm, but is not transported to the nucleus and finally, (iv) even if the virus would be able to enter the nucleus, only a limited level of transcription may take place. The third option as a limiting step is favorable since it seems that although the baculovirus readily enters a number of vertebrate cells, the nuclear entry is restricted (Volkman & Goldsmith 1983, Kukkonen et al. 2003). In addition, the measurement of EGFP expression as a marker for transduction efficiency may not be the optimal method of choice.

Development of targetable baculovirus vectors is important in spite of the possibility that the transduction efficiency cannot be significantly increased above that of the wild type baculovirus. Careful choice of the target receptor, e.g. an actively recycled receptor, such as the transferrin receptor (Ponka & Lok 1999), might allow more efficient internalization of the virus. In addition, pseudotyping of the virus surface with an envelope protein, which binds to a well-known receptor and is known to mediate efficient entry may have a positive effect on baculovirus transduction. Proof of principle of this method has been demonstrated by successful pseudotyping of AcMNPV with the VSV

G protein (Barsoum1997, Mangor2001), although utilization of this particular protein is more likely to broaden the target cell types rather than restricting their number as demonstrated with retroviruses (Burns et al. 1993, Lee et al. 2001). Further, targeting beyond the cell surface by coating the capsid with targeting molecules for efficient transport to the nucleus may be one solution to enhance transduction (Kukkonen et al. 2003).

Moreover, solving the 3D structure of gp64 and the identification of the receptor(s) would allow more accurate design of baculoviral display vectors. Nevertheless, in spite of the many problems still related to the use of baculovirus as a gene delivery tool, the many advantages discussed above favour further development of baculovirus as a future choice for gene therapy applications.

6.6 Synthesis and trafficking of heterologous viral membrane proteins in baculovirus-transduced BHK cells

Although baculoviruses are an efficient gene transfer vehicle in mammalian cells, its feasibility in the expression of other than reporter proteins in vertebrate cells has been reported only in a few studies (Kost & Condreay 1999). The expression of the full length genomes of the hepatitis B (Delaney & Isom 1998) and C (Fipaldini et al. 1999) viruses in hepatic cells by recombinant baculoviruses has shown that correctly processed viral proteins can be produced in baculovirus-transduced mammalian cells. However, the utility of this technology has not been evaluated for the studies of the maturation and localization of foreign proteins in mammalian cells. Thus, we exploited the rubella virus envelope proteins E1 and E2 as model proteins in order to investigate the applicability of baculovirus vectors for this type of studies.

Studies on the rubella virus show that although the two genera of the *Togaviridae* family, namely *Alphaviruses* and *Rubiviruses*, share similarities in their structure and entry mechanisms, their assembly appears to be quite different (Frey 1994, Law et al. 2001). One example is the dominant budding of RV at the intracellular membranes whereas alphaviruses mature exclusively at the plasma membrane (Garbutt et al. 1999). The RV envelope proteins have been studied both individually and as heterodimers for their maturation and transport in infected cells. The studies show that the proteins are translocated to the ER, where they form heterodimers (Frey 1994) after which they are transported to the Golgi complex (Hobman et al. 1993). However, when these proteins are expressed separately, it seems that E1 accumulates in a post-ER/pre-Golgi compartment (Hobman et al. 1992, Hobman et al. 1997), while E2 is arrested in the ER or is transported to the Golgi complex (Hobman et al. 1993).

Here, the E1 and E2 proteins were cloned into the baculovirus genome as fluorescent fusions with the EGFP and RFP, respectively, under the

