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Occlusion-Derived Baculovirus: Interaction with Human Hepatocarcinoma Cells and Application of Baculovirus Display Technology

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Master's Thesis
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PREFACE

This study was carried out at the University of Jyväskylä, Department of Biological and Environmental Science, Division of Biotechnology.

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Abstract:

The baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), is a promising gene delivery vector, which can transiently or stably transduce both dividing and nondividing mammalian cells. As an insect pathogen, *Ac*MNPV is unable to replicate in mammalian cells, rendering it a safe vector candidate for gene therapy purposes. *Ac*MNPV has two distinctive phenotypes, the budded virus (BV) and the occlusion-derived virus (ODV), of which only the former has been studied for gene delivery purposes to mammalian cells. The phenotype of BV has also been modified by envelope and capsid display technologies. Alternatively, ODV might have several advantages as a gene delivery vector in comparison to BV, including its inherent inability to transduce mammalian cells, easy purification, and the ability to simultaneously introduce several genetic payloads to target cells. The objectives of this study were to investigate the entry and gene delivery of ODV to human hepatocarcinoma, HepG2, cells and to apply the baculovirus display technology to include ODV surface display. Two recombinant viruses, namely *Ac*WT and *Ac*ZZp74, were constructed, both containing mammalian cell-active reporter genes. *Ac*ZZp74 ODV was designed to display the IgG-binding ZZ domains of protein A fused to the ODV envelope protein p74 (ZZp74). In principle, the ZZ-displaying ODV should be able to bind to any cell surface antigen, for which an antibody exists. By confocal microscopy, *Ac*WT ODV, possessing wild-type phenotype, was demonstrated to bind to HepG2 cells, and quantitative analysis by flow cytometry showed the binding to be concentration-dependent. *Ac*WT ODV was also detected to enter to the cytoplasm of HepG2 cells at 4 °C, suggesting direct membrane fusion of the virus envelope with the target cell membrane. However, no nuclear-localized capsids or transgene expression were observed in the *Ac*WT or *Ac*ZZp74 ODV-transduced target cells. Unexpectedly, Western blotting showed that the *Ac*ZZp74 BV, in addition to ODV, incorporated the ZZp74 fusion protein. This probably resulted from abnormal localization of ZZp74 in nucleocapsids, and thus, the ZZp74 was most likely not displayed on the *Ac*ZZp74 ODV surface as emphasized. In addition, the *Ac*ZZp74 BV was observed to be normally infective in insect cells, whereas its ability to transduce mammalian cells was abolished. To date, BV has been considered to use similar endocytic route to enter both insect and mammalian cells, whereas the behaviour of *Ac*ZZp74 BV indicates that there are some differences in the entry processes. Thus, *Ac*ZZp74 BV could prove to be a valuable tool for studying BV entry mechanism into mammalian cells. Moreover, the ODV display technology appears possible and holds potential for gene delivery or other purposes.

Keywords: baculovirus, *Ac*MNPV, ODV, p74, Z domain, display, gene delivery, binding, HepG2 cells

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Tiivistelmä:

Bakulovirus (*Autographa californica* multiple nucleopolyhedrovirus) on lupaava geeninsiirtovektori, joka kykenee transdusoimaan pysyvästi tai väliaikaisesti niin jakautuvia kuin jakautumattomiakin nisäkässoluja. Hyönteisiä infektoivana viruksena *Ac*MNPV ei kykene lisääntymään nisäkässoluissa, mikä tekee siitä turvallisen geeniterapiavektoriehdokkaan. *Ac*MNPV:llä on kaksi fenotyyppiä, BV (Budded Virus) eli silmikoituva virus ja ODV (Occlusion-Derived Virus) eli okluusioperäinen virus, joista vain ensin mainitun geeninsiirtokykyä nisäkässoluihin on tutkittu. BV:n fenotyyppiä on myös muunneltu ilmentämällä vieraita proteiineja tai peptidejä viruksen vaipan tai kapsidin pinnalla. ODV:lla puolestaan voisi olla useita etuja geeninsiirtovektorina BV:hen verrattuna, sillä se on luonnostaan kykenemätön transdusoimaan nisäkässoluja, helppo puhdistaa sekä kykenee siirtämään samanaikaisesti useita genomin kopioita kohdesoluihin. Tämän tutkimuksen tarkoituksena oli tutkia ODV:n sisäänmenoa ja geeninsiirtoa ihmisen hepatokarsinoomasoluihin (HepG2) sekä soveltaa bakuloviruksen pintailmennystekniikkaa ODV:hen. Kaksi reportterigeenejä sisältävää rekombinanttivirusta, *Ac*WT ja *Ac*ZZp74, kloonattiin tutkimusta varten. *Ac*ZZp74:n ODV suunniteltiin ilmentämään vaippansa pinnalla proteiini A:n IgG:tä sitovia ZZ-domeeneja fuusioimalla ne ODV:n p74-kalvoproteiiniin (ZZp74). ZZ-domeeneja pinnallaan ilmentävän ODV:n pitäisi periaatteessa kyetä sitoutumaan mihin tahansa solun pinnan antigeeniin, jota vastaan on olemassa vasta-aine. Villityypin fenotyyppiä edustavan *Ac*WT:n ODV:n havaittiin konfokaalimikroskopian avulla sitoutuvan HepG2-soluihin, ja kvantitatiivinen analyysi virtaussytometrillä osoitti sitoutumisen olevan konsentraatiosta riippuvaa. *Ac*WT:n ODV:n havaittiin myös pääsevän HepG2-solujen sytoplasmaan 4 °C:ssa, mikä viittaa suoraan kalvofuusioon viruksen vaipan ja kohdesolun kalvon välillä. Tumaan kulkeutuneita kapsideja tai siirtogeenin ilmentymistä ei kuitenkaan havaittu *Ac*WT:n tai *Ac*ZZp74:n ODV:illa transdusoiduissa kohdesoluissa. Western-blottaus osoitti yllättäen, että ODV:n lisäksi myös *Ac*ZZp74:n BV sisältää ZZp74-fuusioproteiinia. Tämä oli todennäköisesti seurausta ZZp74:n epänormaalista sijoittumisesta nukleokapsideihin, ja näin ollen ZZp74 tuskin ilmentyi suunnitellusti *Ac*ZZp74:n ODV:n pinnalla. Lisäksi *Ac*ZZp74:n BV:n havaittiin olevan normaalisti infektiivinen hyönteissoluissa, mutta menettäneen kykynsä transdusoida nisäkässoluja. Tähän asti BV:n on ajateltu käyttävän samankaltaista endosytoottista reittiä niin hyönteis- kuin nisäkässoluihin päästäkseen, kun taas *Ac*ZZp74:n BV:n käyttäytyminen viittaa jonkinlaisiin eroihin sisäänmenoprosesseissa. *Ac*ZZp74:n BV saattaa siis osoittautua hyödylliseksi työkaluksi tutkittaessa BV:n sisäänmenomekanismia nisäkässoluissa. Myös proteiinien tai peptidien ilmentäminen ODV:n pinnalla vaikuttaa hyvinkin mahdolliselta ja täten lupaavalta tekniikalta geeninsiirtoon tai muihin tarkoituksiin.

Avainsanat: bakulovirus, *Ac*MNPV, ODV, p74, Z-domeeni, pintailmennys, geeninsiirto, sitoutuminen, HepG2-solut

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ABBREVIATIONS

*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
BBMV	brush border membrane vesicle
β-gal	*β-galactosidase*
bp	base pair
BSA	bovine serum albumin
BV	budded virus
CEA	carcinoembryonic antigen
CHO cell	Chinese hamster ovary cell
CMV	cytomegalovirus *immediate early* promoter
Da	dalton
ddH_2O	double-deionized water
DIC	differential interference contrast
E. coli	*Escherichia coli*
EGFP	enhanced green fluorescent protein
FCS	fetal calf serum
GFP	green fluorescent protein
gp64	major envelope glycoprotein 64 of *Ac*MNPV
HIV-1	human immunodeficiency virus type 1
IgG	immunoglobulin G
LB	Luria(-Bertani) Broth
MNPV	multiple nucleopolyhedrovirus
MOI	multiplicity of infection
NPV	nucleopolyhedrovirus
ODV	occlusion-derived virus
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pfu	plaque-forming unit
p.i.	post-infection
pif	*per os* infectivity factor
p.t.	post-transduction
RT	room temperature
scFv	single-chain variable fragment antibody
SDS	sodium dodecyl sulfate
*Sf*9 cell	*Spodoptera frugiperda* 9 cell
SNPV	single nucleopolyhedrovirus
SV40	simian virus 40 promoter
TBS	Tris-buffered saline
vp39	virus structural protein 39 *Ac*MNPV
VSVg	vesicular stomatitis virus envelope G glycoprotein
wt	wild-type
Z domain	synthetic IgG-binding domain from protein A

1 INTRODUCTION

1.1 Baculoviruses

Baculoviruses constitute a large family (*Baculoviridae*) of double-stranded DNA viruses with over 500 different types known so far (Hu, 2005; Summers, 2006). Baculoviruses are invertebrate-specific viruses, which predominantly infect the larval stages of insects in the orders Lepidoptera (butterflies and moths), Diptera (mosquitoes), and Hymenoptera (sawflies) (Blissard and Rohrmann, 1990). Baculovirus genome is circular, 80-180 kb in size (Jehle et al., 2006), and surrounded by a rod-shaped nucleocapsid. Traditionally, baculoviruses have been grouped into two genera: nucleopolyhedroviruses (NPVs) and granuloviruses (Blissard and Rohrmann, 1990). Granuloviruses form small occlusion bodies with a single embedded virion, whereas NPVs form larger occlusion bodies with multiple embedded virions. Moreover, the virions of granuloviruses are enclosed in occlusion bodies composed of granulin matrix, while NPV occlusion bodies are composed of polyhedrin (Rohrmann, 1992). NPVs can be further subdivided to single and multiple NPVs (SNPV and MNPV, respectively) containing either a single nucleocapsid or multiple nucleocapsids within their envelope, respectively. However, a new classification and nomenclature for the genera within the baculovirus family has recently been proposed (Jehle et al., 2006). This classification includes four genera: alphabaculovirus (lepidopteran-specific NPV), betabaculovirus (lepidopteran-specific granuloviruses), gammabaculovirus (hymenopteran-specific NPV) and deltabaculovirus (dipteran-specific NPV) (Jehle et al., 2006).

Originally, baculoviruses were mainly used to control insect pest populations due to their high species-specificity and efficiency against certain pests (Blissard and Rohrmann, 1990). The restriction of baculovirus infection to arthropod hosts renders it a safe pesticide (for review see Maeda, 1995). The most prominent problem with baculovirus pesticides is the slow killing effect, which enables the insect hosts to feed for days or weeks before their death. As the genetic engineering of baculoviruses has been developed, it has also been applied to improve baculoviruses as pesticides. A frequently used approach to increase the pathogenicity of baculovirus is the incorporation of an insect-specific toxin in the viral

genome for expression in the infected host cells. (For review see Inceoglu et al., 2006). However, the expression of the toxin does not necessarily improve viral infectivity (Martens et al., 1995; Ribeiro and Crook, 1993). Incorporation of a toxin protein into polyhedra is more effective because the toxin is thus both delivered directly to its normal site of activity, the gut of the host, as well as expressed in the cells of the host (Chang et al., 2003).

Later on, baculoviruses have become very popular in many molecular biology and biotechnology applications. Accordingly, baculovirus-insect cell expression system was developed for routine production of recombinant proteins during the 1980s, and so far several hundred complex animal, human, and viral proteins have been successfully produced for e.g. diagnostic applications (for review see Summers, 2006). Protein production in insect cells with the aid of baculovirus has the advantage of proper post-translational modification comparable to that of mammalian cells, high insertion capacity for foreign genes, a very high yield of heterologous proteins as a result of using strong baculoviral promoters derived from *polyhedrin* and *p10*, and biosafety due to the inherent inability of baculoviruses to infect mammalian cells (for review see Hu, 2005; Kost et al., 2005). Disadvantages of the baculovirus-insect cell expression system include improper glycosylation in some cases. Moreover, baculovirus infection gradually causes immature post-translational modification and finally termination of protein expression as the cells progress towards their deaths (Hu, 2005; Summers, 2006).

More recently, baculoviruses have emerged as auspicious candidates for gene therapy and for other *in vivo* applications. Baculoviruses appear to be suitable for gene therapy, *inter alia*, due to their inability to replicate in mammalian cells, lack of cytotoxicity and efficient transduction of various mammalian cell lines (see section 1.3). Some challenges also exist, for example the induction of immune responses by baculovirus vectors (section 1.3.3.2), and the inactivation of the vectors by the complement system (section 1.3.3.1). However, the ability of baculovirus to elicit immune responses can be advantageously exploited for vaccine purposes (section 1.3.3.2). A strategy to display foreign peptides or proteins on the surface of baculovirus has been recently invented, having several promising applications (section 1.4).

1.2 *Autographa californica* multiple nucleopolyhedrovirus, *Ac*MNPV

The most widely studied species of baculoviruses is the *Autographa californica* multiple nucleopolyhedrovirus, *Ac*MNPV, which was first isolated from *Autographa californica*, alfalfa looper, but is able to infect at least 40 other species within the Lepidoptera (for review see Bonning, 2005). The genome of *Ac*MNPV (133 894 bp) has been completely sequenced (Ayres et al., 1994).

1.2.1 Infection cycle

*Ac*MNPV produces two types of viral progeny, budded virus (BV) and occlusion-derived virus (ODV), during infection (Blissard and Rohrmann, 1990) (Fig. 1.1). The roles of the phenotypes in the virus life cycle significantly differ from each other: ODV is responsible for the establishment of the first round of infection within the larval host, whereas BV spreads the infection throughout the host (Braunagel and Summers, 1994; Volkman, 1983; Volkman and Summers, 1977; Volkman et al., 1976). Infection ensues when a larval host ingests ODV-containing viral occlusions called polyhedra while feeding. The alkaline environment of the host midgut dissolves the polyhedra releasing ODVs, which

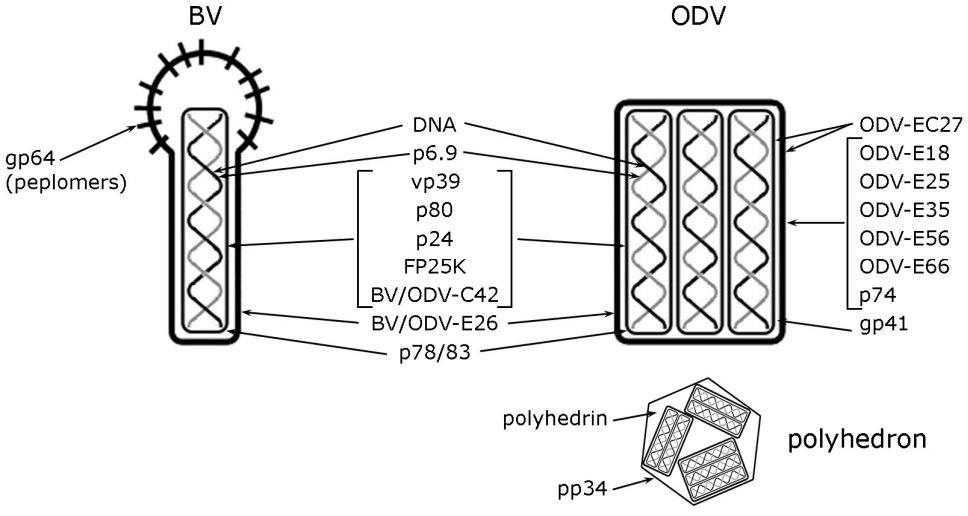

Figure 1.1 The two phenotypes of *Autographa californica* multiple nucleopolyhedrovirus, *Ac*MNPV. Budded virus (BV) and occlusion-derived virus (ODV) have identical nucleocapsids, whereas the protein and lipid (not shown) compositions of their envelopes are different. ODVs are further enclosed in polyhedra.

subsequently enter midgut epithelial cells (Fig. 1.2). BV production begins early in the infection cycle by budding of the progeny nucleocapsids from the nucleus. Subsequently, the nucleocapsids lose their envelope originating from the nuclear envelope and bud through the plasma membrane acquiring their characteristic envelope with virus-encoded proteins (Blissard and Rohrmann, 1990). The budding of BVs through the basal side of the midgut epithelial cells into the hemocoel transmits the infection first to juxtaposed tracheal cells and finally throughout the host (Engelhard et al., 1994; Kirkpatrick et al., 1994; Washburn et al., 1995; Washburn et al., 1999). Approximately 24 h post-infection (p.i.), virus production shifts predominantly from BVs to ODVs (Blissard and Rohrmann, 1990), which are produced by enveloping nucleocapsids with viral-induced membranes in the intranuclear ring zone of the nucleus (i.e. peristromal space) (Stoltz et al., 1973; Summers and Volkman, 1976). Mature ODVs, which generally contain several nucleocapsids, are

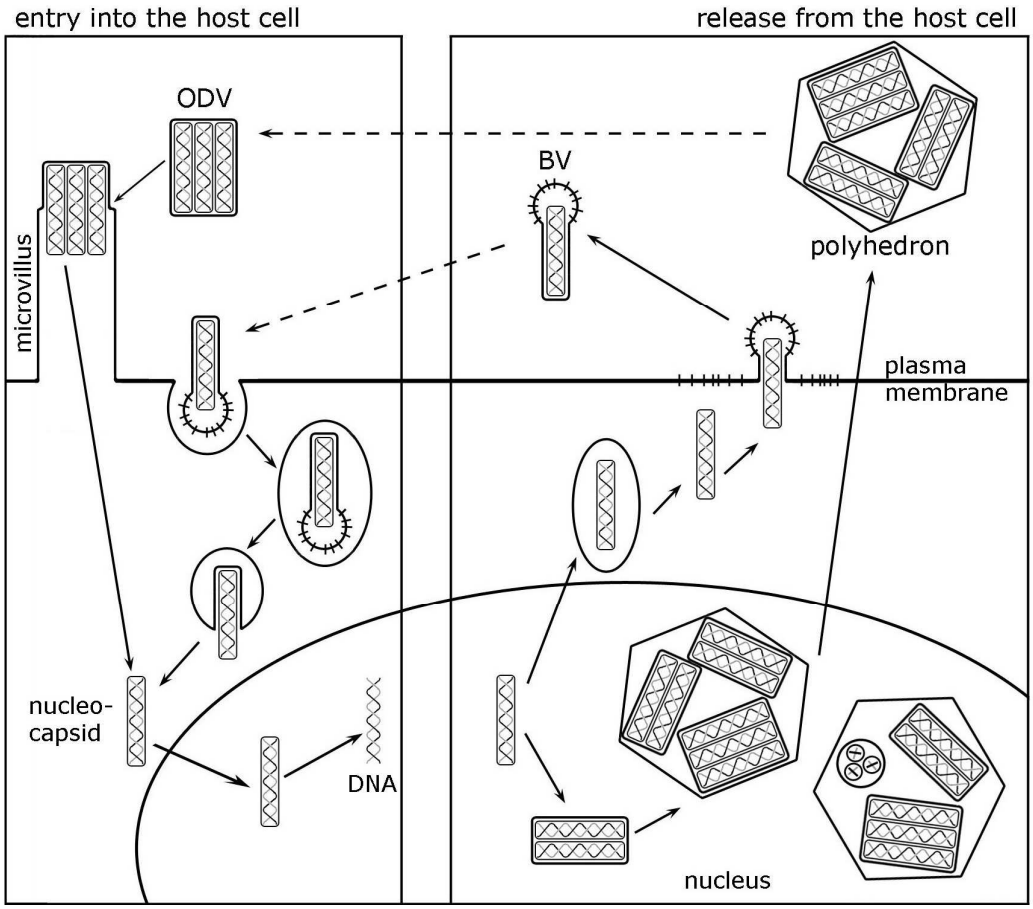

Figure 1.2 The infection cycle of *Autographa californica* multiple nucleopolyhedrovirus, *Ac*MNPV. ODV, occlusion-derived virus; BV, budded virus.

occluded within a proteinaceous matrix composed of the viral protein polyhedrin. Generally, the diameter of the occlusion bodies or polyhedra is 0.5-2 μm, but it can be up to 15 μm (Jehle et al., 2006; Slack and Arif, 2007). Each polyhedron is further enclosed by envelope or calyx, which predominantly comprises of carbohydrates and phosphoprotein pp34, which is linked to polyhedrin and to other pp34 molecules by thiol bonds (Gombart et al., 1989; Whitt and Manning, 1988). Cell lysis in the dying host finally releases the polyhedra into the environment, where they can be ingested by another host (Blissard and Rohrmann, 1990). Polyhedra are very stable and survive extremely well in the environment unless exposed to direct sunlight, the UV radiation of which inactivates the virus (for review see Slack and Arif, 2007). Although the ODV-containing polyhedra have a crucial role in transmitting the infection from host to host, they are not required for the propagation of baculovirus in cell culture (Smith et al., 1983a), and hence in baculovirus expression systems, the non-essential *polyhedrin* gene is often replaced with a foreign gene to exploit the strong *polyhedrin* promoter for heterologous protein expression.

1.2.2 Baculovirus phenotypes: BV and ODV

BV and ODV have identical genomes and rod-shaped nucleocapsids, which are 30-60 nm in diameter and 250-300 nm in length (Jehle et al., 2006), whereas their envelopes differ in lipid and protein compositions due to their different origins (Braunagel and Summers, 1994; Volkman, 1983). Consequently, the phenotypes have different antigenicities, tissue specifities, and entry mechanisms into their host cells (for review see Blissard and Rohrmann, 1990; Volkman, 1997). Mature BV is highly infectious to the tissues of the host and to cultured cells, whereas ODV is generally less infectious, with the exception of the highly differentiated columnar epithelial cells within the larval midgut (Keddie and Volkman, 1985; Volkman and Summers, 1977; Volkman et al., 1976).

The proteins shared by both BV and ODV (Fig. 1.1) include, *inter alia*, the major capsid protein vp39 (viral structural protein 39; Pearson et al., 1988; Thiem and Miller, 1989), DNA-binding protein p6.9 (Wilson et al., 1987), envelope protein E26 (Beniya et al., 1998), and capsid protein FP25K (Beames and Summers, 1988). The FP25K protein is indeed a structural protein of both BV and ODV nucleocapsids, even though a large

fraction of the protein remains in the cytoplasm during the infection (Braunagel et al., 1999; Harrison and Summers, 1995a). The FP25K is not essential *in vitro*, but it is known to be required for virion occlusion and formation of polyhedra, although the exact mechanism has not been determined (Beames and Summers, 1988; Beames and Summers, 1989; Harrison and Summers, 1995b; Wang et al., 1989). Disruption of FP25K expression produces a few polyhedra phenotype, which is, for example, characterized by a decreased polyhedrin production and polyhedra formation as well as increased BV production (Harrison and Summers, 1995b; Hink and Vail, 1973; Ramoska and Hink, 1974). A protein present only in BV is major envelope glycoprotein gp64 (Volkman and Goldsmith, 1984; Whitford et al., 1989), which has several important functions in viral entry and budding (see section 1.2.3). ODV-specific proteins include envelope proteins p74, which is involved in ODV entry into host cells (Faulkner et al., 1997; Kuzio et al., 1989), E66 (Hong et al., 1994), and E25 (Russell and Rohrmann, 1993), as well as gp41, which is located between the nucleocapsid and the envelope of ODV (Maruniak et al., 1979; Stiles and Wood, 1983; Whitford and Faulkner, 1992b). Other baculoviral proteins include polyhedrin and p10, which are expressed very late in the infectious cycle. The expression of these proteins continues until cell death, and consequently, they accumulate in the cell in vast amounts (Smith et al., 1983b). Polyhedrin, which eventually is incorporated into polyhedra, localizes mostly intranuclearly, whereas p10 forms fibrillar bodies both in the cytoplasm and the nucleus of the infected cells (Van Oers et al., 1994; Vlak et al., 1988; Williams et al., 1989). P10 is a nonstructural protein, which is absent from BV and ODV, although some p10 copurifies with polyhedra (Vlak et al., 1981). Deletion of the *p10* has been observed to cause disappearance of the fibrillar bodies and to prevent cell lysis, while the production of polyhedra is not hindered (Williams et al., 1989), albeit the lack of p10 impedes the release of polyhedra from the host and possibly reduces their stability (Gross et al., 1994). However, p10 is not required for cell lysis, but instead appears to have a role in the disintegration of the nuclear matrix, although the fibrillar structure itself is not essential for the disintegration (van Oers et al., 1993). In addition, p10 appears to have a role in the morphogenesis of polyhedra and most likely a binding site for pp34 (for review see Van Oers and Vlak, 1997).

BV exploits adsorptive endocytosis (Volkman and Goldsmith, 1985; Volkman et al., 1984) to enter host cells, while ODV appears to enter midgut epithelial cells via direct membrane fusion at the cell surface (Granados, 1978; Granados and Lawler, 1981; Horton and Burand, 1993; Kawanishi et al., 1972; Tanada et al., 1975). After the nucleocapsids have reached the cytoplasm, they induce formation of actin filaments and are transported towards the nucleus (Charlton and Volkman, 1993; Goley et al., 2006; Lanier and Volkman, 1998; Roncarati and Knebel-Mörsdorf, 1997). Subsequently, the nucleocapsids interact with nuclear pores to enter the nucleus, where they uncoat to release their genomes (Granados, 1978). Transcription, DNA replication and assembly of new nucleocapsids occur within the nucleus (Granados, 1978; Granados and Lawler, 1981; Knudson and Harrap, 1976; Wilson and Price, 1988). Filamentous actin is known to be essential also for the production of viral progeny (Charlton and Volkman, 1991; Ohkawa et al., 2002). Early viral gene products are required to cause accumulation of monomeric actin in the nucleus and late gene products to polymerize the actin into filaments (Charlton and Volkman, 1991; Ohkawa et al., 2002). Supporting evidence has been gathered by using actin-binding drugs (cytochalasin D or latrunculin A), which interfere with actin function, and thus prevent proper intranuclear assembly of viral nucleocapsids and the production of infectious progeny (Charlton and Volkman, 1991; Hess et al., 1989; Kasman and Volkman, 2000; Volkman, 1988; Volkman et al., 1987).

1.2.3 BV entry into host cells

BV is known to exploit adsorptive endocytosis to enter its host cells, although the exact mechanism has not been determined (Volkman and Goldsmith, 1985; Volkman et al., 1984). In addition, BV can enter insect cells at 4 °C by fusion of its envelope with the cell membrane, but entry through this route is exiguous, and it is not known whether BV can establish a productive infection thereafter (Volkman et al., 1986). It has also been suggested that BV primarily uses clathrin-mediated endocytosis to enter insect as well as mammalian cells (Long et al., 2006). Gp64, the major envelope protein of BV, has been shown to be necessary and sufficient for low pH-triggered membrane fusion activity, which is required for BV envelope to fuse with the membrane of an endosome (Blissard and Wenz, 1992; Chernomordik et al., 1995; Leikina et al., 1992; Markovic et al., 1998;

Monsma and Blissard, 1995) (Fig. 1.2). Gp64 is a glycoprotein present on the surface of infected cells and BVs as homo-oligomers (Volkman and Goldsmith, 1984) known as peplomers (Summers and Volkman, 1976). Each peplomer is comprised of three identical phosphorylated, acetylated, and glycosylated gp64 proteins, which are stabilized by intermolecular disulfide bonds formed post-translationally (Markovic et al., 1998; Oomens et al., 1995; Roberts and Faulkner, 1989; Volkman and Goldsmith, 1984; Volkman et al., 1984). The peplomers are restricted to one pole of BV (Summers and Volkman, 1976) (Fig. 1.1). There are two isomeric forms of gp64 trimers, which are thought to differ in the pattern of disulfide bonding (Oomens et al., 1995), although it is not yet known whether the two forms have different functions. Distinct domains of gp64 with different functions have been identified: a fusion domain essential for the pH-dependent membrane fusion, an oligomerization domain required for the production of gp64 trimers (Monsma and Blissard, 1995)), a C-terminal hydrophobic sequence anchoring gp64 on the plasma membrane (Blissard and Rohrmann, 1989), and a cytoplasmic tail domain, which has been suggested to promote efficient budding of BV and is involved in concentrating gp64 within the virus particle (Oomens and Blissard, 1999).

First evidence of the essential role of gp64 in the endocytosis of BV came from studies with anti-gp64 antibodies. A monoclonal antibody, AcV1, directed against gp64 was shown to neutralize the infectivity of BV (Hohmann and Faulkner, 1983; Volkman et al., 1984) by inhibiting fusion of the viral envelope with the membrane of an endosome (Chernomordik et al., 1995; Volkman and Goldsmith, 1985). AcV1 recognizes a conformational, 24-amino acid epitope in the neutral pH conformation of gp64, but does not bind to the low-pH conformation or denatured form of gp64 (Chernomordik et al., 1995; Hohmann and Faulkner, 1983; Monsma and Blissard, 1995; Zhou and Blissard, 2006). The neutralization of gp64 function by AcV1 binding has been suggested to be due to inhibition of a required conformational change (Zhou and Blissard, 2006). Baculovirus-infected cells displaying gp64 on their membranes or uninfected cells transiently expressing gp64 from a plasmid have been shown to be capable of mediating low-pH triggered membrane fusion (Blissard and Wenz, 1992; Leikina et al., 1992). This indicates that gp64 alone is sufficient to enable membrane fusion between the envelope of BV and the membrane of an endosome (Blissard and Wenz, 1992; Monsma and Blissard, 1995).

Interestingly, gp64 can be replaced with vesicular stomatitis virus envelope G glycoprotein (VSVg) (Mangor et al., 2001) or with F proteins, which are fusion proteins mediating pH-triggered membrane fusion of some other baculoviruses (Lung et al., 2002). However, replacement of gp64 with VSVg decreases the infectious virus titers (Mangor et al., 2001) and all F proteins cannot substitute for gp64 (Lung et al., 2002).

The fusogenic activity of gp64 has been observed to be dependent on the presence of stable gp64 trimers on the membrane (Markovic et al., 1998). The monomeric forms of gp64 are not transported to the surface of the infected cell but are degraded (Monsma and Blissard, 1995; Oomens et al., 1995). Moreover, the low pH-induced conformational changes of individual gp64 trimers on the viral envelope have been demonstrated to be required, but not sufficient, to enable membrane fusion (Markovic et al., 1998). A prerequisite for the occurrence of the membrane fusion appears to be assembly of the gp64 trimers into multiprotein complexes, which can have up to 10 peplomers (Markovic et al., 1998; Plonsky and Zimmerberg, 1996). This lateral assembly of gp64 trimers occurs only in the presence of the contacting membrane (Markovic et al., 1998).

In addition to its role as a fusion protein, gp64 has been proven to be a viral host cell receptor-binding protein (Hefferon et al., 1999). A soluble form of gp64, produced by deletion of the transmembrane anchor domain of gp64, has been observed to specifically compete with a recombinant *Ac*MNPV marker virus for binding to host cells (Hefferon et al., 1999). The disulfide bonds of gp64 might be important in maintaining a structure, which efficiently binds the receptor (Hefferon et al., 1999). Moreover, the glycosylation state of gp64 may also affect virion binding to host cells (Jarvis et al., 1998). The host cell receptor for gp64 has not been identified to date, but phospholipids have been shown to have a role in the binding or entry process of BV in both insect and mammalian cells (Tani et al., 2001).

Yet another role for gp64 is in the budding of BV from the infected cells (Fig. 1.2). Gp64 concentrates in discrete areas on the plasma membrane (Blissard and Rohrmann, 1989), and BV budding occurs at these sites (Volkman et al., 1984). By using an *Ac*MNPV with an inactivated *gp64*, Monsma and coworkers (1996) were able to demonstrate that gp64 is

necessary for the propagation of BV infection through cell-to-cell transmission. More recently, the defective infectivity of gp64-null BVs has been detected to be due to lack of virion production (Oomens and Blissard, 1999).

1.2.4 ODV entry into host cells

ODV is known to use direct membrane fusion to enter host midgut cells (Granados, 1978; Granados and Lawler, 1981; Horton and Burand, 1993; Kawanishi et al., 1972; Tanada et al., 1975), although the entry process has not been fully characterized. Polyhedra are pH-sensitive and rapidly dissolve in the highly alkaline digestive fluids of the larval host releasing the ODVs, which subsequently enter the host midgut cells by direct fusion of their envelope with the membrane of the apical microvilli of the columnar epithelial cells (Granados, 1978; Granados and Lawler, 1981; Horton and Burand, 1993; Kawanishi et al., 1972; Tanada et al., 1975) (Fig. 1.2). The columnar cells of the midgut epithelium are lined by a protective extracellular fibrous matrix called peritrophic membrane, which is composed of chitin, glycosaminoglycans, and proteins (for review see Richards and Richards, 1977; Wang and Granados, 2001). It is not thoroughly known how ODV gains access through the peritrophic membrane. The occlusions of some baculoviruses are known to contain metalloprotease called enhancin, which facilitates penetration through the peritrophic membrane by degrading mucin (Bischoff and Slavicek, 1997; Derksen and Granados, 1988; Lepore et al., 1996; Wang and Granados, 1997; Wang et al., 1994). On the other hand, results obtained by Washburn and collagues in 1995 suggested that peritrophic membrane did not significantly impede *Ac*MNPV infection of larvae, even though the enhancin levels were negligible. It is also possible that the peritrophic membrane is not fully formed at the time of infection, which would assist ODV to pass through, or if there are other, yet unknown viral proteins with the ability to degrade the peritrophic membrane (Bonning, 2005). Moreover, Adang and Spence (1981) discovered that columnar cell microvilli penetrate through the peritrophic membrane of *Trichoplusia ni* to the lumen of the midgut, suggesting that ODVs do not have to pass through the membrane for fusion with the microvilli. If the same was valid for other insects as well, it would solve the mystery.

A remarkable feature of ODV entry is the large size of the nucleocapsids compared to the microvilli. Nucleocapsids are 30-60 nm by 250-300 nm (Jehle et al., 2006) and microvilli 100 nm by 1 μm (Danielsen and Hansen, 2003), and the nucleocapsids entering apically are transported down the full length of a microvillus. Moreover, microvilli are packed with cross filaments and a bulky core of filamentous actin (Danielsen and Hansen, 2003), which would be supposed to prevent transport of the large nucleocapsids. Therefore, it is quite likely that the nucleocapsids somehow exploit the actin cytoskeleton for their transport along the microvillus. Baculoviral nucleocapsids are, after all, known to exploit actin fibers during their cytoplasmic transport towards the nucleus (see section 1.2.2).

1.2.4.1 Proteins involved in the entry of ODV to host cells

ODV proteins with fusogenic activity have not been identified so far, and it is not precisely known how the fusion occurs. However, ODV is known to bind some specific protein receptor(s) on the surface of the midgut cells (Haas-Stapleton et al., 2004; Horton and Burand, 1993), but these proteins are yet to be identified. In 1993, Horton and Burand studied the fusion between *Lymantria dispar* MNPV ODV and its host tissues by using brush border membrane vesicles (BBMV) produced from *Lymantria dispar* midgut tissue. ODV was demonstrated to bind to a specific receptor, and protease pretreatment studies suggested that the receptor is a protein (Horton and Burand, 1993). However, the identities of the receptor and the ODV binding protein(s) were not discovered, whereas the amount of ODV receptor sites per cell (in a *Lymantria dispar* cell line) was determined to be approximately 1×10^6 (Horton and Burand, 1993). This result compares well with estimations made by Wickham and coworkers (1990), who determined with the aid of a mathematical model that *Spodoptera frugiperda* 9 (*Sf*9) cells have 1×10^5-10^7 receptor sites for *Ac*MNPV BV. More recently, ODV binding to midgut cells has been suggested to be specific (Haas-Stapleton et al., 2004; Haas-Stapleton et al., 2005). Accordingly, the binding of *Ac*MNPV and *Spodoptera frugiperda* MNPV ODVs to *Spodoptera frugiperda* larvae midgut were compared. Unspecifically bound ODVs were detected to fuse with the microvillus membrane, although a productive infection did not necessarily follow (Haas-Stapleton et al., 2004; Haas-Stapleton et al., 2005). In other words, specific ODV binding is required to readily establish infection.

Several proteins mediating the oral infectivity of ODV have been identified to date. These proteins are called *per os* infectivity factors or pifs and they are p74 (Faulkner et al., 1997; Kuzio et al., 1989), pif-1 (Kikhno et al., 2002), pif-2 (Pijlman et al., 2003), pif-3 (Ohkawa et al., 2005), and 11K proteins Ac145 and Ac150 (Lapointe et al., 2004; Zhang et al., 2005). However, only the first three have been shown to be required for ODV entry into midgut cells (Haas-Stapleton et al., 2004; Ohkawa et al., 2005). The first protein discovered to be involved in oral infectivity by ingestion of polyhedra was the ODV-specific p74 (Faulkner et al., 1997; Kuzio et al., 1989). In 1989, Kuzio and coworkers observed that deletion of the C-terminus of p74 abolishes oral infectivity of ODV, but does not interfere with virus replication in cell culture. More recent studies have also proven p74 to be essential for the infectivity of ODV (Faulkner et al., 1997; Haas-Stapleton et al., 2004; Yao et al., 2004; Zhou et al., 2005). The *p74* gene is only weakly transcribed and expressed between 16 and 20 h p.i. (Kuzio et al., 1989), and thus, due to its low abundance, the protein p74 has been difficult to study in the wild-type (wt) virus. Moreover, overexpression of *p74* does not abolish the problem because the abundance of p74 on the surface of ODV is constant and the excess p74 protein will not be assembled into the envelope of ODVs (Zhou et al., 2005). P74 has been demonstrated to localize on the surface of ODV envelope and to be absent from BV (Faulkner et al., 1997; Haas-Stapleton et al., 2004). The exposure of p74 on the surface of ODV suggests that it has a role in ODV attachment and/or fusion with midgut cell membranes (Slack et al., 2001). Moreover, p74 is a well-conserved ODV protein among baculoviruses, supporting the argument (Slack and Lawrence, 2005).

In 1997, Faulkner and coworkers reported p74 to be an ODV envelope protein with the properties characteristic of a viral attachment protein. More recently, *Ac*MNPV ODV and larval midgut epithelia from its host, *Heliothis virescens*, were used to demonstrate that p74 binds a specific cellular receptor in the host midgut (Haas-Stapleton et al., 2004). P74 has also been shown to specifically bind to its host BBMV (Zhou et al., 2005). Moreover, a 35-kDa binding partner for p74 present in host BBMV extracts but absent from non-host BBMV extracts has been discovered (Zhou et al., 2005). The identity of this protein has not been established to date, but it is likely to be the host midgut receptor for p74 (Zhou et al., 2005).

P74 contains a hydrophobic C-terminus, which has been demonstrated to be essential for the infectivity of ODV (Kuzio et al., 1989). The C-terminus has been proven to function as a transmembrane anchor by partial deletion of the C-terminus, which produced a soluble form of p74 (Slack et al., 2001). P74 has been predicted to have three transmembrane domains in total (Fig. 1.3): one in the middle part of the protein (I) and two at the C-terminus (II and III) (Slack et al., 2001). The transmembrane domain I is situated in a large

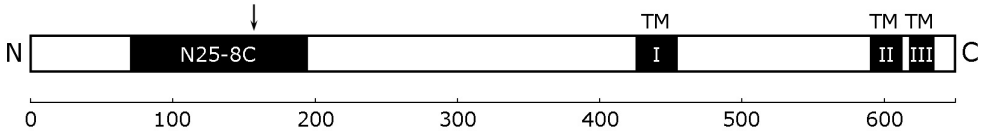

Figure 1.3 Schematic representation of p74 protein of *Autographa californica* multiple nucleopolyhedrovirus, *Ac*MNPV. P74 (645 amino acids) has three predicted transmembrane domains (TM): I (423-452), II (587-609), and III (613-631) (Slack et al., 2001). The sequence containing the antigenic epitope recognized by the antibody N25-8C is illustrated (68-194) (Faulkner et al., 1997; Slack et al., 2001). A predicted, highly conserved trypsin cleavage site (156) (Slack and Lawrence, 2005) is indicated with an arrow. N, N-terminus; C, C-terminus.

hydrophobic region, which might surround the transmembrane domain in the three-dimensional structure of the protein, since the C-terminally truncated p74 is soluble (Slack et al., 2001). The two C-terminal transmembrane domains are highly conserved and are suggested to form a hairpin motif, which anchors the protein in the ODV envelope leaving the N-terminus of p74 exposed on the virion surface (Faulkner et al., 1997; Slack et al., 2001). The transmembrane hairpin has been proposed to also function as a membrane insertion sequence (Slack et al., 2001), which is supported by the observation that p74 protein fed to the larvae with p74-null ODVs can rescue oral infectivity (Yao et al., 2004; Zhou et al., 2005). Other ODV envelope proteins, such as E66 and E25, are also known to contain hydrophobic domains that induce post-translational membrane insertion (Hong et al., 1997). Intranuclear microvesicles in the intranuclear ring zone are the proposed source of ODV envelopes, and several ODV envelope proteins have been observed to be directed there (Hong et al., 1997). The N-terminal hydrophobic domains of E66 and E25 have been shown to be sufficient to target reporter genes into the intranuclear microvesicles (Hong et al., 1997). Accordingly, it has been observed that the hydrophobic C-terminus of p74 is sufficient to direct green fluorescent protein (GFP) into the intranuclear microvesicles, and thus, the C-terminus has been suggested to be required for intranuclear localization (Slack et al., 2001). Gp64 is the only other baculoviral protein, which has been reported to have a

C-terminal transmembrane anchor (Slack et al., 2001), and accordingly, both p74 and gp64 can be solubilized by the deletion of their C-termini (Kuzio et al., 1989; Oomens and Blissard, 1999; Slack et al., 2001). The N-terminus of p74 might be required for nuclear import, since N-terminally truncated p74 proteins do not enter the nucleus, except for the fusion protein containing the hydrophobic C-terminus of p74 and GFP, which is predicted to passively enter the nucleus due to its small size (Slack et al., 2001). However, it is also possible that the transmembrane domain I of the N-terminally truncated proteins is exposed and interacts nonspecifically with cytosolic membranes (Slack et al., 2001).

P74 may be N-terminally cleaved in the host midgut. In 1997, p74 was demonstrated to be susceptible to proteolytic digestion (Faulkner et al., 1997), and more recently, studies conducted with fusion protein containing a C-terminally truncated, soluble p74 fused to GFP, have shown that under alkaline conditions p74 is cleaved by BBMV-specific trypsins (Slack and Lawrence, 2005). Accordingly, one predicted trypsin cleavage site, which is highly conserved among p74 homologues from various baculoviruses, was identified (Fig. 1.3) (Slack and Lawrence, 2005). It was proposed that alkaline conditions prime p74 for N-terminal cleavage by favoring a peptide region to become more hydrophilic and thus exposed on the protein surface (Slack and Lawrence, 2005). However, it is not yet known whether this cleavage is significant for the function of p74, although encountering active trypsins is unavoidable for p74 in natural infection by ODV (Slack and Lawrence, 2005).

The second protein involved in the *per os* infectivity of ODV was identified in studies conducted with *Spodoptera littoralis* NPV deletion mutant, which lacked oral infectivity but retained BV infectivity (Kikhno et al., 2002). A deletion within the *Not*I D fragment was discovered, and subsequently, open reading frame (ORF) 7 in this fragment was determined to be responsible for the lack of oral infection (Kikhno et al., 2002). ORF 7 of *Spodoptera littoralis* NPV is homologous to ORF 119 in *Ac*MNPV and has homologues in all sequenced baculoviruses (Kikhno et al., 2002). This protein with its homologues was named *per os* infectivity factor or pif (Kikhno et al., 2002), and later specified to be pif-1 (Ohkawa et al., 2005). More recently, the necessity of pif-1 in oral infectivity has been confirmed by deleting the corresponding gene from the genome of *Ac*MNPV (Gutierrez et

al., 2005). Pif-1 has been identified to be an ODV envelope protein with an N-terminal hydrophobic sequence, which is predicted to act as a signal peptide (Kikhno et al., 2002).