transcriptional regulation of the CMV-IE promoter (V). The expression and localization of the proteins in transduced BHK cells was examined at 24, 36 and 48 h post transduction. The results were mostly in agreement with previous studies, since individually expressed CE1-EGFP was retained in the ER or *cis*-Golgi and CE2-RFP was shown to partially move from the ER to the TGN. The CI-MPR, which normally is located in the late endosomes, has been shown to localize primarily in the TGN in BHK cells (Juuti-Uusitalo et al. 2000). Thus, it can be assumed that co-localization of CE1-EGFP and CE2-EGFP with the anti-CI-MPR antibody is detected within TGN rather than late endosomes. Identification of the exact region for CE1-EGFP retention was not studied here. However, the ER marker, PDI, has been shown to be able to locate to the pre-Golgi compartment (Hobman et al. 1993) and hence it is possible that CE1-EGFP is mainly in that area. Although the same study by Hobman et al. demonstrated that the E1 was not arrested in the Golgi complex and thus not in the *cis*-Golgi either, our fusion protein showed clear overlap with the *cis*-Golgi marker. Further, some of the protein was transferred to the TGN and also to the lysosomes at later time points. These locations may result from the fluorescent fusion protein, which can give rise to conformational changes in the E1, leading to retargeting. The EGFP could also mask the retention signal that is known to reside in the C-terminus of E1 (Hobman et al. 1997) and therefore would enable the movement of CE1-EGFP from the ER. Moreover, it has been demonstrated that overproduction of E1 can cause its mislocation due to the saturation of the protein retention systems (Hobman et al. 1997). The studies by Hobman and co-workers were further conducted in CHO cells, which differ morphologically from BHK cells. This may be a reason for the differences in protein trafficking and localization. Some of the CE2-RFP was observed in lysosomes at 48 h p.t., which may be a consequence of similar overproduction as in the case of E1 or due to the aberrant conformations caused by the RFP fusion. Interestingly, CE2-RFP was always seen as large aggregates, which also seemed to cause enlargement of the respective cell organelles. The reason for this is unclear, but the aggregates can, as previously shown, be due to morphological changes in cells caused by RV proteins themselves (Frey 1994). The aggregates may also arise from the fusion protein, but as the RFP used in this study, DsRed2-N1, is a new DsRed variant that has been engineered to lower non-specific aggregation, this does not seem probable. It has been shown that EGFP is able to form dimers (Tsien 1998) and that mature RFP is a tetrameric protein (Yarbrough et al. 2001). Thus, it is also possible that the oligomerization of these fluorescent proteins may have an influence on the movement and localization of the RV envelope proteins. However, there are studies demonstrating that the fluorescent fusion did not change the behavior of the chimeric proteins. For example, fusion of either EGFP or RFP to the N- or C-terminus of Epstein-Barr virus latent membrane protein 2B did not change the localization pattern of this protein (Lynch et al. 2002). Nevertheless, the effect of the oligomerization cannot be ruled out and experiments with unfused proteins would give additional information on E1 and E2 trafficking in baculovirus-transduced cells.

When the proteins were co-expressed, they were mostly seen to translocate from the ER (24 h) through TGN (36 h) to the lysosomes (48 h), although a large quantity of CE1-EGFP did not proceed beyond the *cis*-Golgi. Again, the lysosomal location can again be explained by overproduction or retargeting caused by the fusion proteins. Otherwise, the ER and TGN locations are in agreement with previous studies (Hobman et al. 1993, Frey 1994). No fusion proteins were seen on the plasma membrane at any time point, which is consistent with the intracellular assembly demonstrated for rubella virus. The overall retention of some of the fusion proteins in the ER, irrespective of whether they were expressed individually or together, was very clear and may be due to misfolding or oligomerization and assembly defects, since these reasons heve been shown to cause ER retentions of other viral proteins (Hurtley & Helenius 1989, Klausner & Sitia 1990). In addition, possible continuous synthesis of CE1-EGFP and CE2-RFP may result in prolonged ER detection. The lack of glycosylation has also demonstrated to cause ER-retention (Qiu et al. 1992, Frey 1994), but this is probably not the reason for retention of our fusion proteins, since the genes used for the virus constructs were authentic.

These experiments show that trafficking of fluorescent RV envelope proteins expressed in baculovirus-transduced cells resembled the results of other reports, although some influence of the fluorescent molecules on protein behavior in this study cannot be ruled out. However, the results suggest that baculoviral transduction could be applied for studies on detection of protein synthesis and trafficking in mammalian cells. In the future, this type of expression methods should be applicable also to a variety of other proteins. Moreover, the efficiency and ease of baculoviral transduction can be regarded beneficial when compared to the usually low transfection efficiencies obtained with e.g. liposomes.

7 CONCLUSIONS

1. Various proteins could successfully be displayed on the surface of the baculovirus AcMNPV. However, the number of fusion proteins on the surface of the baculoviral particles was quite low when the display was performed to a second copy of the AcMNPV gp64 protein.

2. The displayed proteins retained their ligand-binding activity and were thus functional on the surface of the baculovirus particles.

3. The use of a heterologous membrane anchor derived from the VSV G protein increased the number of fusion proteins on the baculovirus surface, resulting in enhanced binding activity of the recombinant virus.

4. Certain recombinant display baculoviruses can be bound to specific mammalian cell types either directly by displaying a targeting motif or via an intermediate antibody. Baculoviruses readily transduce a wide range of cells, which challenges their further development as targetable gene transfer vectors.

5. Baculoviruses have applications also in studies related to synthesis and intracellular trafficking of foreign proteins in mammalian cells.