Pijlman and coworkers (2003) were able to identify a third protein required for the oral infectivity process by studying *Spodoptera exigua* MNPV deletion mutants. A deletion of ORF 35 was observed to abolish the oral infectivity of polyhedra but not to affect the infectivity of BV (Pijlman et al., 2003). This gene, like *p74* and *pif-1*, has a homologue in *Ac*MNPV (ORF 22) and in other sequenced baculoviruses (Herniou et al., 2001). The protein was named *per os* infectivity factor 2 or pif-2 (Pijlman et al., 2003). Pif-2 is predicted to have a strong N-terminal hydrophobic domain and an N-terminal signal peptide similar to pif-1 (Pijlman et al., 2003). This suggests that pif-2 might be associated with membranes, although it is not yet definitely known whether pif-2 indeed is an ODV-specific structural protein (Ohkawa et al., 2005; Pijlman et al., 2003). The N-terminal sequences of pif-1 and pif-2 do not only remind each other but they also are similar to the N-terminal sequences of *Ac*MNPV ODV envelope proteins E66 and E25 (Braunagel et al., 2004; Hong et al., 1997; Pijlman et al., 2003). These other two ODV proteins are conserved among all lepidopteran baculoviruses (Herniou et al., 2001), and it has been shown that their N-terminal hydrophobic domains are sufficient to direct reporter proteins to the nuclear envelope, intranuclear microvesicles, and the ODV envelope within infected cells (Hong et al., 1997). E66 and E25 are not cleaved (Hong et al., 1997), whereas pif-1 and pif-2 have a predicted cleavage site after the hydrophobic domain, even though it is not known whether a cleavage occurs (Pijlman et al., 2003).

Ohkawa and coworkers (2005) used an improved *in vivo* fluoresecence-dequenching assay to demonstrate that pif-1 and pif-2, like p74, have a role in the specific binding of ODV to midgut cells. Moreover, a fourth *pif* gene, *pif-3*, which is ORF 115 in *Ac*MNPV and its homologues in other baculoviruses, was identified (Ohkawa et al., 2005). However, unlike pif-1, pif-2, and p74, pif-3 is not involved in the specific binding of ODV to midgut cells, but mediates another critical, yet unidentified event, during the early primary infection (Ohkawa et al., 2005). Deletion of *pif-1*, *pif-2*, or *p74* have been observed to decrease the binding of ODV to midgut cells but not to diminish the occurrence of fusion among the bound ODVs, which strongly suggest that none of the pifs are fusogenic proteins (Haas-

Stapleton et al., 2004; Ohkawa et al., 2005). It is quite likely that either the ODV fusion proteins are yet to be identified or the fusion mechanism is something unforeseen or both (Ohkawa et al., 2005). The ODV fusion event is quite unique also in other ways. Although the fusion can occur in basic or neutral conditions, it prefers highly alkaline environment and can also occur at 4 °C (Horton and Burand, 1993), whereas most viruses require temperatures of at least 22 °C for the fusion event (for review see White, 1990). *Ac*MNPV BV and influenza virus are both able to penetrate host cell membranes at 4 °C, although it is not known whether they are able to establish an infection thereafter (Stephenson et al., 1978; Volkman et al., 1986).

The identities of the cellular receptors for ODV binding and entry remain a mystery. However, integrins are alluring candidates due to their function as cell surface signaling molecules, which can upon ligand binding trigger changes in the cytoskeleton (for review see Hynes, 2002). Moreover, microvilli are rich in lipid rafts containing cholesterol and sphingolipids, which recruit integrins and other signaling molecules (for review see Danielsen and Hansen, 2003; Del Pozo, 2004). According to Ohkawa and coworkers (2005), the most likely integrin ligand among the known pif proteins is pif-1, which has an RGD minimal integrin recognition motif and also a homologous region with both laminin and tenascins, which signal through RGD motifs (for review see Chiquet-Ehrismann and Tucker, 2004). However, not a lot is known about integrins in lepidopteran larvae, and the possible role of integrins in ODV entry remains to be elucidated. Ohkawa and coworkers (2005) also pondered the possibility of brush border microvilli being the fusogenic apparatus for ODV entry, due to the central role they have in cell fusion events during embryogenesis and after. Consequently, brush border microvilli have also been described as cell-fusion organelles (Wilson and Snell, 1998).

1.2.4.2 Multiple versus single nucleocapsid strategy

Considering the large size of the nucleocapsids compared to microvilli, it seems peculiar that *Ac*MNPV, among other lepidopteran-specific MNPVs, have evolved ODVs, which simultaneously deliver multiple nucleocapsids to the same microvillus, especially when only a single nucleopcapsid per cell would suffice (Washburn et al., 1999). However, ODVs with multiple nucleocapsids have been shown to be more efficient in establishing

systemic infection than genetically identical ODVs with single nucleocapsids, whereas the transport time for both the multiple- and single-nucleocapsid ODVs is similar (Washburn et al., 1999). The efficiency of multiple-nucleocapsid ODVs is mainly based on the acceleration of the onset of the secondary infection by direct repacking of some of the parental ODV-derived nucleocapsids as BVs, which directly bud from the cell without the nucleocapsids entering the nucleus (Granados and Lawler, 1981; Washburn et al., 1999; Washburn et al., 2003b). The budding of BV is possible only when the viral protein gp64 has been synthesized and transported to the cell membrane (Oomens and Blissard, 1999; Volkman et al., 1984; Washburn et al., 2003a; Zhang et al., 2004), and gp64 indeed has the unusual feature of its synthesis being regulated by both an early and a late promoter (Blissard and Rohrmann, 1989; Jarvis and Garcia, 1994; Oomens et al., 1995; Whitford et al., 1989). Therefore, gp64 is produced already early in the infection cycle before any progeny BVs are constructed. The repackaging of ODV-derived nucleocapsids as BVs within the primary target cell before viral replication is completed enables rapid spread of viral infection into the secondary target cells. This speed is especially important for the spread of infection, because sloughing of infected midgut cells has been suggested to be the first line of defense against *Ac*MNPV ODV infection by the host (Engelhard and Volkman, 1995; Granados and Lawler, 1981; Keddie et al., 1989; Kirkpatrick et al., 1998; Washburn et al., 1998). Moreover, sloughing appears to be a major force shaping *Ac*MNPV infection strategies (Washburn et al., 1999).

Washburn and coworkers (2003b) have compared the infectivity of *Ac*MNPV and *Helicoverpa zea* SNPV ODVs in *Heliothis virescens* larvae and observed that ODVs with single nucleocapsids initiate infections more quickly and more numerously than ODVs with multiple nucleocapsids. However, MNPV is more rapid in establishing secondary infection due to its ability to infect secondary target cells with parental ODV nucleocapsids repackaged as BVs, whereas SNPV has to synthesize BVs from the beginning. Nevertheless, due to the rapid initial infection by SNPVs, both *Helicoverpa zea* SNPV and *Ac*MNPV infect secondary target cells at the same time. Moreover, the cells with primary infection by MNPV were observed to be sloughed more frequently than the cells with SNPV infection (Washburn et al., 2003b).

1.3 Baculovirus and mammalian cells

1.3.1 Baculoviral transduction *in vitro*

The host specificity of baculovirus was long supposed to be restricted to cells derived from arthropods, until in 1983, Volkman and Goldsmith demonstrated that both *Ac*MNPV BV and ODV were able to enter certain cell lines derived from vertebrate species (Volkman and Goldsmith, 1983). However, no evidence of viral gene expression was detected (Volkman and Goldsmith, 1983), and later studies have corroborated the results of BV entry into mammalian cells without virus replication (Carbonell et al., 1985; Carbonell and Miller, 1987; Gröner et al., 1984). A decade later, two groups reported that recombinant BVs containing mammalian cell-active promoters can efficiently transduce mammalian cells. Hofmann and coworkers (1995) used recombinant baculoviruses harboring a cytomegalovirus promoter-*luciferase* gene cassette and Boyce and Bucher (1996) baculoviruses with a Rous sarcoma virus long terminal repeat promoter-*β-galactosidase (β-gal)* cassette to transduce primary hepatocytes and a variety of non-hepatic cell lines. Primary hepatocytes and hepatoma cells were efficiently transduced, whereas in many other cell lines the reporter gene activity was significantly lower or not detectable at all. Moreover, the block of expression appeared to be due to events subsequent to viral entry, since cell lines expressing high and low amounts of reporter genes internalized similar amounts of virus (Boyce and Bucher, 1996). More recently, the block to efficient transduction in the less susceptible cells has been suggested to lie in the cytoplasmic trafficking or nuclear import of the viral nucleocapsids (Kukkonen et al., 2003). Promoter strength has also been observed to be an important factor affecting the transduction efficiency. By using a strong CAG promoter, which is a composite promoter consisting of the cytomegalovirus *immediate early* enhancer, chicken *β-actin* promoter, and rabbit *β-globin* polyadenylation signal, efficient transduction of several of the less susceptible cells has been achieved (Shoji et al., 1997). For instance, *luciferase* expression in HeLa cells was detected to be weak with cytomegalovirus *immediate early* (CMV) promoter (Hofmann et al., 1995), whereas with CAG promoter the expression was 10-fold higher (Shoji et al., 1997).

Subsequent studies have identified numerous cells that can be transduced by *Ac*MNPV or other baculovirus vectors. These include human, rodent, porcine, bovine, and even fish cell lines (for review see Hu, 2006; Kost and Condreay, 2002; Kost et al., 2005), as well as primary cells such as human neural cells (Sarkis et al., 2000), human and mouse pancreatic islet cells (Ma et al., 2000), rat Schwann cells (Kenoutis et al., 2006), and rat articular chondrocytes (Ho et al., 2004). Baculovirus has also been shown to be capable of transducing non-dividing cells (van Loo et al., 2001). However, cell lines of hematopoietic origin cannot be efficiently transduced (Cheng et al., 2004; Condreay et al., 1999). Moreover, it has been demonstrated that cells can be simultaneously transduced with two different baculovirus vectors (Yap et al., 1997).

Factors affecting transduction efficiency. In addition to promoter strength and choice of promoter, several other factors have also been shown to influence the efficiency of BV transduction, including addition of histone deacetylase inhibitors, such as sodium butyrate, trichostatin A (Condreay et al., 1999), or valproic acid (Hu et al., 2003a). These compounds prevent histone deacetylation, which results in increased level of transcription. Disadvantageously, these compounds are cytotoxic and induce cell cycle arrest (Kim et al., 2000). In some cases the transduction efficiency can be enhanced by merely altering the transduction protocol (e.g. temperature, medium components, or the surrounding solution) (for review see Hu, 2005). Retroviruses pseudotyped with VSVg have been shown to have enhanced transduction efficiency and broader host range than the original retroviruses (Burns et al., 1993; Emi et al., 1991). Accordingly, Barsoum and collagues (1997) constructed a VSVg-pseudotyped baculovirus and detected enhanced transduction and broader host range. It was suggested that the increased transduction efficiency was due to the ability of VSVg protein to increase the efficiency of baculovirus escape from endosomes (Barsoum et al., 1997). However, vesicular stomatitis virus is known to interact with phosphatidylserine, which is a widely distributed cellular component (Carneiro et al., 2006; Coil and Miller, 2004; Schlegel et al., 1983; Schlegel and Wade, 1983), and the enhancement in transduction efficiency might alternatively result from increased virus binding onto the cell surface.

Insertion of various DNA elements into baculovirus genome have been shown to increase the expression of reporter genes in insect cells (for review see Hu, 2005; Kost et al., 2005). Accordingly, insertion of an additional copy of baculovirus *homology region 1* into the baculoviral genome has been shown to be yet another way to increase the transduction efficiency of BV (Viswanathan et al., 2003). One problem associated with the use of baculovirus as a gene delivery vector is confronted already during purification. BV is generally purified by ultracentrifugation (O'Reilly et al., 1994), which induces virus aggregation (Barsoum, 1999) in addition to being a time-consuming, laborious, and difficult process to scale up. To alleviate the problem, cation-exchange chromatography (Barsoum, 1999) and size-exclusion chromatography (Transfiguracion et al., 2007) methods for BV purification have been developed as well as a hexahistidine tag-displaying recombinant baculovirus, which can be readily purified by immobilized metal affinity chromatography (Hu et al., 2003b).

Transient versus stable transduction. Transduction by baculovirus vectors generally lasts for approximately 1-2 weeks (Hu, 2005). The diminution of the transduction results from cell division as well as degradation of baculoviral DNA (Ho et al., 2004; Wang et al., 2005). However, it has been demonstrated that if Chinese hamster ovary (CHO) cells are transduced with a *GFP*-containing baculovirus vector expressing *neomycin phospho-transferase II*, and selected by using antibiotic G418, the result is CHO cells stably expressing GFP (Condreay et al., 1999). This method yields approximately one stable cell line for every 50 to 100 transiently transduced CHO cells (Condreay et al., 1999). Later studies with four independent CHO cell clones showed that discrete portions of baculovirus DNA are randomly integrated in the cell genome as single-copy fragments ranging in size from 5 to 18 kb (Merrihew et al., 2001). Moreover, two of the four cell clones maintained starting levels of reporter gene expression over a 5 month period (Merrihew et al., 2001). Earlier, a baculovirus-based vector containing two expression cassettes with *β-gal* and *hygromycin* resistance genes, flanked by the inverted terminal repeats of adeno-associated virus, had been constructed (Palombo et al., 1998). With this hybrid vector human fibroblast were stably transduced in the presence of the *rep* gene product responsible for the site-specific integration of adeno-associated virus DNA (Palombo et al., 1998). More recently, two Epstein-Barr virus elements, which are

responsible for the extachromosomal maintenance of the viral genome in host cells, were used to generate baculovirus vectors with prolonged and enhanced reporter gene expression in mammalian cells (Shan et al., 2006).

1.3.2 Baculovirus entry into mammalian cells

For baculovirus to be used as a gene delivery vector for gene therapy, it is important to precisely know how it enters mammalian cells. However, the exact entry mechanism for BV is still poorly characterized, and the entry process of ODV has not been studied at all. BV enters insect cells via an endosomal pathway and is supposed to exploit a similar endocytic route for entry into mammalian cells. This has been proved with electron microscopy as well as with lysosomotropic agents, which block endosomal maturation by preventing the acidification of endosomes, and consequently prevent endosomal release of BV in mammalian cells (Boyce and Bucher, 1996; Hofmann et al., 1995; van Loo et al., 2001). Recently, baculovirus has been suggested to use clathrin-mediated endocytosis and macropinocytosis to enter mammalian cells (Long et al., 2006; Matilainen et al., 2005). Interestingly, caveolae might have a role in BV entry into mammalian cells as well, since transduction is enhanced in the presence of genistein, which inhibits caveola-mediated endocytosis (Long et al., 2006). This was suggested to be due to BVs funneling from the blocked caveola pathway to another entry route (Long et al., 2006). The pH-dependent fusion protein gp64 is supposed to be important in the entry of BV into mammalian cells similar to its role in insect cell entry. This was concluded from evidence showing that monoclonal antibodies against gp64, which inhibit infection of insect cells, also inhibit transduction of mammalian cells (Hefferon et al., 1999; Hofmann et al., 1998; van Loo et al., 2001; Volkman and Goldsmith, 1985). Moreover, BV vectors with increased levels of gp64 on their membrane exhibit enhanced levels of transduction (Tani et al., 2001). However, more studies are required to determine the detailed mechanisms for the binding and entry of BV into mammalian and insect cells, as well as the precise mechanisms for intracellular movement and nuclear entry.

Considering the wide range of mammalian cells that can be transduced by baculovirus vectors (see section 1.3.1), it is likely that the cellular target molecule for BV binding is a

rather widely distributed cell surface component (Duisit et al., 1999). Interactions of gp64 and phospholipids on the cell surface have been suggested to be important in binding or penetration of baculoviruses both in mammalian and insect cells (Tani et al., 2001). At first, BV transduction was suggested to be liver-specific and asialoglycoprotein receptor was supposed possibly to be involved in virus binding (Boyce and Bucher, 1996; Hofmann et al., 1995). However, more recent data have shown that BV transduction is not restricted to liver-derived cells (Condreay et al., 1999; Shoji et al., 1997; van Loo et al., 2001), and further, that asialoglycoprotein receptor is not necessary for transduction, since cells not expressing the receptor can be successfully transduced (Hofmann et al., 1995; van Loo et al., 2001). Moreover, it has been suggested that electrostatic interactions and heparan sulfate moieties might be necessary for BV binding to mammalian cells, and furthermore, that the electrostatic interactions are not necessarily cell type-specific (Duisit et al., 1999).

Studies with primary hepatocytes have shown that only peripheral cells in the culture are transduced by BV, and that transient disruption of paracellular junction complexes by calcium depletion enhances the transduction efficiency (Bilello et al., 2003; Bilello et al., 2001). Disruption of intercellular junctions exposes the basolateral surface of the cell, and hence it has been suggested that the basolateral surface might have an important role in the BV transduction process (Bilello et al., 2003; Bilello et al., 2001). However, based on studies conducted in another laboratory, it has been reported that the transient disruption of intercellular junctions does not effectively enhance the transduction of chondrocytes and HepG2 hepatocarcinoma cells, which implies that other factors might also have important roles in the BV transduction of these cells (Hu, 2005).

1.3.3 Baculoviral transduction *in vivo*

Since BV was discovered to efficiently transduce a wide range of mammalian cells *in vitro*, it has also been shown to transduce mammalian cells of diverse origins *in vivo* (Airenne et al., 2000; Haeseleer et al., 2001; Hofmann et al., 1998; Hüser et al., 2001; Lehtolainen et al., 2002; Pieroni et al., 2001; Sarkis et al., 2000; Shoji et al., 1997; Tani et al., 2003). Although not mammalian, even embryos of zebrafish have been shown to be readily transduced *in vivo* (Wagle and Jesuthasan, 2003). The ability of baculovirus to

efficiently transduce mammalian cells while being unable to replicate in these cells (Carbonell and Miller, 1987; Döller et al., 1983; Gröner et al., 1984; Hartig et al., 1992; Tjia et al., 1983; Volkman and Goldsmith, 1983) renders it an attractive candidate for gene therapy and for other *in vivo* applications. Moreover, baculovirus is rather nontoxic to mammalian cells even at high multiplicity of infection (Gao et al., 2002; Hofmann et al., 1995; Kenoutis et al., 2006; Sandig et al., 1996; Shoji et al., 1997; Tani et al., 2003; Yap et al., 1997), and its ability to also transduce nondividing cells (van Loo et al., 2001) is an important feature for both *in vivo* and *in vitro* approaches. Other advantageous characteristics of baculovirus include the ability to accommodate large foreign DNA inserts (Cheshenko et al., 2001; O'Reilly et al., 1994), the rather easy construction and propagation of recombinant vectors (O'Reilly et al., 1994), and the ability to transduce cells both transiently and stably (Condreay et al., 1999; see also section 1.3.1).

Recently, the biodistribution of baculovirus has been studied *in vivo* in rats by using magnetic resonance imaging (Räty et al., 2006) and combined microSPECT/CT (micro single-photon emission computed tomography/computed tomography) device (Räty et al., 2007). Intrafemoral, intraperitoneal, intramuscular (Räty et al., 2007), and intracerebroventricular administrations of an avidin-displaying BV vector were studied (Räty et al., 2007; Räty et al., 2006). The results indicated that baculovirus might spread via the lymphatic system (Räty et al., 2007). Moreover, following systemic (intrafemoral) administration, the baculovirus vector was observed to accumulate in the nasal area (Räty et al., 2007). Kidneys appeared to be very susceptible to BV transduction, since they were detected to be transduced by all but intracerebroventricular administration (Räty et al., 2007), whereas injection into the brain ventricles of rat resulted in the accumulation of baculovirus vectors in choroid plexus cells (Räty et al., 2006).

Baculovirus has also potential for *ex vivo* therapy, although the possibility has not been thoroughly studied. In the *ex vivo* approach, cells removed from the patient are genetically modified and subsequently transplanted back into the patient, compared to the *in vivo* strategy of directly transferring the genetic material into the patient (for review see Mountain, 2000). Sandig and collagues (1996) have shown that baculovirus can deliver genes into *ex vivo* perfused human liver tissue, and more recently, Ho and coworkers

(2005) demonstrated baculovirus vectors to have potential for *ex vivo* gene therapy to genetically modify human mesenchymal stem cells.

1.3.3.1 Baculovirus vector inactivation by complement system

An expected challenge in the use of baculoviruses for *in vivo* purposes is their inactivation by the serum complement (Hofmann and Strauss, 1998; Sandig et al., 1996). Several approaches have been attempted to solve this problem. The most straightforward application is to try to avoid direct contact of baculovirus vectors with the components of the complement system. This approach has been applied to transduce adventitial cells of rabbit carotid artery by using a silastic collar (Airenne et al., 2000). Transient expression with an efficiency comparable to that of adenoviral vectors was achieved, although inflammation was detected as well (Airenne et al., 2000). Efficient transduction has also been achieved by direct injection of the baculovirus vector into the brains of mice and rats (Sarkis et al., 2000). The injection was conducted very carefully to avoid hemorrhage, which would lead to exposure of the baculovirus vector to complement (Sarkis et al., 2000). Similar results were also provided by another research group. Baculovirus was directly injected into the brains of rats and detected to preferentially transduce choroid plexus epithelial cells and microvascular endothelial cells (Lehtolainen et al., 2002). Similarly, Räty and collagues (2006) also injected baculovirus vectors into rat brain ventricles and observed them to accumulate in choroid plexus cells. Based on these results, *in vivo* transduction of brain appears to be a rather promising approach, mostly due to the absence of the complement system from the brain. Furthermore, for instance mouse retinal pigment epithelial cells have been successfully transduced by direct injection of baculovirus vectors (Haeseleer et al., 2001).

Another option to avoid the inactivation of baculovirus vectors, is to inhibit the activation of the complement system. Treatment of human serum with antibodies against complement component 5 as well as treatment of both human plasma and blood with cobra venom factor, a complement-blocking agent, have been demonstrated to prevent inactivation of baculovirus vectors (Hofmann and Strauss, 1998). In addition, recombinant soluble complement receptor type 1 has been shown to protect baculovirus vectors from

inactivation by the complement system (Hofmann et al., 1999). Moreover, Hüser and collagues (2001) developed yet another approach to surmount the baculovirus vector inactivation problem. A complement-resistant baculovirus vector was generated by incorporating a human decay-accelerating factor, which inhibits the various pathways of the complement system, on the viral envelope by fusing it to gp64 (Hüser et al., 2001). Direct intrahepatic injection of this display vector resulted in enhanced transient gene transfer into the livers of neonatal rats (Hüser et al., 2001).

Yet one more approach to avoid baculovirus inactivation, is display of VSVg on the baculovirus envelope. This idea was inspired by VSVg-pseudotyped retroviruses, which have been shown to be more resistant against the complement system than wt retroviruses (Ory et al., 1996). Moreover, the display of VSVg on the baculovirus envelope has also the advantage of enhanced transduction and expanded host range of the vector (see section 1.3.1). VSVg-displaying baculovirus vectors have been used to transduce mouse hepatocytes by intravenous injection into the tail veins of mice (Barsoum et al., 1997). Furthermore, VSVg-pseudotyped BV has also been demonstrated to enhance gene delivery to mouse skeletal muscle by direct intramuscular injection, which in fact partially bypasses the complement system (Pieroni et al., 2001). Significantly higher reporter gene expression was observed with VSVg-modified virus compared to unmodified virus, and moreover, the expression levels were similar in both immune-competent and complement-deficient animals (Pieroni et al., 2001). The effect of animal sera on the transduction of VSVg-pseudotyped and unmodified viruses has been investigated, and VSVg-displaying viruses have been detected to exhibit resistance to serum treatment (with the exception of rat serum) conducted prior to transduction (Tani et al., 2003). Moreover, cerebral cortexes and testes of mice have also been transduced with direct injection of the pseudotyped virus into the organs (Tani et al., 2003). In addition, a complement pathway inhibitor FUT-175, which is known to protect retrovirus vectors against serum inactivation (Miyao et al., 1997), has been observed to inhibit complement inactivation of baculovirus as well (Tani et al., 2003). However, FUT-175 has a short half-life, and therefore it was suggested that combination of FUT-175 treatment with VSVg-pseudotyping might be efficacious (Tani et al., 2003).

1.3.3.2 Baculovirus-mediated antibody production

The potential virus replication is not the only concern for the safety of baculovirus in *in vivo* applications, for baculovirus can also elicit immune responses. Very little is known about the inadvertent expression of baculoviral genes in mammalian cells, but most likely the transcription of baculoviral genes is limited to immediate early genes, promoters of which are recognized by host RNA polymerase (Kost and Condreay, 2002). The presence of mRNA transcripts from baculovirus early genes *ie0*, *ie1*, and *he65* (Kenoutis et al., 2006; Murges et al., 1997) has been reported in transduced mammalian cells, whereas the differentiation or transcription of the mammalian cells were not observed to be affected (Kenoutis et al., 2006). However, Beck and collagues (2000) have discovered baculovirus to disrupt the *phenobarbital* gene induction process characteristic of highly differentiated hepatocytes and repress expression of the gene *albumin* (Beck et al., 2000). In a study conducted in 1999, the host responses to baculovirus transduction were examined, and baculovirus was observed to be able to stimulate interferon production in human and murine cell lines both *in vitro* and *in vivo* (Gronowski et al., 1999). Similarly, more recent studies have demonstrated that baculovirus is able to induce expression of inflammatory cytokines (Abe et al., 2003; Beck et al., 2000; Hervas-Stubbs et al., 2007), such as tumor necrosis factor α, interleukin-1α, and interleukin-1β in primary hepatocytes, which is most likely due to the presence of Kupffer cells in the culture populations (Beck et al., 2000). The induction of inflammatory cytokine production by baculovirus has been demonstrated to occur via Toll-like receptor 9 signaling pathway (Abe et al., 2003). Furthermore, Airenne and collagues (2000) observed signs of inflammation when transducing carotid arteries of rabbits, and Pieroni and coworkers (2001) detected neutralizing antibodies against baculovirus after intramuscular injection of VSVg-pseudotyped baculovirus (see section 1.3.3.1). In conclusion, these findings show that host responses to baculovirus must be further studied to ensure the safety of baculovirus vectors for *in vivo* applications.

The ability of baculovirus to induce immune responses through expression or by display of desired antigens can, however, be exploited to develop vaccine vectors. Intramuscular inoculation of mice with a BV vector expressing the *glycoprotein B* gene of pseudorabies virus has been observed to induce production of anti-glycoprotein B antibodies (Aoki et

al., 1999). Thus, this vector is a potential vaccine vector candidate for pseudorabies virus. Similarly, human factor IX-expressing BV vector has been shown to cause production of antibodies against human factor IX (Hüser et al., 2001). Furthermore, an intranasal inoculation of mice with a baculovirus vector constructed to express the *hemagglutinin* gene of influenza virus has been demonstrated to induce a strong innate immune response, which protects the mice against influenza virus (Abe et al., 2003). Moreover, baculoviruses expressing either *carcinoembryonic antigen* (*CEA*) or *E2 glycoprotein* gene of hepatitis C virus have been shown to elicit antigen-specific immune responses (Facciabene et al., 2004). In addition, pseudotyping the *E2*-expressing baculovirus with VSVg was detected to cause 10-fold decrease in the minimal dose of virus required to induce a measurable T-cell response (Facciabene et al., 2004). The difference in the immunogenic efficiencies between the two vectors is most likely due to the enhanced transduction potential of the VSVg-pseudotyped baculovirus vector (Facciabene et al., 2004; see section 1.3.1).

BV vectors displaying foreign proteins on their surface can be exploited for antibody production and vaccination purposes as well. This strategy has been used to produce monoclonal antibodies against nuclear receptors LXRβ and FXR (Lindley et al., 2000), and human peroxisome proliferator-activated receptors (Tanaka et al., 2002), as well as to induce antibody responses for example to *Theileria parva* sporozoite surface antigen p67 (Kaba et al., 2003), rodent and human malaria *Plasmodium berghei* and *Plasmodium falciparum* circumsporozoite proteins, respectively (Strauss et al., 2007; Yoshida et al., 2003), hemagglutinin protein of Rinderpest virus, fusion glycoprotein of *Peste des petitis ruminants* virus (Rahman et al., 2003), foot-and-mouth disease virus proteins (Tami et al., 2000; Tami et al., 2004), SARS-associated coronavirus spike protein (Feng et al., 2006), and bovine herpesvirus-1 envelope glycoprotein D (Peralta et al., 2007). The choice of the cytoplasmic domain of the display protein has been shown to affect the immunogenicity of the corresponding virus, since hemagglutinin protein of avian influenza virus displayed with gp64 cytoplasmic domain was detected to be more immunogenic in mouse than hemagglutinin displayed with its native cytoplasmic domain (Yang et al., 2007). Moreover, even multipass membrane proteins PepT1 (peptide transporter) and CCR2 (chemokine receptor) have been used as immunogens by displaying them on BV surface (Saitoh et al., 2007). Monoclonal antibodies against the native conformations of the multipass proteins

were successfully produced, although the strong antigenicity of gp64 compelled the usage of gp64 transgenic mice for the antibody production (Saitoh et al., 2007). Interestingly, it might also be possible to display antigenic epitopes on the surface of polyhedra and thus elicit specific immune responses (McLinden et al., 1992; see section 1.4.2). Together, these studies demonstrate that immunogen-displaying baculoviruses are promising candidates for vaccine purposes.

1.4 Baculovirus display strategies

1.4.1 BV display

1.4.1.1 BV surface display strategies

Display of foreign proteins or peptides on the surface of the budded phenotype of *Ac*MNPV is a very attractive approach, which already has many applications, such as cell- and tissue-specific targeting of BV vectors (see below), enhancement of transduction (section 1.3.1), production of specific antibodies against a displayed immunogen (section 1.3.3.2), and protection of the virus vector from complement system inactivation *in vivo* (section 1.3.3.1). Moreover, the display vectors enable easy construction and screening of eukaryotic display libraries (for review see Mäkelä and Oker-Blom, 2006; Oker-Blom et al., 2003).

Different strategies have been developed for the display of foreign proteins on the surface of BV. One strategy is to fuse the foreign gene to the entire *gp64* gene. In 1995, gp64 fusions with glutathione-S-transferase and human immunodeficiency virus type 1 (HIV-1) envelope protein gp120 were displayed on the envelope of BV (Boublik et al., 1995) (Boublik et al., 1995). These fusion proteins were generated by inserting the foreign gene between the signal sequence of *gp64* and the complete *gp64* gene (Boublik et al., 1995). The gp64-fusion proteins were expressed along with the wt gp64. It was suggested that the incorporation of the fusion proteins into the virion results from oligomerization of the fusion proteins with wt gp64 (Boublik et al., 1995). Moreover, gp120 displayed on the virion surface was observed to retain its functional activity, since it was still able to bind its ligand (Boublik et al., 1995). More recently, many other proteins have been displayed in

the same or similar context (for review see Mäkelä and Oker-Blom, 2006; Oker-Blom et al., 2003). For instance, HIV-1 envelope protein gp41 (Grabherr et al., 1997), GFP, and rubella virus envelope proteins E1 and E2 (Mottershead et al., 1997) have been displayed on the surface of baculovirus and infected insect cells, as well as antigens or antigenic epitopes (see section 1.3.3.2), immunoglobulin-binding domains or single-chain variable fragment antibodies (see section 1.4.1.3), and a complement system inactivating factor (Hüser et al., 2001; section 1.3.3.1).

The above-mentioned *gp64* fusions were constructed to the second copy of the gene, but it is also possible to directly fuse short peptides to the native copy of *gp64* (Ernst et al., 2000; Spenger et al., 2002). However, the insertion site must be carefully chosen to guarantee proper presentation of the epitope and to avoid disruption of the gp64 conformation, which could compromise the function of the protein in fusion and budding (Spenger et al., 2002). So far, only rather short peptides (23 amino acids) have been successfully inserted into the *gp64* gene (Ernst et al., 2000; Spenger et al., 2002), and the potentiality of different insertion sites has been examined (Spenger et al., 2002). A 23 amino acids long integrin-binding RGD motif-containing peptide, derived from VP1 protein of foot-and-mouth disease virus, has been displayed on the surface of BV by insertion of the corresponding DNA sequence in the second or the only copy of *gp64* (Ernst et al., 2006). Interestingly, the display viruses enhanced the specific uptake of BV by mammalian cells only when the virus had both the intact and the recombinant gp64 on its surface (Ernst et al., 2006). Yet another strategy for the surface display is to fuse the foreign gene into the C-terminal 43-amino acid transmembrane anchor domain of gp64. This approach was used to display the envelope protein gp41 of HIV-1 on the surface of BV by fusing the *gp41* gene between *gp64* signal sequence the anchor domain (Grabherr et al., 1997). When only the membrane anchor of gp64 is used instead of the complete gp64, the construct is smaller in size, which eases the incorporation of the fusion protein into the viral envelope (Toivola et al., 2002).

The choice of promoter for *gp64* fusion proteins is an important factor affecting the expression levels and post-translational processing of the fusion proteins (Grabherr et al., 1997). *Polyhedrin* promoter has been shown to yield very high levels of expression, but the fusion proteins are only partially processed and glycosylated, while the use of *gp64*

promoter results in lower levels of expression but with more complete post-translational processing, including complete glycosylation (Grabherr et al., 1997).

Furthermore, envelope proteins of other viruses can be displayed on the surface of BV. In 1997, BV was pseudotyped with VSVg, and several advancements were detected in viral transduction, including improved gene delivery and a wider tropism (see section 1.3.1; Barsoum et al., 1997). The foreign protein to be displayed can also be fused directly to the membrane anchor of VSVg as was demonstrated by Chapple and Jones (2002), who constructed a display vector by fusing *GFP* between the signal sequence of *gp64* and the membrane anchor of *VSVg*. A difference between VSVg-anchored and gp64-based fusion proteins is in their distribution, since gp64 fusion proteins are restricted to one pole of the virus like wt gp64 (see section 1.2.3), whereas VSVg-anchored fusion proteins are spread uniformly on the virus envelope (Chapple and Jones, 2002). Analogously to the VSVg membrane anchor approach, also the membrane anchor of neuraminidase protein from influenza virus has been used for EGFP (enhanced GFP) display (Borg et al., 2004). Moreover, histidine-tagged hemagglutinin protein of avian influenza virus has been displayed on BV surface either with the cytoplasmic domain of hemagglutinin or gp64 (Yang et al., 2007). The display protein with the gp64 cytoplasmic domain was demonstrated to be incorporated into the virions more effectively, and the corresponding virus had elevated transduction and immunogenicity levels when compared to the virus displaying hemagglutinin with its native cytoplasmic domain (Yang et al., 2007).

Membrane proteins, *per se*, have also been displayed on the BV surface. BVs functionally displaying plasma membrane proteins, including β2-adrenergic receptor (Loisel et al., 1997), trimeric G protein, or a leukotriene B4 receptor BLT1 (Masuda et al., 2003), have been recovered after infection of *Sf*9 cells with the corresponding recombinant baculoviruses. Furthermore, SREBP-2 and SCAP (Urano et al., 2003), membrane proteins of endoplasmic reticulum, as well as PepT1 and CCR2 (Saitoh et al., 2007), multipass membrane proteins of the cell surface, have been displayed in the same context. The SCAP-, PepT1- and CCr2-displaying viruses were also used to induce immune responses in mice for production of specific antibodies (Saitoh et al., 2007; Urano et al., 2003; for the latter two viruses, see also section 1.3.3.2).

1.4.1.2 Capsid display strategy

Recently, Kukkonen and coworkers (2003) demonstrated a different baculovirus display strategy by fusing EGFP to the baculovirus major capsid protein vp39. It was shown that foreign proteins can be displayed on the surface of the capsid either as N- or C-terminal fusions to vp39 (Kukkonen et al., 2003). Size restriction has not yet been determined for the capsid display system, but proteins up to 41 kDa have been successfully displayed, and it is likely that larger proteins can also be incorporated, since the baculoviral nucleocapsid can freely extend (Kukkonen et al., 2003). The capsid display system differs from the gp64 display strategy in several aspects. First of all, vp39 has not been demonstrated to contain any structural motifs for association with molecules in the nucleus or for capsid assembly (Russell et al., 1991), which reduces the possibility of the fusion to disrupt the infectivity of the virus. In addition, the vp39 is uniformly distributed on the surface of the capsid (Russell et al., 1991), whereas gp64 is restricted to one pole of the virus envelope (see section 1.2.3). Moreover, only the N-terminus of gp64 can be exploited for fusions, whereas both termini of vp39 can be used (Kukkonen et al., 2003). Finally, the capsid-displayed proteins avoid contact with the acidic environment of endosomes and are transported along with the capsid into the cytoplasm and the nucleus, whereas gp64 fusion proteins remain at the endosomes (Oker-Blom et al., 2003). The differencies in the BV envelope and nucleocapsid display strategies render them applicable to different purposes. Kukkonen and collagues (2003) demonstrated that the capsid display approach can be used to study the transduction route of baculovirus in mammalian cells as well as to observe the biodistribution of baculoviruses in rat brain *in vivo* (Kukkonen et al., 2003). Furthermore, it has also been suggested that in the field of gene delivery, the capsid display strategy could be used to target the virus intracellularly (Kukkonen et al., 2003). This could be worthwhile, since capsid transport occurs efficiently only in some cell types (Volkman and Goldsmith, 1983). Moreover, the intracellular targeting of the virus could be combined with envelope display strategy, which can be used to target BV to specific tissues or cells (see below).

1.4.1.3 Targeting of BV transduction

The display of specific ligand-binding moieties on the surface of BV have potential to enable cell- or tissue-specific delivery of the target gene. An RGD motif recognized by αV integrin has been displayed on the BV surface by fusing RGD motifs from human parechovirus 1 VP1 and coxsackie virus A9 proteins to the N-terminus of gp64 (Matilainen et al., 2006). Enhanced binding of the display virus to human lung carcinoma cells known to contain αV integrins, as well as increased transduction efficiency, was detected (Matilainen et al., 2006). Moreover, specific ligand-directed gene delivery into baby hamster kidney cells expressing measles virus envelope glycoproteins was detected with gp64-null baculoviruses pseudotyped with measles virus receptors (Kitagawa et al., 2005). The transduction was inhibited by pretreatment of the viruses with specific antibodies against the displayed measles virus receptors (Kitagawa et al., 2005). Furthermore, tumor-homing peptides have also been displayed on the surface of BV by fusing the peptides to the transmembrane anchor of VSVg (Mäkelä et al., 2006). Enhanced binding to and transduction of human breast carcinoma and hepatocarcinoma cells by the modified viruses were detected (Mäkelä et al., 2006). More recently, a BV vector displaying a chimeric gp64 protein, containing a CD21-binding motif of Epstein-Barr virus gp350/220 protein, was generated (Ge et al., 2007). CD21 is a surface protein of B lymphocytes, and increased transduction of several B lymphocyte cell lines by the display virus was indeed observed as well as inhibition of the transduction after CD21 antibody treatment of the cells (Ge et al., 2007).

The specificity of BV can also be enhanced by displaying single-chain variable fragment antibodies (scFvs) or synthetic immunoglobulin G (IgG) -binding Z domain on the virus surface. Mottershead and collagues (2000) displayed a murine scFv specific for the hapten 2-phenyloxazolone and a human scFv specific for CEA on the surface of BV. The scFvs were fused to the N-terminus of gp64, and the display viruses were shown to specifically bind to their relevant antigens in enzyme-linked immunosorbent assays. The anti-CEA scFv-displaying BV was also demonstrated to directly bind to mammalian adenocarcinoma cells expressing CEA on their surface (Ojala et al., 2001). Z domains have been displayed on the surface of BV as well by fusing them to the N-terminus of gp64 (Mottershead et al.,

2000; Ojala et al., 2001). The Z domain is derived from *Staphylococcus aureus* protein A and binds especially strongly the Fc domains of IgG antibodies (Löwenadler et al., 1987; Nilsson et al., 1987). Due to the IgG-binding ability of the Z domain, it should be possible to target Z-displaying viruses to any cells for which an IgG antibody against a cell-specific antigen is available. Specific binding of the Z/ZZ-displaying viruses was demonstrated with enzyme-linked immunosorbent assays (Mottershead et al., 2000), and accordingly, ZZ-displaying virus was shown to be able to bind specifically to baby hamster kidney cells via an anti-α5β1 integrin IgG antibody and to readily transduce the cells, although transduction efficiency was not enhanced (Ojala et al., 2001). More recently, Ojala and collagues (2004) improved the ZZ display strategy by fusing the ZZ domains to the transmembrane anchor of VSVg, which resulted in enhanced ZZ domain display and vector binding to IgG antibodies, although transduction efficiency did not increase. Both targeted and enhanced transduction has been obtained by avidin-displaying BV vector, which was constructed by fusing avidin to the N-terminus of a second copy of gp64 (Räty et al., 2004). Avidin as such was already demonstrated to enhance gene delivery (Räty et al., 2004). In addition, transduction of biotinylated mammalian cells was observed to be enhanced due to the very strong receptor-ligand interaction between avidin and biotin (Räty et al., 2004). Targeting of the virus with biotinylated ligands was also shown to be possible (Räty et al., 2004). In conclusion, there are several encouraging results regarding BV targeting, but no successful *in vivo* targeting of BV transduction has yet been reported.

1.4.2 Polyhedra and ODV display

In contrast to the BV display technology, the display of foreign proteins or epitopes on the surface of polyhedra or ODV has not been considerably studied. In 1992, an influenza hemagglutinin epitope was inserted into polyhedrin protein and displayed on the surface of polyhedra (McLinden et al., 1992). Five recombinants with varying epitope locations within the polyhedrin gene were constructed, and three of them were observed to form occlusion bodies and to display the foreign epitope on the polyhedron surface (McLinden et al., 1992). Moreover, a monoclonal antibody against the used epitope was shown to specifically recognize its antigenic site in the denaturated form of the recombinant polyhedrin and when the recombinant polyhedrin was displayed on the surface of

polyhedra (McLinden et al., 1992). In addition, the recombinant polyhedrin proteins were shown to elicit immune response against the incorporated influenza epitope in rabbits, which suggests that it might be possible to use polyhedra as carriers of antigenic epitopes for the production of specific antibodies (McLinden et al., 1992). More recently, a system for the incorporation of entire foreign proteins into baculovirus polyhedra has been developed (Je et al., 2003). First it was detected that fusion of GFP to polyhedrin abolishes the production of occlusion bodies, and subsequently, that coexpression of the polyhedrin-GFP fusion with native polyhedrin results in recombinant viruses capable of producing polyhedra displaying the fusion protein on their surface (Je et al., 2003). Accordingly, the interaction between the native polyhedrin and the polyhedrin fusion protein have been suggested to be crucial for the incorporation of a foreign protein into occlusion bodies (Je et al., 2003). The GFP-displaying occlusion bodies were shown to incorporate virions normally and retain their infectivity (Je et al., 2003). Later on, *Bacillus thuringiensis* insect toxin was incorporated into the fusion protein between polyhedrin and GFP for insecticide purposes (Chang et al., 2003; Seo et al., 2005).