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YHTEENVETO (Résumé in Finnish)

Bakulovirukset ovat hyönteisiä infektoivia viruksia, joiden kaksijuosteinen DNA on pakattu sauvamaisen kapsidin sisään. Bakulovirusten elinkierron aikana muodostuu proteiinimatriksiin kiedottuja viruksia (occlusion derived virus, ODV) sekä silmikoituvia viruksia (budded virus, BV). ODV-muodot ovat luonnossa kestäviä ja aiheuttavat hyönteistoukkien primääri-infektoitumisen. Seuraavassa infektiovaiheessa tuottuu silmikoituvia viruksia, jotka levittävät bakulovirusinfektion toukan kaikkiin soluihin. Bakuloviruksia on käytetty vuosikymmenien ajan tuhohyönteisten luonnonmukaiseen torjuntaan viljelyksillä. Nykyisin bakuloviruksia hyödynnetään myös laajalti biotekniikassa vieraiden proteiinien tuotossa. Bakulovirusten suosio proteiinintuotossa perustuu virusten ja käytettävien hyönteissolujen eukaryoottiseen alkuperään, mikä mahdollistaa tuotettavien proteiinien monipuolisen muokkauksen ja siten edesauttaa toimivien proteiinien syntymistä. Viimeisen kymmenen vuoden aikana bakulovirusten käyttö on laajentunut käsittämään myös ”display”-tekniikan, jolla tarkoitetaan vieraiden peptidien tai proteiinien ilmentämistä bakuloviruksen pinnalla osana viruksen vaipan luonnollista gp64-pintaproteiinia.

Bakulovirusten on useissa tutkimuksissa havaittu kykenevän tunkeutumaan moniin erilaisiin nisäkässolutyyppeihin. Vaikka bakulovirukset eivät lisäännykään kyseisissä soluissa, voivat ne saada aikaan siirtogeenien ilmentymisen, mikäli geenien yhteyteen on liitetty nisäkässoluissa toimiva promoottorialue. Bakulovirukset ovat lisänneet suosiotaan tutkittaessa potentiaalisia geeninsiirtäjiä geeniterapeuttisiin sovelluksiin, sillä bakulovirukset ovat vaarattomia ihmisille, niitä pystytään tuottamaan nopeasti suuria määriä ja niiden genomiin voidaan liittää suuriakin määriä vierasta DNA:ta.

Kohdennettavien virusvektoreiden kehittäminen geeniterapiaa varten on osoittautunut tärkeäksi, sillä kohdennuksen avulla spesifinen geeninsiirto haluttuihin soluihin tai kudoksiin olisi mahdollista. Tämän tutkimuksen tavoitteena oli kehittää sellaisia bakuloviruksia, jotka ilmentäessään pinnallaan vieraita proteiineja, kohdentuisivat näiden proteiinien avulla toivottuihin solutyyppeihin. Lisäksi haluttiin selvittää, siirtyvätkö nämä rekombinanttiset virukset paremmin solujen sisään kuin ns. villin tyypin bakulovirus ja saataisiinko tällöin aikaan tehokkaampi geenin ilmentyminen. Ensisijaisena tavoitteena oli kehittää bakulovirusta siten, että sen käyttö geeniterapiassa olisi mielekästä.

Tutkimuksessa kehitettiin bakuloviruksia, joiden pinnalla ilmentyy joko erääseen syöpäantigeeniin (carcinoembryonic antigen, CEA) sitoutuva vasta-aine tai immunoglobuliineihin sitoutuva proteiini A:n ZZ-domeeni (I). Virusten genomiin liitettiin myös fluoresoivaa proteiinia koodaava geeni, jonka avulla voitiin seurata viruksen kykyä aiheuttaa siirtogeenin ilmeneminen kohdesoluissa (II). Useiden kokeiden avulla voitiin osoittaa, että virusten tuottaminen onnistui hyvin ja niiden pinnalla ilmentyi toimivia, sitoutumiskykyisiä fuusioproteiineja. Gp64-fuusioproteiinin määrä oli kuitenkin selvästi vähäisempi kuin villityypin gp64-proteiinin, mikä voitiin todeta muun muassa fluoresenssi-