Actual display of foreign proteins or epitopes on the surface of ODV has not been reported to date, but foreign genes have been fused to certain ODV proteins or parts thereof. In 1996, a fusion protein, in which the C-terminal portion of the ODV envelope protein E56 was replaced with β-gal, was constructed (Braunagel et al., 1996). The hydrophobic domain of E56 is in the C-terminus, but replacement of this domain with β-gal did not disrupt the targeting of the protein to the nucleus (Braunagel et al., 1996). However, the fusion protein did no longer associate with intranuclear microvesicles and ODV envelope, but rather associated with viral nucleocapsids (Braunagel et al., 1996). Another fusion protein was constructed by replacing the C-terminal half of FP25K with β-gal to produce FP-β-gal (Braunagel et al., 1999). The FP-β-gal as well as an N-terminally truncated FP25K were both observed to affect the accumulation of several viral structural proteins, and in addition, the FP25K was showed to be a structural protein of the nucleocapsid (Braunagel et al., 1999). Moreover, GFP has been fused to the C-termini of the N-terminal amino acid sequences of ODV envelope proteins E66 and E25, and similarly, β-gal has been fused to the C-terminus of the E66 N-terminal sequence (Hong et al., 1997). Both E66 and E25 are suggested to be oriented with their N-termini on the virion surface, and

thus GFP and β-gal probably were not exposed on the surface of ODV (Slack and Arif, 2007). The study concentrated on the intracellular trafficking of the fusion proteins, and the N-terminal sequences of E66 and E25 (23 and 24 amino acids, respectively) were shown to be sufficient to direct native and fusion proteins to viral-induced intranuclear microvesicles and ODV envelope (Hong et al., 1997). The fusion protein with the N-terminus of E66 fused to GFP on its envelope was confirmed to be incorporated into polyhedra in a normal manner (Hong et al., 1997). More recently, another group studied the fusion of GFP and the N-terminus of E66 as well, but 33 N-terminal amino acids alongside with 23 amino acids were used, and similar results were attained with both fusion proteins (Braunagel et al., 2004). The 33 N-terminal amino acids were shown to function as a sorting motif directing the protein to intranuclear membranes and ODV envelope (Braunagel et al., 2004). It was also suggested that the N-terminus of E66 interacts with FP25K and/or E26 during trafficking from endoplasmic reticulum to inner nuclear membrane (Braunagel et al., 2004). In 2001, the ODV-specific envelope protein p74 was studied by fusing GFP to the C-terminus of either the entire p74 or after various N-terminal truncations (Slack et al., 2001; see section 1.2.4.1). In addition, a similar p74-GFP fusion protein, but this time with C-terminal deletion of p74, was generated and detected to be soluble (Slack et al., 2001). Again, the C-terminus of p74, and thus also the GFP fused to it, does not reside on the virion surface. With the aid of the GFP, the trafficking of p74 was studied in infected *Sf*9 cells, and the hydrophobic C-terminus of p74 was detected to have an important role in protein localization and maybe also in the transmembrane anchoring and insertion of the protein into the membrane (Slack et al., 2001; see section 1.2.4.1).

2 AIM OF THE STUDY

The aims of the present study were:

1. To construct and characterize an occlusion-positive baculovirus equipped with a mammalian expression cassette encoding reporter genes *EGFP* and firefly *luciferase*.
2. To study the binding and transduction potentials of the occlusion-derived phenotype of baculovirus (ODV) in human cancer cells.
3. To construct and characterize an ODV displaying a ZZp74 fusion protein.

3 MATERIALS AND METHODS

Occlusion-positive recombinant baculoviruses *Ac*WT and *Ac*ZZp74 were generated. *Ac*ZZp74 occlusion-derived virus (ODV) was designed to display a fusion protein on its surface. Both viruses were equipped with reporter genes encoding enhanced green fluorescent protein (EGFP) and North American firefly (*Photinus pyralis*) luciferase to enable fluorescent and luminescent detection of transgene delivery in mammalian cells.

3.1 Construction of pWT

Cloning of pWT (Fig. 3.1 A) was conducted by using a pFastBac™Dual vector (Invitrogen Life technologies, Carlsbad, CA), pF3-VP39 (Mäkelä et al., manuscript; kindly provided by Anna Mäkelä from the University of Jyväskylä), as a vector backbone. pF3-VP39 contained reporter genes *luciferase* and *EGFP* under simian virus 40 (SV40) and human cytomegalovirus *immediate early* (CMV) promoters, respectively, as well as an *F3-VP39* fusion gene under the *polyhedrin* promoter. The reporter genes and their corresponding

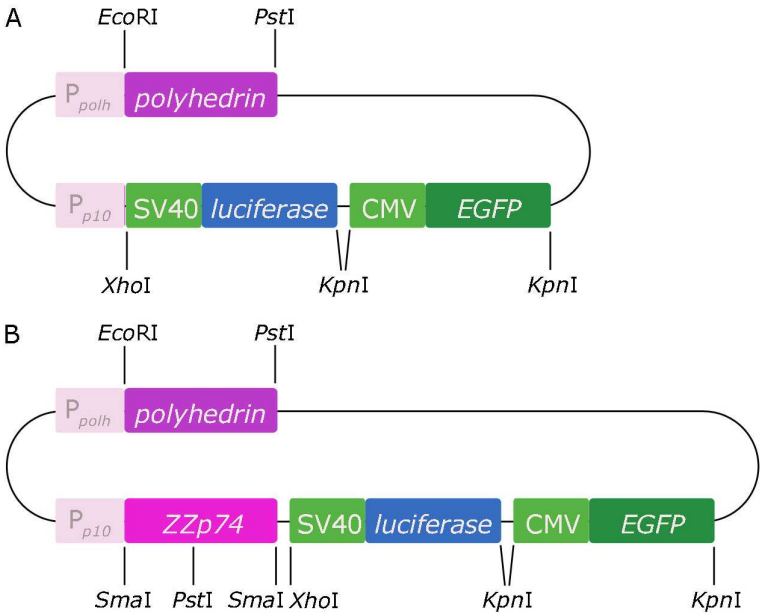

Figure 3.1 The pFastBac™Dual vector constructs. A) pWT and **B)** pZZp74. CMV, cytomegalovirus *immediate early* promoter; *EGFP*, *enhanced green fluorescent protein*; P_{p10}, *p10* promoter; P_{polh}, *polyhedrin* promoter; SV40, simian virus 40 promoter; *ZZp74*, a fusion gene consisting of IgG-binding ZZ domains of protein A and *Ac*MNPV *p74*.

promoters were previously cloned into the vector under the strong baculoviral *p10* promoter: SV40-*luciferase* downstream the *p10* promoter (p10SV40-luc; Mäkelä et al., 2006) and CMV-*EGFP* downstream the SV40-*luciferase* using *Xho*I / *Kpn*I and *Kpn*I / *Kpn*I restriction enzymes, respectively.

3.1.1 Polymerase chain reaction (PCR)

Cloning of the pWT was begun by replacing the *F3-VP39* gene under the *polyhedrin* promoter with a gene encoding *polyhedrin* (Fig. 3.1 A). The *polyhedrin* gene (738 bp; see Appendix II) was amplified by polymerase chain reaction (PCR) using wt baculovirus-containing medium as a template. The forward (*Polh*Forw) and reverse (*Polh*Rev) primers (the primer sequences are shown in Appendix I) contained *Eco*RI and *Pst*I restriction enzyme sites, respectively. In addition to the template (approximately 500 ng) and primers (100 pmol/primer), the PCR reaction mixture contained the following reagents: 10 x DyNAzyme reaction buffer (1 x in the final reaction solution; Finnzymes, Espoo, Finland), deoxynucleotide triphosphate mixture (dNTP Mix; 200 µM / dNTP; Finnzymes), and 2 U of DyNAzyme II DNA polymerase (Finnzymes), all dissolved in sterile double-deionized water (ddH_2O). The PCR was conducted with a thermal cycler (T3 Thermocycler, Biometra, Göttingen, Germany) using the PCR program shown in Table 3.1 below.

Table 3.1 PCR program for the amplification of *polyhedrin*.

Step	Temperature (°C)	Time (min)
1. Initial denaturation	94	4
2. Denaturation*	94	1
3. Annealing*	52	1
4. Extension*	72	1
5. Final extension	72	10

*Steps 2-4 were repeated 30 times before proceeding to step 5.

3.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis was employed to concentrate the PCR product to a discrete band on the gel, from where it could be subsequently isolated. The gel was prepared by dissolving an appropriate amount of agarose (Multi ABgarose, ABgene House, Epsom, Surray, UK) in 1 x Tris acetate – EDTA buffer (40 mM Trizma base, 1 mM EDTA

[ethylenediaminetetraacetic acid], and 0.11% acetic acid) to achieve a final agarose concentration of 0.8%, and by finally adding 0.4 μg/ml ethidium bromide (Sigma, Saint Louis, MO). Prior to the run, 6 x loading buffer (Mass Loading Dye Solution; MBI Fermentas, Vilnius, Lithuania) was added to the samples to obtain a 1 x final concentration. 8-10 μl of a molecular-weight marker (GeneRuler™ 1 kb: 0.5 μg/μl; MBI Fermentas), a horizontal gel, and an electrophoresis apparatus (MINI-SUB® CELL GT: Bio-Rad Laboratories, Hercules, CA) were used for each run. After a 45-60 min run at 80 V, the DNA bands were visualized using ultraviolet transillumination. Desired DNA bands were excised from the gel and isolated using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) according to manufacturer's instructions. All the following agarose gel electrophoresis runs and DNA isolations from the gel were similarly conducted unless otherwise stated.

3.1.3 Restriction enzyme digestion and ligation reactions

The *polyhedrin* PCR product and the pF3-VP39 were digested with *Eco*RI and *Pst*I restriction enzymes. The reactions were conducted by mixing the DNA to be digested, the restriction enzymes (MBI Fermentas), and an optimal buffer (MBI Fermentas) for both of the enzymes in sterile ddH_2O according to manufacturer's instructions. The reaction mixtures were incubated at 37 °C for a minimum of 2 h, and subsequently run on an agarose gel, followed by the isolation of the vector and insert DNAs. T4 ligase and an appropriate buffer (both from MBI Fermentas) were used for the ligation reaction according to manufacturer's instructions. Following agarose gel electrophoresis, the relative concentrations of the vector and insert DNAs were estimated from the band intensity. Approximately three-fold molar excess of insert DNA relative to vector DNA was used. The ligation was performed overnight at 6 °C. All the following restriction enzyme digestions and ligations were conducted similarly unless otherwise stated.

3.1.4 Transformation

The ligation product was transformed into electrocompetent *Escherichia coli* (*E. coli*) cells (strain JM109; Stratagene, La Jolla, CA) by electroporation. The cells were first thawed on

ice and then mixed with the ligation product. Electroporation was performed with BTX electro cell manipulator® 600 electroporation system (BTX Inc., San Diego, CA) using an appropriate electroporation cuvette (BTX Electroporation cuvette Plus™: SC Bio Express, Kaysville, UT). After the electrical shock (2.5 kV, 129 Ω), the cells were immediately suspended in prewarmed (37 °C) SOC medium (10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ + $MgSO_4$, and 20 mM glucose [ICN Biomedicals Inc., Aurora, OH], with 20 mg/ml Bacto Tryptone and 5 mg/ml Yeast Extract [both from Becton, Dickinson and Company, Difco Laboratories Inc., Sparks, MD]) and grown in a shaking incubator for 1 h (37 °C, 225 rpm). The cells were plated on Luria(-Bertani) Broth (LB) agar plates (10 mg/ml Bacto Agar [Becton, Dickinson and Company, Difco Laboratories Inc.] diluted in LB medium; LB medium [pH 7]: 10 mg/ml Bacto Tryptone, 5 mg/ml Yeast Extract, and 0.17 M NaCl) containing 50 μg/ml ampicillin (D-[–]-α-aminobenzylpenicillin: Sigma-Aldrich, St. Louis, MO). After a 16-18 h incubation at 37 °C, a maximum of 10 colonies were selected and grown in a suspension culture containing LB medium supplemented with ampicillin (100 μg/ml). The suspension cultures were grown for 16-18 h in a shaking incubator (37 °C, 225 rpm) followed by plasmid isolation using NucleoSpin® Plasmid QuickPure Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. All the following transformations, platings, suspension cultures, and plasmid isolations were performed similarly unless otherwise stated.

3.1.5 Sequence verification

*Eco*RI / *Pst*I restriction enzyme digestions were used to confirm the presence of the *polyhedrin* insert in the plasmids. Digestion products were run on an agarose gel, and the plasmids producing proper-sized digestion products were sequenced to verify a correct reading frame of the insert. The sequencing was performed using fluorescent IRD700 (pentamethine carbocyanine dye) and IRD800 (heptamethine cyanine dye) labeled primers (PolhIRD700 and PolhIRD800; Appendix I), SequiTherm EXCEL™ II DNA Sequencing Kit-LC (Epicentre Techologies, Madison, WI), and an automatic sequencing device with a dual laser system (LI-COR® 4200-2, LI-COR Biosciences, Lincoln, NE) for simultaneous detection of both the IRD700 and IRD800 dyes. The sequencing was performed by laboratory technician Eila Korhonen (Division of Biotechnology, University of Jyväskylä).

3.2 Construction of pZZp74

3.2.1 Cloning of *ZZp74* fusion gene

The IgG-binding *ZZ* domains (360 bp) of protein A from *Staphylococcus aureus* were amplified by PCR similarly as described for *polyhedrin* in section 3.1.1, with the following exceptions: pZZVSVgTM (Ojala et al., 2004) was used as a template, the primers were *ZZ*Forw and *ZZ*Rev (Appendix I), and extension was performed for 30 s at 72 °C. *Xba*I and *Pst*I restriction enzyme sites were included in the forward and reverse primers, respectively (Appendix I). Subsequently, the PCR products were run on an agarose gel and isolated from the gel. The *ZZ* PCR product and an empty pFastBac™Dual vector were digested with *Xba*I and *Pst*I restriction enzymes. Subsequent to agarose gel electrophoresis and DNA isolation from the gel, the *ZZ* insert was ligated into the vector under the *polyhedrin* promoter, resulting in an intermediate plasmid, pZZ. The ligation product was transformed into *E. coli* cells, followed by plating of cells, suspension cultures, and plasmid isolation. The presence and orientation of the *ZZ* insert in pZZ was confirmed by *Eco*RI / *Pst*I restriction enzyme digestion.

PCR was again employed to amplify baculoviral *p74* gene (1938 bp). The PCR was conducted as described in section 3.1.1 with the following exceptions: primers were *p74*Forw and *p74*Rev (Appendix I), template was the WT bacmid (section 3.3.1), annealing temperature was 50 °C, and the extension time was 2 min. The forward and reverse primers contained *Pst*I and *Hind*III restriction enzyme sites, respectively (Appendix I). The *p74* PCR product was purified from the PCR reaction mixture using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's instructions. Both the *p74* PCR product and the p*ZZ* were digested with *Pst*I and *Hind*III. The digestion products were run on an agarose gel followed by gel extraction and ligation, producing pZZp74-intermediate. Subsequently, the ligation products were transformed into *E. coli* cells, the cells were plated, grown in suspension cultures, and the plasmids isolated. The presence and orientation of the *ZZp74* insert in the pZZp74-intermediate was confirmed by *Xba*I / *Hind*III restriction enzyme digestion, and the correct reading frame of the insert was verified by sequencing (see section 3.1.5).

3.2.2 Cloning of the final pZZp74

The *ZZp74* fusion gene (2304 bp; Appendix II), including the SV40 polyadenylation signal from the pFastBac™Dual, was amplified by PCR using *ZZp74*Forw and SV40polyARev primers containing *Sma*I restriction enzyme sites (Appendix I). The PCR was performed similarly as described in section 3.1.1 with the exceptions of using p*ZZp74*-intermediate as a template and a 2 min extension time. The PCR product (2574 bp without the flanking restriction enzyme sites) purified from the PCR reaction mixture and the pWT (section 3.1) were subsequently digested with *Sma*I for 2 h at 30 °C.

A thermal cycler (T3 Thermocycler) was employed to add an A overhang to the *Sma*I-digested *ZZp74* and a T overhang to the *Sma*I-digested pWT. Overhangs were synthesized for 30 min at 70 °C in the presence of 10 mM dATP for the insert and 10 mM dTTP for the vector, as well as 2 U of DyNAzyme II DNA polymerase (Finnzymes) and DyNAzyme reaction buffer (Finnzymes). Subsequently, the products were run on an agarose gel and isolated from the gel. The *ZZp74* with an A overhang and the pWT with a T overhang were ligated together, transformed into *E. coli* cells, and plated. Positive colonies were screened by colony PCR using the same primers and reaction as described above for *ZZp74*. Positive clones were grown in suspension cultures followed by plasmid isolation. For further screening, two different PCRs were conducted for the isolated plasmids: one like described above for the colonies and one using the *ZZ*Forw and *p74*Rev primers (Appendix I). The latter PCR program was performed as described for *polyhedrin* in section 3.1.1 with the exceptions of using 50.5 °C annealing temperature and a 2 min extension time.

Later, after the recombinant baculovirus *Ac*ZZp74 had been cloned, produced, and purified (see section 3.3), the presence of the gene encoding *polyhedrin* was confirmed by PCR. pWT (section 3.1), pZZp74 (section 3.2), ZZp74 bacmid (section 3.3.1), and *Ac*ZZp74 (section 3.3.3) were used as templates. The primers and the program were the same as described for the *polyhedrin* PCR previously (Appendix I and section 3.1.1). The PCR-products were run on an agarose gel to monitor whether the *polyhedrin* gene is present in the template DNAs.

3.3 Production and purification of the recombinant viruses

The recombinant baculoviruses were generated with Bac-to-Bac® Baculovirus Expression System (Invitrogen Life technologies), which is based on a method developed by Luckow and coworkers (1993), and offers an efficient method to generate recombinant baculoviruses (Ciccarone et al., 1997). In pFastBac™Dual vector, the inserted genes are flanked by the left and right arms of the Tn7 transposon (Tn7L and Tn7R, respectively). Subsequently, the vector is transformed into electrocompetent DH10Bac™ (Invitrogen Life technologies) strain of *E.coli*, which contains a baculovirus shuttle vector (bacmid) and a helper plasmid. With the aid of site-specific transposition of the Tn7 transposon, the part of pFastBac™Dual containing the inserted gene(s) is incorporated into the bacmid, which contains target sites for the transposon. The helper plasmid provides enzymes needed in the transposition reaction. The selection for the recombinant clones on plate cultures and in suspension cultures is conducted by using antibiotics gentamicin, kanamycin, and tetracycline, due to the presence of the respective resistance genes in the pFastBac™Dual (within Tn7L and Tn7R sites), the bacmid, and the helper plasmid, respectively. The bacmid also contains DNA encoding for LacZα peptide, into which, without any disruption of the reading frame, the Tn7 target site has been inserted. Thus, in the absence of transposition, the bacmid can complement a *lac*Z deletion present on the *E. coli* chromosome in the presence of Bluo-gal (5-bromo-3-indolyl-β-D-galactopyranoside) and an inducer of its promoter, IPTG (isopropyl-β-D-thiogalactopyranoside), to produce blue colonies. However, if transposition occurs, the β-galactosidase peptide cannot be expressed and the ensuing colonies are white. Finally, the bacmid DNA is isolated from *E. coli* cells and used to transfect insect cells for the generation of recombinant baculoviruses.

3.3.1 Generation of recombinant bacmids

After sequence verification, both pWT and pZZp74 were transformed into electrocompetent DH10Bac™ cells. Transformation was conducted as described for the JM109 strain of *E. coli* (section 3.1.4) with the exception of growing the cells in SOC medium for 4 h after the electrical shock. The cells were diluted in SOC medium (1:1 000, 1:2 500, and 1:5 000), and 100 µl of each dilution was plated on LB agar plates containing 7 µg/ml

gentamicin (gentamicin sulfate: Sigma-Aldrich), 50 μg/ml kanamycin (kanamycin monosulfate: MP Biomedicals, LLC, Eschwege, Germany), 10 μg/ml tetracycline (tetracycline hydrochloride: Sigma-Aldrich), and IPTG–Bluo-gal mixture (40 μg/ml IPTG: MBI Fermentas; and 100 μg/ml Bluo-gal [in 100% dimethylformamide]: Sigma-Aldrich) to enable blue-white selection. After 48 h incubation at 37 °C, positive white clones were selected and grown in a shaking incubator for 18 h (37 °C, 225 rpm) in suspension cultures containing 7 μg/ml gentamicin, 50 μg/ml kanamycin, and 10 μg/ml tetracycline in LB medium. Finally, the bacmids were isolated according to Bac-to-Bac® Baculovirus Expression System manual.

3.3.2 Virus production

Virus production was conducted using *Spodoptera frugiperda* 9 insect cells (*Sf*9, Gibco-BRL, Grand Island, NY) grown in a suspension culture in a shaking incubator at 28 °C using insect cell culture medium (HyQ SFX-Insect culture medium, HyClone, Logan, UT). The purified bacmid DNAs were transfected to *Sf*9 cells according to the instructions of the Bac-to-Bac® Baculovirus Expression System manual. The protocol uses Cellfectin® Reagent (Invitrogen Life technologies), which is designed for the optimal transfection of DNA into insect cells. An exception from the protocol was to leave the DNA-lipid transfection mixture on the cells during the whole procedure. The state of the infection was observed with a phase contrast microscope, and as the cells demonstrated signs of later stages of infection, a primary budded virus (BV) stock was collected, 4 days post-transfection at the latest. To remove cells and large debris, the virus-containing medium was clarified by centrifugation (10 min, 1 000 x g; Hermle Z 513 K, Hermle Labortechnik GmbH, Wehingen, Germany), and subsequently supplemented with fetal calf serum (FCS; PAA Laboratories GmbH, Austria) to a final concentration of 2.5% to facilitate storage at 4 °C. For characterization of the virus and infected cells by Western blotting and luminescence measurements, samples of 1 x 10^6 *Sf*9 cells were collected. After removal of the virus-containing medium, the cells were detached from the culture dish by scraping, diluted in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 ; pH 7.4), and pelleted for 3 min at 500 x g (Heraeus Pico Biofuge, Heraeus instruments GmbH, Osterode am Harz, Germany).

3.3.3 Virus stock amplification

Western blotting and/or luminescence measurements were used to choose the correct *Ac*WT and *Ac*ZZp74 clones for further amplification to obtain high titer virus stocks. *Ac*ZZp74 bacmid isolation, transfection of *Sf*9 cells, and the subsequent amplification of the *Ac*ZZp74 virus stock were performed by Anna Mäkelä from the University of Jyväskylä. Two successive amplifications were conducted to obtain secondary and tertiary virus stocks. 15 x 10^6 *Sf*9 cells in 30 ml of culture medium were allowed to attach on a culture flask (175 cm^2) for 30 min at 28 °C. The attached cells were infected with 0.5-1.0 ml (0.5-1.0 multiplicity of infection, MOI) of the primary virus stock and incubated for 3-4 days at 28 °C. The secondary virus stock was isolated as described above for the primary virus stock, and the infected cells were similarly characterized with luminescence measurements and/or Western blotting. The final round of amplification was conducted by infecting 200 ml of *Sf*9 cells (2 x 10^6 cells/ml) with 2 ml (approximately 0.5 MOI) of the secondary virus stock, followed by isolation of the tertiary stock three days post-infection (p.i.) by centrifugation (Hermle Z 513 K: first 10 min, 4 °C, 1 000 x g and subsequently 20 min, 4 °C, 4 500 x g). The secondary and tertiary virus stocks were supplemented with 2.5% FCS and stored at 4 °C. Prior to collecting the tertiary virus stock, samples of 2 x 10^6 *Sf9* cells were obtained from the suspension culture and pelleted for 3 min at 500 x g (Heraeus Pico Biofuge) for luminescence measurements and Western blotting (sections 3.4.1 and 3.4.2, respectively). The titer of *Ac*ZZp74 was determined to be 1.17 x 10^8 plaque-forming units (pfu)/ml by Anna Mäkelä (from the University of Jyväskylä).

3.3.4 Production and purification of ODV

All the steps during ODV production and purification were performed under sterile conditions, and all the centrifugations were conducted at 4 °C and at 2 000 x g (Hermle Z 513 K). For *Ac*WT ODV production, 8 x 10^8 *Sf*9 cells (2 x 10^6 cells/ml) were infected with tertiary *Ac*WT BV virus stock approximately at a MOI of 1-2 at 28 °C. *Ac*ZZp74 ODV was produced by coinfection with both *Ac*ZZp74 and *Ac*WT BVs, both at 1-2 MOI, due to low polyhedra production by *Ac*ZZp74. At 5 days p.i., the state of the infection was observed with a phase contrast microscope, a sample of 2 x 10^6 cells was collected and

pelleted for 3 min at 500 x g (Heraeus Pico Biofuge) for luminescence measurement and Western blotting (sections 3.4.1 and 3.4.2, respectively), and the remaining cells were pelleted by a 10 min centrifugation. The BV-containing supernatant was either discarded or stored at 4 °C for later use. The cell pellet was resuspended in 30 ml of sterile ddH_2O and vortexed. Subsequently, the cells were either stored at 4 °C for later purification or directly lysed to release the polyhedra from the nuclei of the infected cells. Lysis was performed by adding 20% sodium dodecyl sulfate (SDS: BDH Laboratory Supplies, Poole, England) to a final concentration of 0.5%, followed by 30 min shaking at room temperature (RT) with an orbital shaker (250 rpm). After vigorous vortexing, the polyhedra were collected by a 30 min centrifugation, after which the pellet was resuspended in 40% sucrose by vortexing. Following 30 min centrifugation, the polyhedra were washed with 35 ml of sterile ddH_2O. The wash suspension was vortexed, and the polyhedra pelleted with a 20 min centrifugation. The polyhedra-containing pellet was either directly resuspended in 3.2 ml of sterile ddH_2O for further purification or resuspended in a small volume of 60% (v/v) glycerol (J.T.Baker, Deventer, Holland) and stored at 4 °C. Samples were collected from the purified polyhedra for Western blotting (section 3.4.2), transmission electron microscopy (section 3.4.5), and differential interference contrast (DIC) microscopy (section 3.4.3).

To isolate ODVs from the occlusion bodies, the polyhedra were lysed by adding 1/5 volume of alkaline solution (500 mM Na_2CO_3 and 250 mM NaCl; pH 10.9), vortexed vigorously, and incubated for 1 h on ice in an orbital shaker (250 rpm). Next, the suspension was neutralized with 1/10 volume of 1 M Tris-HCl (pH 7.6) (1 M Trizma base and HCl to adjust the pH) and incubated for 1 h at RT in an orbital shaker (250 rpm). Following 20 min centrifugation, the supernatant containing the ODVs was loaded to a Millipore Amicon Ultra-15 centrifugal filter device (nominal molecular weight limit 10 000 Da; Millipore corporation, Bedstone, MA) and centrifuged for 15 min. Centrifugation was continued as long as required after addition of 10 ml of Tris-EDTA buffer (pH 8.0; 10 mM Trizma base and 1 mM EDTA) or PBS to achieve a final ODV stock volume of about 0.25-1 ml. The concentrated ODV stock was stored at 4 °C. The total protein concentration of the ODV stock was measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) at 280 nm using lysozyme, IgG, and bovine serum

albumin (BSA) as references. IgG and BSA, when used as references, produced approximately 1.7- and 3.2-fold higher concentrations than lysozyme, respectively. The concentrations acquired with lysozyme as a reference were chosen to be applied. In 1976, Volkman and coworkers determined the particle per µg ratios for *Ac*MNPV ODV. Electron microscopic studies revealed the *Ac*WT ODVs to predominantly contain only a single nucleocapsid per virion (section 4.2.3), and thus the ratio determined for single-nucleocapsid ODVs (1.2 x 10^9 particles per µg of ODV protein; Volkman et al., 1976) was exploited to estimate the amount of ODV particles.

3.4 Characterization of the recombinant viruses

3.4.1 Luminescence measurements

Luminescence measurements were used to assess the expression and enzymatic activity of the luciferase protein. For this purpose, the *Sf9* cell pellets (1-2 x 10^6 cells), acquired during virus production (sections 3.3.2 and 3.3.3), were resuspended in 100 µl of PBS. 100 µl of the substrate, 1 mM D-luciferin (Sigma-Aldrich; in 0.1 M sodium citrate buffer, which was prepared by mixing 0.1 M trisodium citrate [Riedel-deHaën, Seelze, Germany] and 0.1 M citric acid [Merck, Darmstadt, Germany] to obtain pH 5) was added to the cells immediately before the measurement. Luminescence measurements were performed on white 96-well plates with a multilabel counter Wallac 1420 Victor$^{2™}$ (PerkinElmer Wallac Oy, Turku, Finland) using software version 2.0.

3.4.2 SDS-PAGE and Western blotting

For SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), the *Sf9* cell pellets, acquired during virus production (sections 3.3.2 and 3.3.3), were resuspended in 2 x Laemmli buffer with β-mercaptoethanol (2 x Laemmli buffer + β-mercaptoethanol: 63 mM Tris-HCl, 25% [v/v] glycerol, 2% SDS, 0.01% bromophenol blue [Sigma-Aldrich], and 5% β-mercaptoethanol [Merck]) to obtain a final cell concentration of 1 x 10^4 cells/µl. Polyhedra samples taken during ODV purification as well as the purified ODVs (section 3.3.4) were suspended in 4 x Laemmli buffer with β-mercaptoethanol to obtain 1 x final

concentration. BV samples for SDS-PAGE were prepared by first pelleting cell debris by centrifugation of the tertiary virus stock (Hermle Z 513 K: 15 min, 4 °C, 4 500 x g), and subsequently pelleting the BVs by ultracentrifugation (rotor SW 40 Ti; Beckman L-70 ultracentifuge, Beckman Coulter, Inc., Fullerton, CA: 1 h, 4 °C, 111 000 x g). Finally, the concentrated BVs were resuspended in 2 x Laemmli buffer with β-mercaptoethanol to obtain a final concentration of 2.5 x 10^8 pfu/µl. The SDS-PAGE samples were stored at –20 °C, and prior to the run, the samples were denaturated by heating for 5 min at 95 °C and kept on ice.

2 x 10^5 mock-, *Ac*WT-, and *Ac*ZZp74-infected *Sf9* cells; 2.3 x 10^{10} particles of *Ac*WT ODV; 4.5 x 10^9 particles of *Ac*ZZp74 ODV; 1 mg of *Ac*WT and *Ac*ZZp74 polyhedra, and 1 x 10^9 *Ac*WT and *Ac*ZZp74 BV particles per well were loaded to the gels. The amounts of ODV and polyhedra were calculated from the total protein concentrations of the stocks. Precision Plus Protein™ dual color standard (5 µl; Bio-Rad Laboratories) was used to facilitate the estimation of the molecular weights of the sample proteins. The proteins were separated using vertical, 1.5 mm thick gels composed of 5% stacking gel (5% acrylamide/Bis solution [29:1] [Bio-Rad Laboratories], 0.125 M Tris-HCl [pH 6.8], 0.1% SDS, 0.1% [w/v] ammoniumpersulfate [Bio-Rad Laboratories], and 0.1% [v/v] TEMED [N,N,N',N'-tetramethylene diamine; Bio-Rad Laboratories]) and 12% resolving gel (12% acrylamide/Bis solution [29:1], 0.375 M Tris-HCl [pH 8.8], 0.1% SDS, 0.1% [w/v] ammoniumpersulfate, and 0.04% [v/v] TEMED). Electrophoresis was conducted with a Mini 2-D Electrophoresis Cell apparatus (Bio-Rad Laboratories) in the presence of electrode buffer (25 mM Trizma base, 0.2 M glycine [Riedel-deHaën], and 3.5 mM SDS). The samples were concentrated at the boundary of the stacking and resolving gels by using a 100 V current for approximately 15 min. Subsequently, the current was increased to 180 V and the run continued for 45 to 60 min until the dye front reached the bottom of the gel.

Western blotting was performed to transfer the proteins from the gels to nitrocellulose paper (pore size 0.45 µm). A Mini-PROTEAN 3 Cell electroblotting apparatus (Bio-Rad Laboratories) filled with ice-cold transfer buffer (25 mM Trizma base, 0.2 M glycine, and 20% [v/v] methanol) was used for the blotting procedure. The blotting was conducted with a 100 V current for 1 h. To visualize total protein, the blots were subsequently stained with

0.2% Ponceau S (0.2% [w/v] Ponceau S [Sigma], and 5% [v/v] acetic acid) for approximately 5 min at RT and washed with sterile ddH_2O. The blots were blocked with 5% milk diluted in Tris-buffered saline (TBS: 20 mM Trizma base, and 0.5 M NaCl) supplemented with 0.05% Tween® 20 (Fluka Chemie GmbH, Buchs, Switzerland) (milk-TBS-Tween) for 1 h at RT with rocking to prevent unspecific binding of antibodies.

Immunolabeling was performed to detect specific bands of proteins. Primary antibodies used were mouse monoclonal anti-vp39 diluted 1:1 000, mouse monoclonal anti-p74 diluted 1:500, rabbit polyclonal anti-polyhedrin diluted 1:1 000, and rabbit IgG diluted 1:1 000 (for details of the primary antibodies, see Appendix III). All the antibodies were diluted in milk-TBS-Tween and incubated for 1.5 h at RT with rocking, followed by washes (3 x 5 min) with TBS-Tween. Secondary antibodies used were alkaline phosphatase-conjugated goat anti-mouse IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG (Appendix III) diluted in milk-TBS-Tween. The blots were incubated with the secondary antibodies for 1 h at RT with rocking. After washes (3 x 5 min) with TBS-Tween, the blots were equilibrated in alkaline phosphatase assay buffer (0.1M Trizma base, 0.1 M NaCl, and 5 mM $MgCl_2$) for 5 min. The protein bands were subsequently detected by incubating the blots with an alkaline phosphatase-substrate solution containing 0.33 mg/ml NBT (nitro blue tetrazolium [Sigma-Aldrich] in 70% dimethylformamide [Merck]) and 0.165 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt [Sigma-Aldrich] in 100% dimethylformamide) diluted in alkaline phosphatase assay buffer. The blots were incubated until the bands became visible, washed with sterile ddH_2O to stop the reaction, and air-dried.

3.4.3 Immunolabeling of infected *Sf*9 cells

To analyze the infectivities of *Ac*WT BV and ODV, *Sf*9 cells were allowed to attach to glass coverslips for 2 h at 28 °C. The cells were either infected with 6×10^4 ODV particles per cell or with 10 MOI of BV. Both ODV and BV were diluted in insect cell culture medium, and control cells were incubated only with the medium. At 72 h p.i., the cells were fixed with 4% paraformaldehyde (PFA: Merck) in PBS for 20 min at RT. The cells were subsequently double-immunolabeled to visualize viral proteins gp64 and polyhedrin.

The labeling was initiated by permeabilizating the cells with Triton X-100 (Sigma-Aldrich) diluted in 1.5% BSA-PBS (BSA: Roche Diagnostics corporation, Indianapolis, IN) for 20 min at RT. Primary antibodies mouse monoclonal anti-gp64 and rabbit polyclonal anti-polyhedrin (Appendix III) were then added to the cells for 1 h at RT. After washes (3 x 15 min) with 1.5% BSA-PBS, the cells were incubated with secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) and Alexa Fluor® 633 goat anti-rabbit IgG conjugate (purple) (Appendix III) for 30 min at RT in the dark. All antibodies were diluted in 1.5% BSA-PBS-0.1% Triton X-100. Subsequently, the cells were washed (3 x 15 min) with 1.5% BSA-PBS, rinsed once with PBS, and finally the coverslips were mounted on microscope slides with Mowiol (Mowiol 4-88 [Calbiochem, Darmstadt, Germany] in glycerol and Tris-HCl) supplemented with Dabco (25 mg/ml; 1,4-diazobicyllo-[2.2.2]-octane; Aldrich, Steinheim, Germany), and stored at 4 °C.

To study the cellular localization of the fusion protein ZZp74 expressed by the *Ac*ZZp74, *Sf9* insect cells were allowed to attach to glass coverslips for 30 min at 28 °C. The cells were infected with approximately 10 MOI of BV of the following viruses: *Ac*WT, *A*cZZp74, or *Ac*ZZVSVgTM (*Ac*ZZVSVgTM-EGFP [Ojala et al., 2004] without the EGFP). All the viruses were diluted in insect cell culture medium, and control cells were incubated only with the medium. The cells were fixed at 48 h p.i. for 20 min with 4% PFA in PBS at RT. Subsequently, the cells were immunolabeled using the same procedure as described above, this time using rabbit IgG together with a primary antibody against either BV-specific gp64 or ODV-specific p74 (Appendix III). The secondary antibodies used were Alexa Fluor® 555 goat anti-rabbit IgG conjugate (red) and Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) (Appendix III). The coverslips were mounted on microscope slides with Prolong Gold antifade reagent with DAPI (Invitrogen Molecular Probes). Samples of polyhedra were prepared for DIC by mounting purified polyhedra-containing solution (section 3.3.4) in Mowiol (1/6 ratio).

3.4.4 Confocal microscopy

The immunolabeled samples were observed with Carl Zeiss Axiovert 100 M inverted fluorescence microscope (Carl Zeiss Inc., Jena, Germany) equipped with an LSM 510

laser-scanning confocal module. The microscope contained a 488 nm argon laser, as well as 543 nm and 633 nm helium-neon lasers. Plan-Apochromat 63x/1.40 and Plan-Neofluar 40x/1.30 oil objectives were used. The fluorescence and DIC images were acquired and processed using LSM 510 software versions 3.0 and 3.5 using a multitracking mode. Some of the images were acquired together with Anna Mäkelä from the University of Jyväskylä.

3.4.5 Preparation of ODV and polyhedra samples for transmission electron microscopy

*Ac*WT ODV samples for transmission electron microscopy were prepared by diluting an ODV stock (6.4 x 10^8 ODV particles/µl) 1:10, 1:100, and 1:1 000 in sterile PBS filtered through a 0.2 µm (pore size) filter. Subsequently, the ODVs were allowed to attach on Formvar-coated copper grids for 20 min at RT, washed 5 times with sterile ddH_2O, stained with uranyl oxalate (Electron Microscopy Sciences, Hatfield, PA) for 10 min, and air-dried. The samples were inspected at 60 kV by using a JEOL JEM-1200 EX transmission electron microscope (Jeol Ltd., Tokyo, Japan). *Ac*WT polyhedra samples were similarly prepared by diluting a polyhedra stock 1:100, 1:1 000, and 1:10 000.

3.5 Binding and transduction experiments with human cancer cells

3.5.1 Mammalian cell culture

Human hepatocarcinoma HepG2 cells (American Type Culture Collection, Manassas, VA; number HB-8065) were maintained as a monolayer culture using Minimum Essential Medium (Gibco® Invitrogen Life technologies, Paisley, UK) supplemented with L-glutamine and Earle's salts (Gibco®), 10% (v/v) heat-inactivated FCS, 1% (v/v) penicillin - 1% (v/v) streptomycin mixture (Gibco®), 0.1 mM non-essential amino acids (Gibco®), and 1 mM sodium pyruvate (Gibco®). Human lung carcinoma A549 cells (American Type Culture Collection; number CCL-185) were cultured as a monolayer using Dulbecco's Modified Eagle Medium (Gibco®) supplemented with L-glutamine, D-glucose (Gibco®), 10% (v/v) FCS, and 1% (v/v) penicillin - 1% (v/v) streptomycin mixture. Both mammalian cell lines were grown at 37 °C in a humidified atmosphere of 5% CO_2.

3.5.2 ODV binding and entry experiments with HepG2 cells

Time series of ODV binding to HepG2 cells. The binding and transduction potentials of *Ac*WT ODV were examined with HepG2 cells using time points 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h. HepG2 cells were allowed to attach on coverslips overnight at 37 °C, and subsequently incubated in PBS with 1.9 x 10^5 ODV particles per cell. *Ac*WT BV (20 pfu/cell) and mock-transduced cells were used as controls. After 1 h incubation on ice with rocking, the 0 h time point cells were fixed (as described in section 3.4.3), and for the other samples, the PBS was replaced with complete growth medium, and the cells were incubated at 37 °C until fixed at the indicated time points. Immunolabeling was conducted as described in section 3.4.3 with the exception of using a mouse monoclonal vp39 antibody and a secondary antibody Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) (Appendix III).

Transmission electron microscopy of AcWT ODV binding to HepG2 cells. HepG2 cells were allowed to attach on culture dish overnight at 37 °C, followed by incubation with ODV (1.9 x 10^5 ODV particles per cell in PBS) for 1 h at 4 °C. Mock-transduced cells were used as controls. Prior to fixation, the cells were quickly washed three times with phosphate buffer (0.1 M Na_2HPO_4, pH 7.4) at 4 °C, and then fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M phosphate buffer for 1 h at 4 °C. The cells were washed (3 x 10 min) with 0.1 M phosphate buffer and left in the final wash overnight for convenience. The following day, the cells were further fixed with 1% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M phosphate buffer for 1 h at 4 °C. Subsequently, the cells were washed with 0.1 M phosphate buffer (3 x 10 min), dehydrated first with 70% ethanol (3 x 5 min) and then with 96% ethanol (3 x 5 min). The cells were stained *en block* with 2% uranyl acetate (Electron Microscopy Sciences, Washington, PA) in absolute ethanol for 30 min to enhance the electron density of the sample, followed by further dehydration with absolute ethanol (3 x 5 min). The cells were embedded in Epon LX-112 (Ladd Research Industries, Williston, VT) by laying Epon-containing gelatin capsels upside down on the culture dishes, followed by polymerization of the epon for 48 h at 60 °C. To facilitate removal of the capsels, the temperature was briefly (15-30 min) raised to 100 °C. Ultrathin sections (50 nm) were cut from the samples

with an ultramicrotome (Ultracut 8008; Reichert-Jung, Iowa City, IA), and stained with toluidine blue (Electron Microscopy Sciences, Hatfield, PA). Subsequently the sections were positioned on a copper grid and stained with 2% uranyl acetate and lead citrate (Electron Microscopy Sciences, Washington, PA). The ultramicrotome sectioning and the following stainings were performed by laboratory technician Raija Vassinen from the University of Jyväskylä. The samples were examined at 60 kV with a JEOL JEM-1200 EX transmission electron microscope.

Flow cytometric analysis of AcWT ODV binding to HepG2 cells. For the quantification of *Ac*WT ODV binding to HepG2 cells, 2 x 10^5 HepG2 cells per 1.5 ml tube were counted. The cells were pelleted by centrifugation at 500 x g (Heraeus Pico Biofuge) for 5 min at 4 °C, and all the subsequent centrifugations were similarly conducted. All the following steps were conducted at 4 °C and the incubations and washes with swinging. After pelleting, the cells were incubated with increasing concentrations of ODVs diluted in PBS (3.8 x 10^4, 9.4 x 10^4, 1.9 x 10^5, 2.8 x 10^5, 3.8 x 10^5, 9.4 x 10^5, and 1.9 x 10^6 ODV particles per cell). Two parallel samples were used for each concentration and for the virus-free cell control. The cells were incubated with the virus for 1.5 h, after which the cells were pelleted and resuspended in monoclonal p74 antibody (Appendix III) diluted 1:20 in 1.5% BSA-PBS. Following 1 h incubation, the cells were washed once with 1.5% BSA-PBS (10 min), pelleted, and resuspended in secondary antibody Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) (Appendix III) diluted in 1.5% BSA-PBS for 1 h incubation in the dark. Following a final pelleting, the cells were resuspended in 0.5 ml of PBS and stored on ice in the dark until 3 x 10^4 cells per sample were analyzed with FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest software.