spektroskopia-mittauksissa (III). Virukset kohdentuivat soluihin, joiden pinnalla oli fuusioproteiineihin sitoutuvia proteiineja. CEA–vasta-ainetta ilmentävä virus kohdentui CEA-antigeeniä ilmentäviin syöpäsoluihin (PC-3) ja ZZ-domeeneja ilmentävä virus pystyttiin kohdentamaan anti-integriini vasta-aineen avulla BHK-solujen pintaan. Koska ZZ-domeeni sitoo useimpia immunoglobuliini G-alaluokkia, voidaan sitä ilmentävä virus kohdentaa mihin tahansa soluun, mikäli solulle spesifinen vasta-aine on olemassa. Vaikka virukset kohdentuivat odotetulla tavalla, eivät kokeet kuitenkaan osoittaneet tehostunutta fluoresoivan geenin ilmentymistä kohdesoluissa. Tämä oli jossain määrin yllättävää, koska display–virukset kuitenkin sitoutuvat selvästi paremmin solujen pintaan kuin kontrollivirus. Tämän perusteella olisi voitu olettaa, että display-virukset olisivat myös tunkeutuneet tehokkaammin solujen sisään. Yhtenä mahdollisena syynä tähän pidettiin sitä, että fuusioproteiini haittaa gp64:n toimintaa ja siten estää viruksen vapautumisen solun sytoplasmaan.

Jotta gp64 pystyisi toimimaan normaalisti, kehitimme viruksen, jossa ilmennettävät ZZ-domeenit liitettiin vierasta alkuperää olevaan viruksen pintaproteiiniin, vesicular stomatitis viruksen (VSV) G-proteiiniin. Siten viruksen pinnalla ilmentyi sekä villityypin gp64-proteiinia että ZZ-VSV G-fuusioproteiinia (IV). Tuotettu bakulovirus osoittautui tehokkaaksi vasta-aineiden sitojaksi, jonka pinnalla ilmentyi selvästi enemmän fuusioproteiinia kuin gp64-fuusion yhteydessä. Uusi virus sitoutui myös kohdesolujensa pintaan, vaikka aiheuttikin korkeampaa epäspesifistä taustaa kuin kontrollivirukset. Tämä johtui mahdollisesti VSV:n G-proteiinista, joka edesauttaa viruksen sitoutumista useisiin solutyyppeihin. Kyseinen display-virus ei muiden viruskonstruktien tavoin parantanut fluoresoivan geenin ilmentämistä kohdesoluissa. Aikaisemmat tutkimukset ovat osoittaneet, että bakulovirukset kykenevät pääsemään vain heikosti nisäkässolujen tumaan, jossa siirtogeenin ilmentyminen tapahtuu, mikä on todennäköinen syy matalaan geeniekspressioon myös tässä tutkimuksessa.

Näiden tulosten mukaan bakulovirusten pinnalla voidaan ilmentää toimivia peptidejä tai proteiineja ja saadut rekombinanttiset display–virukset sitoutuvat haluttuihin soluihin. Vaikka siirrettävän geenin ilmentyminen kohdesoluissa ei tehostukaan, spesifinen sitoutuminen parantaa bakulovirusten soveltuvuutta geeniterapiaan. Kehittämällä rekombinanttisia bakuloviruksia ja koeolosuhteita edelleen, ovat nämä virukset tulevaisuudessa varteenotettava vaihtoehto terapeuttisten geenien siirtämisessä haluttuihin soluihin.

Yllä mainittujen tutkimusten lisäksi haluttiin osoittaa bakuloviruksen käyttömahdollisuudet proteiinitutkimuksissa nisäkässoluissa (V). Tutkimuksessa käytettiin malliproteiineina vihurirokkoviruksen (rubella virus, RV) pintaproteiineja E1 ja E2, sillä vihurirokkoviruksen biologia on melko huonosti tunnettu. Tämän tutkimuksen tarkoituksena oli lisätä tietämystä viruksen pintaproteiinien tuotosta ja lokalisaatiosta kohdesoluissa. E1 ja E2 vaippaproteiinit ilmennettiin fluoresoivina fuusioproteiineina nisäkässoluissa siirtämällä proteiineja ilmentävät geenit kohdesoluihin bakuloviruksen avulla. Fluoresoivien fuusioproteiinien avulla osoitettiin, että tuotettaessa niitä BHK-soluissa, niiden kulkeutumista ja sijaintia eri soluelinten välillä voidaan seurata.

Fluoresoivien molekyylien vaikutusta vaippaproteiinien kulkeutumiseen ei kuitenkaan voitu sulkea pois. Tässä tutkimuksessa kehitetyt fluoresoivat fuusioproteiinit voivat tulevaisuudessa avata mahdollisuuden proteiini-proteiini –vuorovaikutusten kineettisiin tutkimuksiin sekä osoittavat, että bakuloviruksia voidaan hyödyntää kimeeristen virusproteiinien solukäyttäytymisen seurannassa.

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