3.5.3 Transduction experiments with HepG2 cells

HepG2 cells were attached on coverslips and subsequently incubated with a minimum volume of *Ac*WT ODV (3.4 x 10^6 or 7.1 x 10^6 ODV particles per cell) or with 200 pfu/cell of *Ac*WT or *Ac*ZZp74 BVs diluted in complete growth medium. Virus-free medium was used as a control. The cells were incubated with the virus dilutions for 1 h at RT with rocking, followed by addition of fresh complete growth medium. Thereafter, the

transduction was continued for 24 h at 37 °C. Finally, the cells were fixed and the coverslips mounted and stored as described in section 3.4.3. Similar transduction experiments were also performed by incubating the viruses and cells in PBS with different pH values (7.4, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0) as well as by using different liposomes to aid the *Ac*WT ODV transduction of HepG2 cells. The liposomes used were Cellfectin® Reagent (Invitrogen Life technologies), Oligofectamine™ Reagent (Invitrogen, Life technologies), FuGENE 6 transfection reagent (Roche Diagnostics corporation), and self-made unilamellar liposomes containing DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Sigma-Aldrich) and DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phospocholine; Sigma-Aldrich). For detection of transduction, EGFP expression was monitored by confocal microscopy. The occurrence of transduction was also assessed with luminescence measurements. For this purpose, the transduction was performed as described above with 400 pfu/cell of *Ac*WT or *Ac*ZZp74 BVs. After the 24 h incubation, the cells were detached by scraping, pelleted, and analyzed for luciferase activity (see section 3.4.1).

3.5.4 *Ac*ZZp74 BV entry into HepG2 and A549 cells

Time series of AcZZp74 BV entry into HepG2 and A549 cells. The entry of *Ac*ZZp74 BV into HepG2 and A549 cells was examined with a time series using 0 h, 4 h, 8 h, and 24 h time points. A549 and HepG2 cells were allowed to attach on coverslips for 24 h and 48 h at 37 °C, respectively. The cells were incubated with *Ac*ZZp74 BV (200 pfu/cell) in PBS for 1 h on ice. A recombinant baculovirus, *Ac*-luc (Mäkelä et al., 2006; 200 pfu/cell), possessing wt phenotype and the reporter gene *luciferase*, was used as a control. Subsequently, the cells were washed with PBS (15 min, RT), the 0 h time point cells were fixed for 15 min with 3% PFA in PBS at RT, and for the rest of the cells, complete growth medium was added and the cells incubated at 37 °C until fixed at the indicated time points. The immunolabeling was conducted as described in section 3.4.3. First, monoclonal vp39 antibody (Appendix III) diluted 1:50 was used to label virus particles without permeabilization. After a secondary antibody Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) (Appendix III) incubation, a 15 min permeabilization was performed. Subsequently, the virus particles were similarly labeled again, this time using Alexa Fluor® 555 goat anti-mouse IgG conjugate (red) (see Appendix III) as the secondary antibody. All

the antibodies were diluted in 3% BSA-PBS, which was also used for the washes. This labeling protocol should allow the virus inside the cell to be seen as red and the virus on the cell surface as yellow in the confocal microscope merge image.

Time series of AcZZp74 BV entry into early and late endosomes and lysosomes of HepG2 cells. The *Ac*ZZp74 BV entry into both early endosomes as well as into late endosomes and lysosomes of HepG2 cells was examined with time series by using time points 0 h, 2 h, 6 h, and 24 h. *Ac*WT BV was used as a positive control. HepG2 cells were allowed to attach on coverslips overnight at 37 °C, followed by a 1 h incubation at 4 °C with 1 000 pfu/cell of *Ac*ZZp74 or *Ac*WT BVs in PBS. Subsequently, the cells were washed (3 x 10 min, 4 °C), the 0 h time point cells were fixed (as described in section 3.4.3), and complete growth medium was added to the rest of the cells and they were further incubated at 37 °C until fixed at the indicated time points. The immunolabeling was conducted as described in section 3.4.3. For the study of *Ac*ZZp74 BV entry into early endosomes, the primary antibodies used were mouse monoclonal anti-vp39 and rabbit monoclonal anti-EEA-1 (early endosome antigen 1) (Appendix III), and the secondary antibodies were Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) and Alexa Fluor® 555 goat anti-rabbit IgG conjugate (red) (Appendix III), respectively. The *Ac*ZZp74 BV entry into late endosomes and lysosomes was studied by using primary antibodies rabbit polyclonal anti-BV and mouse monoclonal anti-LAMP-2 (lysosome associated membrane protein 2) (Appendix III), as well as secondary antibodies Alexa Fluor® 555 goat anti-rabbit IgG conjugate (red) and Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) (Appendix III), respectively.

4 RESULTS

4.1 Construction of the recombinant baculovirus vectors

Two occlusion-positive recombinant baculoviruses were generated: *Ac*WT, a control virus, and *Ac*ZZp74 with a surface-modified occlusion-derived virus (ODV). Both viruses were generated to contain reporter genes *luciferase* and *EGFP* to enable luminescent and fluorescent detection of viral transduction in mammalian cells.

4.1.1 Cloning of pWT

The cloning of *Ac*WT was initiated by generating a transfer plasmid, pWT (see Fig. 3.1 A). The gene encoding *polyhedrin* (738 bp) was successfully amplified by PCR using wt baculovirus as a template (Fig. 4.1 A). The primers were designed to produce a PCR product flanked by *Eco*RI and *Pst*I restriction enzyme sites. pWT was successfully created by replacing the *F3-VP39* fusion gene in pF3-VP39 (Mäkelä et al., manuscript) with the *polyhedrin* gene (not shown). The presence and orientation of the *polyhedrin* insert in the pWT was verified by *Xho*I / *Pst*I and *Eco*RI / *Pst*I restriction enzyme digestions (Fig. 4.1 B), producing the expected 1 055 bp (lane 1) and 738 bp (lane 2) bands (excluding the flanking restriction enzyme sites) for the correct clones, respectively. Moreover, the sequence was confirmed by sequencing (section 3.1.5; not shown).

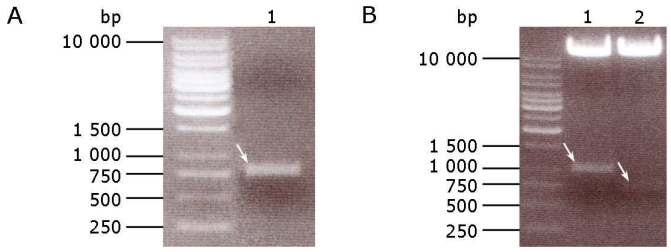

Figure 4.1 PCR amplification of *polyhedrin* and restriction enzyme analysis of pWT. A) PCR-amplified *polyhedrin* gene (738 bp, arrow) after an *Eco*RI / *Pst*I restriction enzyme digestion. **B)** Restriction enzyme analysis of pWT with *Xho*I and *Pst*I (1 055 bp; lane 1, arrow) as well as with *Eco*RI and *Pst*I (738 bp; lane 2, arrow) to confirm the presence and orientation of the *polyhedrin* insert. The lengths of the DNAs are stated without the restriction enzyme sites.

4.1.2 Cloning of pZZp74

The *Ac*ZZp74 was generated by first creating a pZZp74 transfer plasmid (see Fig. 3.1 B). For the cloning of the fusion gene *ZZp74*, the *ZZ* domains (360 bp) were amplified by PCR using pZZVSVgTM (Ojala et al., 2004) as a template (Fig. 4.2 A). The upper band in Figure 4.2 A represents ZZ domains (indicated with an arrow), whereas the lower band contains single Z domains. The primers were designed to produce a PCR product flanked by *Xba*I and *Pst*I restriction enzyme sites. Subsequently, the *ZZ* PCR product was successfully introduced into an empty pFastBac™Dual vector under the *polyhedrin* promoter, producing pZZ. The presence and orientation of the *ZZ* insert in the pZZ was validated by *Eco*RI / *Pst*I restriction enzyme digestion (Fig. 4.2 B), producing a 410 bp bands (excluding the flanking restriction enzyme sites) for the correct clones.

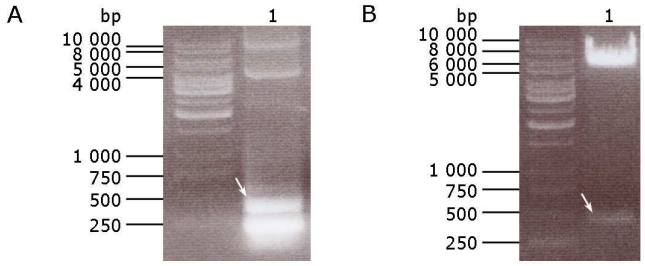

Figure 4.2 PCR amplification of *ZZ* domains and restriction enzyme analysis of pZZ. A) PCR-amplified *ZZ* domains (360 bp, arrow). **B)** The presence and orientation of the *ZZ* insert in the pZZ was confirmed with *Eco*RI / *Pst*I restriction enzyme analysis producing the expected 410 bp band (arrow). The lengths of the DNAs are stated without the restriction enzyme sites.

The *p74* (1 938 bp) was also amplified by PCR using the pWT-based bacmid (see section 4.1.3) as a template (Fig. 4.3 A). The primers were designed so that the PCR product was flanked by *Pst*I and *Hind*III restriction enzyme sites. pZZp74-intermediate was subsequently generated by cloning the *p74* in frame into pZZ, downstream of the *ZZ* domains. *Xba*I / *Hind*III restriction enzyme digestion was used to verify the presence and orientation of the *ZZp74* insert in the pZZp74-intermediate (Fig. 4.3 B). The digestion produced the expected 2 304 bp band (excluding the flanking restriction enzyme sites) for the correct clones. The correct sequence of *ZZp74* was also verified by sequencing (section 3.1.5; not shown).

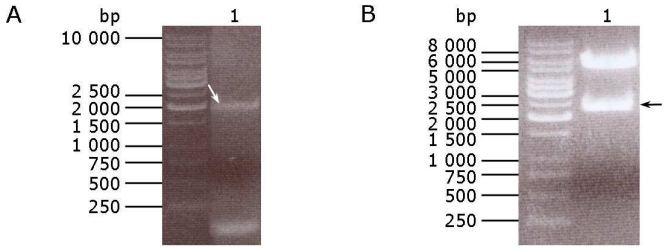

Figure 4.3 PCR amplification of *p74* and restriction enzyme analysis of pZZp74-intermediate. A) PCR-amplified *p74* (1 938 bp, arrow). **B)** Restriction enzyme analysis of pZZp74-intermediate with *Xba*I / *Hind*III (2 304 bp, arrow) to confirm the presence and orientation of the *ZZp74* insert. The lengths of the DNAs are stated without the restriction enzyme sites.

To create the final pZZp74, the *ZZp74* fusion gene was amplified by PCR and inserted into the pWT. The reverse primer was designed to include the pFastBac™Dual-derived SV40 polyadenylation signal downstream the *ZZp74* fusion gene. Moreover, the primers were designed to produce PCR products flanked by *Sma*I restriction enzyme sites. Accordingly, *ZZp74* with the polyadenylation signal (2 568 bp without the flanking restriction enzyme sites) was amplified by PCR (Fig. 4.4 A), followed by addition of an A overhang to the *Sma*I-digested insert and a T overhang to *Sma*I-digested pWT vector. Since the *Sma*I restriction sites were not regenerated in the following ligation reaction, colony PCR was used to identify positive clones containing the *ZZp74* insert (not shown). The presence of the insert was further verified by amplifying the *ZZp74* with and without the polyadenylation signal by PCR, using the isolated plasmids as templates. The expected 2 568 bp (Fig. 4.4 B) and 2 304 bp (not shown) bands (excluding the flanking restriction enzyme sites) were detected, respectively.

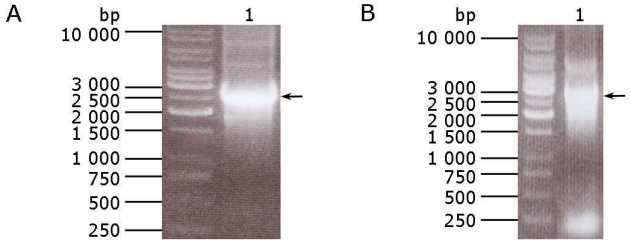

Figure 4.4 PCR amplification of *ZZp74* and verification of the presence of *ZZp74* insert in pZZp74 by PCR. A) PCR-amplified *ZZp74* (2 568 bp with polyadenylation signal, arrow). **B)** The presence of *ZZp74* insert (2 568 bp, arrow) in the isolated pZZp74 was verified with PCR. The lengths of the PCR products are stated without the restriction enzyme sites.

4.1.3 Production of the recombinant viruses

After the sequences of pWT and pZZp74 had been verified, the plasmids were transformed into DH10Bac™ strain of *E. coli* for the generation of recombinant bacmids. Subsequently, positive white colonies were selected using blue-white screening, and the bacmids were isolated from *E. coli* suspension cultures. Primary BV stocks were produced by transfecting *Sf*9 insect cells with the isolated bacmids. For the production of secondary virus stocks, correct *Ac*WT clones were selected by blotting the infected cells (not shown) and by monitoring the enzymatic activity of luciferase in the cells (not shown). The correct *Ac*ZZp74 clones were selected by using only Western blotting (not shown), for the insertion of the *ZZp74* fusion gene abolished the activity of the *luciferase* in insect cells. Subsequently, the correct primary stocks were further amplified to generate secondary BV stocks, and finally, similar procedures were performed to produce tertiary BV stocks. ODV stocks were generated by purifying ODV from *Sf*9 cells infected with *Ac*WT and/or *Ac*ZZp74 BVs (see section 3.3.4).

4.2 Characterization of the recombinant viruses

4.2.1 Western blot analysis

To characterize *Ac*WT and *Ac*ZZp74, Western blotting analysis was performed with mock- and virus-infected cells, polyhedra, ODV, and BV of both viruses using *Ac*MNPV-specific antibodies raised against viral structural proteins polyhedrin, vp39 (not shown), and p74. In addition, cellular expression and incorporation of the ZZp74 fusion protein into virus particles was analyzed using rabbit IgG. As a result, labeling with polyhedrin antibody identified 29-kDa bands characteristic for polyhedrin protein in *Ac*WT-infected *Sf*9 cells (Fig. 4.5 A, lane 2), polyhedra (lane 3), and ODV (lane 4) as well as in the corresponding *Ac*ZZp74 samples (Fig. 4.5 B, lanes 2-4). However, it is important to notice, that the *Ac*ZZp74 polyhedra and ODV were, in fact, produced by coinfection of *Sf9* cells with *Ac*WT and *Ac*ZZp74 (see section 3.3.4), due to lack of sufficient expression of polyhedrin. The polyhedrin antibody also identified approximately 88 kDa bands in the *Ac*ZZp74-infected cells (Fig. 4.5 B, lane 2), polyhedra (lane 3), and ODV (lane 4) (weakly observed). These bands correspond for the ZZp74 fusion protein, which was labeled due to

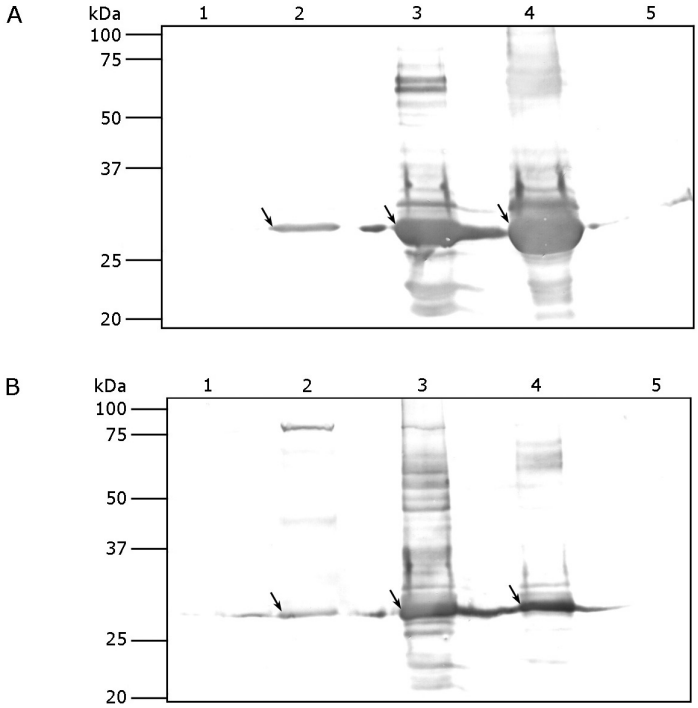

Figure 4.5 Western blot analysis to detect polyhedrin in *Ac*WT- and *Ac*ZZp74-infected *Sf*9 cells, polyhedra, ODV, and BV. Western blots for *Ac*WT (A) and *Ac*ZZp74 (B) samples labeled with polyclonal polyhedrin antibody and an alkaline phosphatase-conjugated secondary antibody. Both blots contain 2 x 10^5 mock-infected *Sf*9 cells (lane 1) as a control, as well as 2 x 10^5 virus-infected *Sf*9 cells (lane 2), 1 mg polyhedra (lane 3), 2.3 x 10^{10} *Ac*WT ODV particles or 4.5 x 10^9 *Ac*ZZp74 ODV particles (lane 4), and 1 x 10^9 BV particles (lane 5). Polyhedrin bands are indicated with arrows.

the high affinity of the IgG-binding Z domains for rabbit IgG antibodies, such as the polyhedrin antibody used here. Moreover, the strong polyhedra band in the ODV sample indicates that some polyhedrin protein remains in the ODV stock after ODV purification.

Anti-p74 labeling produced 74 kDa bands corresponding to ODV envelope protein p74 in all the samples excluding the mock-infected *Sf*9 cells (Fig. 4.6 A, lane 1) and the *Ac*WT BV (lane 8). The p74 stain is weak in the *Ac*WT samples (lanes 2, 4, 6, and 8) due to the inherently low amount of wt p74 protein expressed in insect cells. The *Ac*ZZp74 samples have more intense bands, probably because *Ac*ZZp74 contains both wt p74 and the fusion protein ZZp74, and the resolution in the immunoblot was not high enough to discriminate between 74 kDa of wt p74 and 88 kDa of ZZp74. No further effort was put within this study to improve the resolution of the p74 blot. Moreover, the ZZp74 is under the strong *p10* promoter, and thus produced in vaster amounts than wt p74, which has a weak promoter. Unexpectedly, a band labeled by p74 antibody was also detected in *Ac*ZZp74

BV (Fig. 4.6 A, lane 9). Moreover, a similar band was also observed in the corresponding sample, when IgG labeling produced an 88 kDa band representing the ZZp74 fusion protein (Fig. 4.6 B, lane 9). IgG was used to label the ZZ domains, and it produced no bands for the wt virus as expected (Fig. 4.6 B, lanes 2, 4, 6, and 8). In *Ac*ZZp74-infected *Sf*9 cells (lane 3), polyhedra (lane 5), ODV (lane 7), and BV (lane 9) the 88 kDa bands were detected. The ZZp74 band observed in *Ac*ZZp74 BV by two different labelings suggest incorporation of the ZZp74 fusion protein into budded virions.

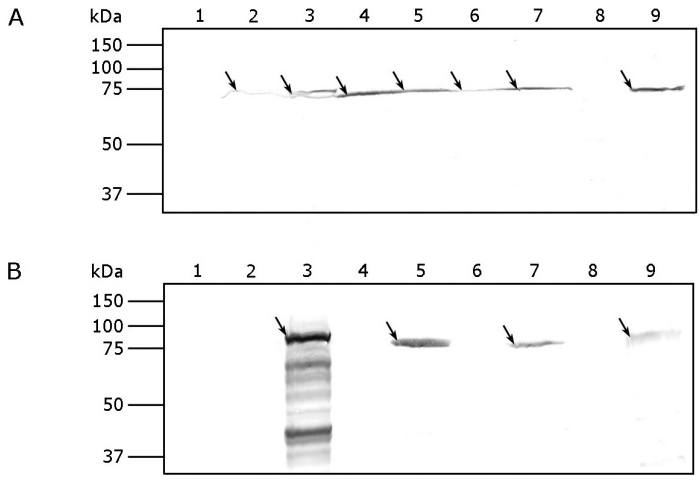

Figure 4.6 Western blot analysis to detect p74 and ZZ domains in *Ac*WT- and *Ac*ZZp74-infected *Sf*9 cells, polyhedra, ODV, and BV. Western blots for the detection of p74 (A), and ZZ domains (B) with monoclonal p74 and rabbit IgG antibodies, respectively, followed by alkaline phosphatase-conjugated secondary antibodies. Both blots contain 2 x 10^5 mock-infected *Sf*9 cells (lane 1) as a control, as well as 2 x 10^5 *Ac*WT- (lane 2) and *Ac*ZZp74-infected *Sf*9 cells (lane 3); 1 mg *Ac*WT (lane 4) and *Ac*ZZp74 polyhedra (lane 5); 2.3 x 10^{10} *Ac*WT ODV particles (lane 6) and 4.5 x 10^9 *Ac*ZZp74 ODV particles (lane 7); and 1 x 10^9 *Ac*WT (lane 8) and *Ac*ZZp74 BV particles (lane 9). The specific bands are indicated with arrows.

4.2.2 Confocal microscopy studies

Infectivity of AcWT BV and ODV. To confirm the infectivity of *Ac*WT as well as its ability to express polyhedrin, *Sf*9 insect cells were infected with either *Ac*WT BV or ODV. In the BV-infected *Sf*9 cells, both gp64 (Fig. 4.7, green) and polyhedrin (purple) were clearly detectable by confocal microscopy. Gp64 was detected to normally localize on the plasma membrane, and accordingly, polyhedrin, which normally resides primarily in the nuclei of infected cells, was observed inside the cells. Polyhedra can be detected in the nuclei of few of the BV-infected cells in the DIC image (black arrows) and as unlabeled spots among the polyhedrin label (white arrows). ODV infects *Sf*9 cells more inefficiently and slower than

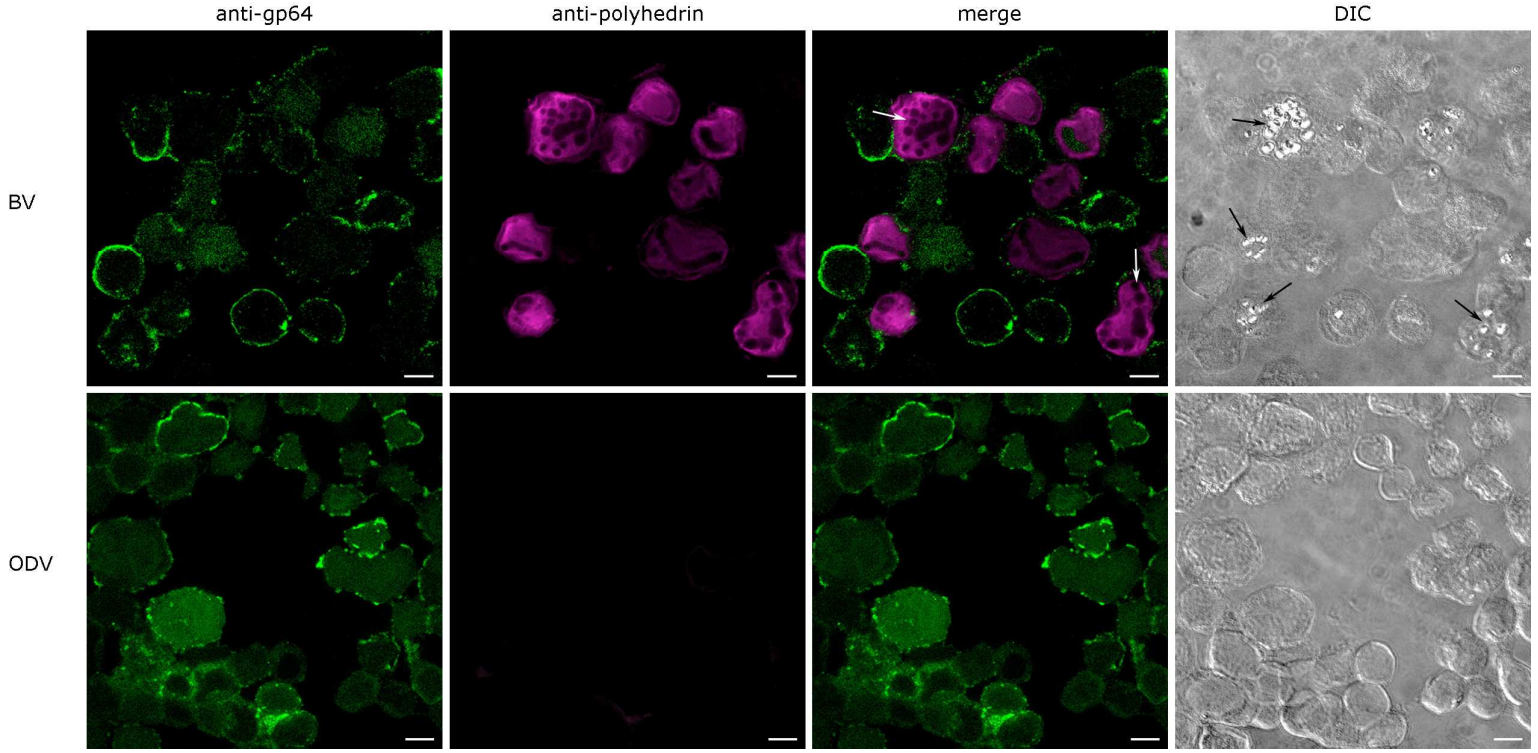

Figure 4.7 *Sf*9 insect cells infected with *Ac*WT BV or ODV. The infectivities of *Ac*WT phenotypes were examined by infecting *Sf*9 insect cells with BV (10 MOI) or ODV (6 x 10^4 particles/cell). Expression of gp64 and polyhedrin were detected 72 h p.i. with monoclonal gp64 antibody and polyclonal polyhedrin antibody, as well as with secondary antibodies Alexa Fluor® 488 (green) and Alexa Fluor® 633 (purple), respectively. Polyhedra are indicated with arrows. The images represent single slices of z-stacks. Scale bar 10 µm. DIC, differential interference contrast.

BV, due to its less infectious nature towards these cells (Volkman et al., 1976). Thus, in the ODV-infected *Sf*9 cells, the infectious cycle has not proceeded as far, and accordingly, only gp64 can be detected (Fig. 4.7). From these results, it can be concluded that the recombinant virus, *Ac*WT, expresses polyhedrin, and that both phenotypes are infective.

Characterization of polyhedra. DIC images of polyhedra were obtained to confirm their normal morphology and the functionality of the polyhedra purification protocol. Similar polyhedra could be seen in both *Ac*WT and *Ac*ZZp74 samples (Fig. 4.8 A and B, respectively). Moreover, *Ac*WT polyhedra samples were also prepared for analysis by transmission electron microscopy (not shown), confirming the results.

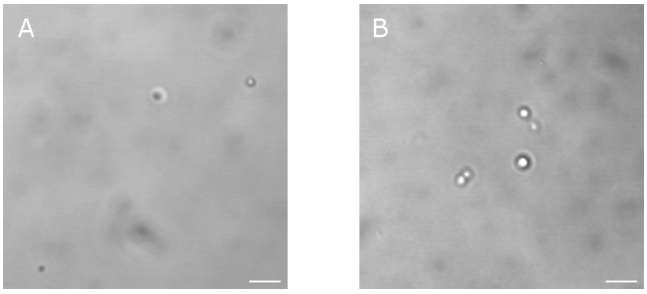

Figure 4.8 Differential interference contrast images of polyhedra. *Ac*WT (A) and *Ac*ZZp74 (B) polyhedra. Scale bar 5 μm.

Cellular localization of ZZp74. The localization of the fusion protein ZZp74 expressed by *Ac*ZZp74 was studied by infecting *Sf*9 cells with *Ac*ZZp74 BV. *Sf*9 cells infected with *Ac*WT and *Ac*ZZVSVgTM BVs served as negative and positive controls, respectively. Gp64 was detected to be expressed on the plasma membrane (Fig. 4.9 A) and p74 inside the cells (Fig. 4.9 B). The p74 label is slightly diffuse, possibly due to only a rather short period of low expression of wt p74 during baculovirus infection (Kuzio et al., 1989). Gp64 and ZZVSVgTM are both plasma membrane-residing proteins, and they were indeed detected on the plasma membrane of the infected *Sf*9 cells (Fig. 4.9). Accordingly, in the infected cells, ZZVSVgTM can be observed to be located outside of wt p74, and gp64 outside of ZZp74, which suggest normal localization for both wt p74 and ZZp74 on the nuclear membrane and in the intranuclear vesicles. Moreover, ZZp74 and p74 partially colocalize with each other, indicating similar intracellular localization.

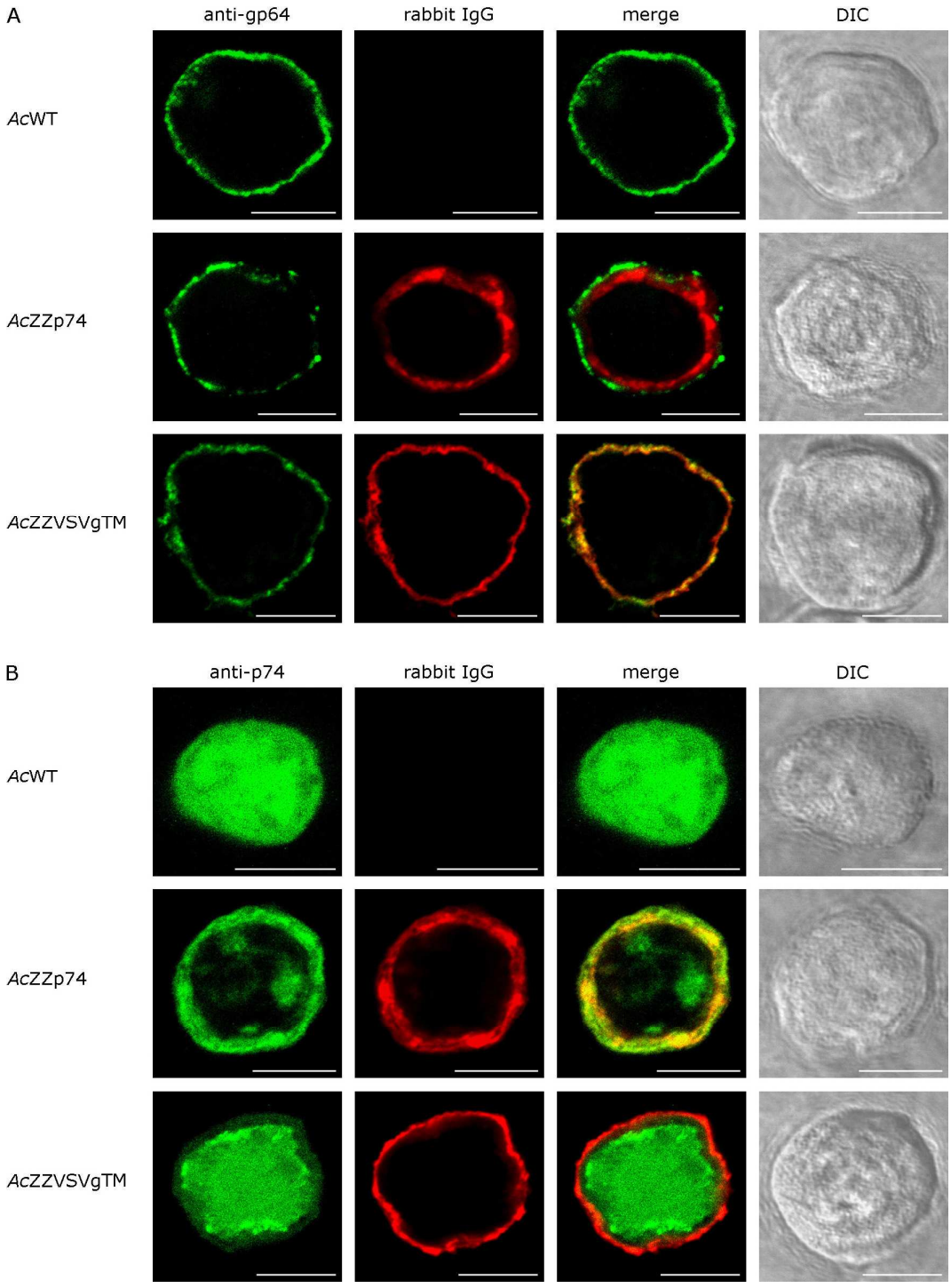

Figure 4.9 *Sf*9 insect cells infected with *Ac*WT, *Ac*ZZp74, or *Ac*ZZVSVgTM BVs. To study the localization of the ZZp74 fusion protein, *Sf*9 cells were infected with 10 MOI of *Ac*WT, *Ac*ZZp74, or *Ac*ZZVSVgTM BVs. At 48 h p.i., the ZZ domains were labeled with rabbit IgG and secondary antibody Alexa Fluor® 555 (red), and, in addition, either gp64 (A) or p74 (B) was labeled with a specific monoclonal antibody and Alexa Fluor® 488 (green). The images are 3D projections of 3 to 4 individual slices of z-stacks in the middle of the cell. Scale bar 10 µm. DIC, differential interference contrast.

4.2.3 Transmission electron microscopy of ODVs

To confirm normal morphology of *Ac*WT ODVs and to study the amount of nucleocapsids in ODV, transmission electron microscopy samples of ODVs were prepared. Plenty of single-nucleocapsid ODVs could be detected (Fig. 4.10 A, solid arrows), whereas ODVs containing multiple nucleocapsids were absent, suggesting that predominantly single-nucleocapsid ODVs are produced by the *Ac*WT. Some ODVs were attached to darker, electron-dense substance likely to be polyhedrin (Fig. 4.10 A, dashed arrows; B, dark substance), indicating that the purification protocol could be rendered more efficacious. Utilization of saccarose gradient ultracentrifugations would have improved the purity of the stock, but simultaneous decrease in the yield would have resulted.

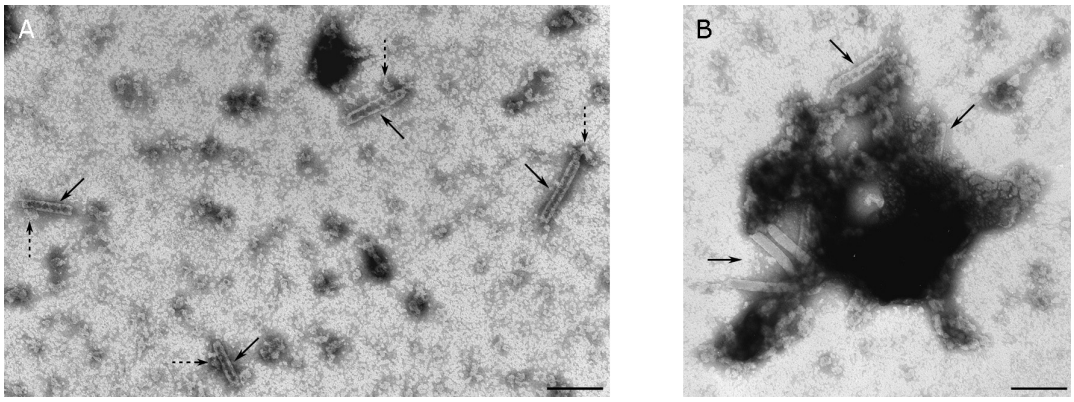

Figure 4.10 Transmission electron microscopy images of *Ac*WT ODV. Transmission electron microscopy samples were prepared from a diluted *Ac*WT ODV stock (6.4 x 10^8 ODV particles/µl diluted 1:10, 1:100, and 1:1 000) by attaching ODVs on copper grids and subsequently staining with uranyl oxalate. *Ac*WT ODVs are indicated with solid arrows. Most of the ODVs are still attached to polyhedrin (A, dashed arrows; B, black substance). Scale bar 200 nm.

4.2.4 PCR analysis

Since the polyhedra production of *Ac*ZZp74 was detected to be low or absent, the presence of the *polyhedrin* gene in pZZp74, ZZp74 bacmid, and *Ac*ZZp74 was examined by amplifying the *polyhedrin* (738 bp) by PCR using pWT as a positive control. A 738 bp band representing *polyhedrin* could be detected when pWT (Fig. 4.11, lane 1), pZZp74 (lane 2), and ZZp74 bacmid (lane 3) were used as templates. Unspecific PCR products were also observed. No *polyhedrin* band could be detected for the *Ac*ZZp74 virus (Fig. 4.11, lane 4), which, however, does not reliably confirm the absence of the *polyhedrin*

from the *Ac*ZZp74 genome, since the virus-containing medium used as a template might interfere with the amplification reaction. No further efforts were conducted within this study to optimize the reaction. However, it is generally possible to successfully amplify genes by PCR using nonpurified baculovirus-containing medium as a template (an example of this is the *polyhedrin* PCR described in sections, 3.1.1 and 4.1.1).

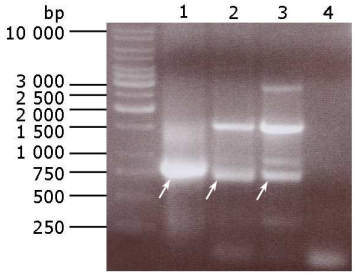

Figure 4.11 Verification of the presence of *polyhedrin* in pZZp74, ZZp74 bacmid, and *Ac*ZZp74 with PCR. The presence of the *polyhedrin* insert in pZZp74 (lane 2), ZZp74 bacmid (lane 3), and *Ac*ZZp74 (lane 4) was confirmed by PCR amplification of *polyhedrin*. pWT was used as a control (lane 1). The *polyhedrin* PCR products (738 bp) are indicated with arrows.

4.3 Binding and transduction experiments with human cancer cells

4.3.1 Binding

AcWT ODV binding and entry to HepG2 cells. To study the ability of *Ac*WT ODV to bind to and enter HepG2 cells, a time series was performed and examined by confocal microscopy. *Ac*WT BV was used as a positive control, since it is known to bind to HepG2 cells (Hofmann et al., 1998; Hofmann et al., 1995). ODV and BV were allowed to bind to the cells for 1 h on ice. The low temperature prevents BV internalization by endocytosis (Volkman and Goldsmith, 1985), whereas it does not completely prevent ODV internalization, since direct membrane fusion of ODV with its target membranes is known to occur also at 4 °C (Horton and Burand, 1993). The entry of ODV into HepG2 cells was qualitatively assessed at 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h post-transduction (p.t.). *Ac*WT ODV was observed to bind to HepG2 cells at all time points (Fig. 4.12, time points 0 h, 4 h, and 24 h are shown) and the amount of bound virus did not appear to change significantly over time. As can be observed in the orthogonal cut of a HepG2 cell in Figure 4.13, small amount of ODVs have entered the cytoplasm of HepG2 cells already at 0 h

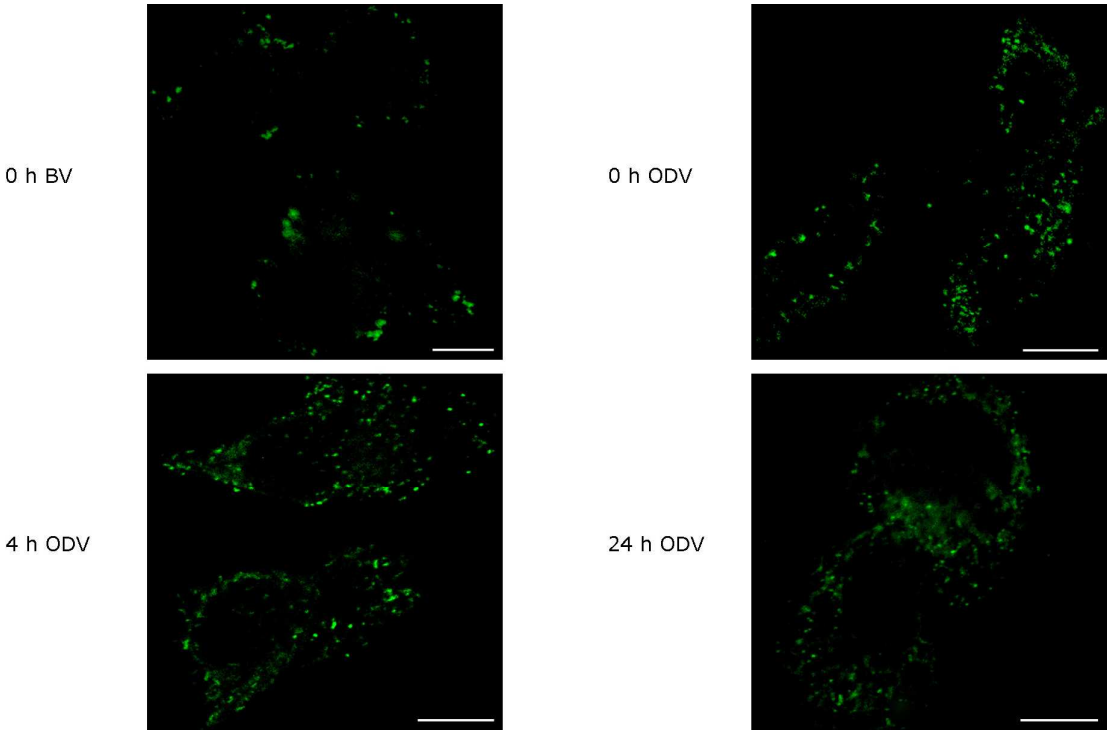

Figure 4.12 *Ac*WT ODV binding and entry to HepG2 cells. A time series of *Ac*WT ODV binding and entry to human HepG2 hepatocarcinoma cells was performed using *Ac*WT BV (0 h) as a control. BV (20 pfu/cell) and ODV (1.9 x 10^5 particles/cell) were allowed to bind to HepG2 cells for 1 h on ice, after which the cells were fixed either immediately (0 h time point) or after further incubation at 37 °C (time points 2 h, 6 h, and 8 h are not shown). The virus particles were labeled with a monoclonal vp39 antibody and secondary antibody Alexa Fluor® 488 (green). The images represent single slices of z-stacks from the middle of the cell. Scale bar 10 µm. DIC, differential interference contrast.

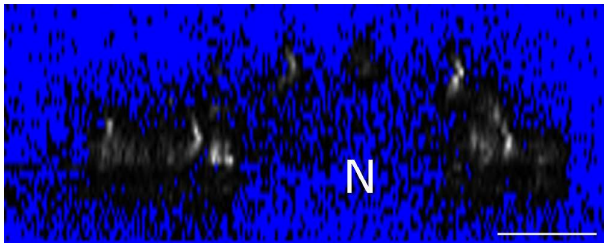

Figure 4.13 Entry of ODV into HepG2 cells. Orthogonal cut of a HepG2 cell after 1 h incubation with *Ac*WT ODVs (white) on ice. ODVs were labeled with a monoclonal vp39 antibody and secondary antibody Alexa Fluor® 488. The image is acquired from the z-stacks of the cell with the LSM 510 software using range indicator style from color palette. Scale bar 5 µm. N, nucleus.

time point. A similar ODV entry pattern was also detected in the later time points (not shown). In addition, the colocalization of *Ac*WT ODV with two cellular plasma membrane proteins, integrin α2 and transferrin receptor, was studied by labeling them and ODVs bound to the surface of HepG2 cells. No significant colocalization was observed (data not shown).

Quantification of AcWT ODV binding to HepG2 cells by flow cytometry. In order to study the kinetics of ODV binding to HepG2 cells and to determine the saturation point, the binding of ODV to HepG2 cells was quantified with a flow cytometer. Increasing concentrations of ODVs (3.8×10^4, 9.4×10^4, 1.9×10^5, 2.8×10^5, 3.8×10^5, 9.4×10^5, and 1.9×10^6 ODV particles per cell) were allowed to bind to HepG2 cells by incubating for 1.5 h at 4 °C. Subsequently, the virus particles were detected by labeling the viral protein p74. The binding of ODV to HepG2 cells appeared to be concentration-dependent, although no clear saturation was achieved (Fig. 4.14).

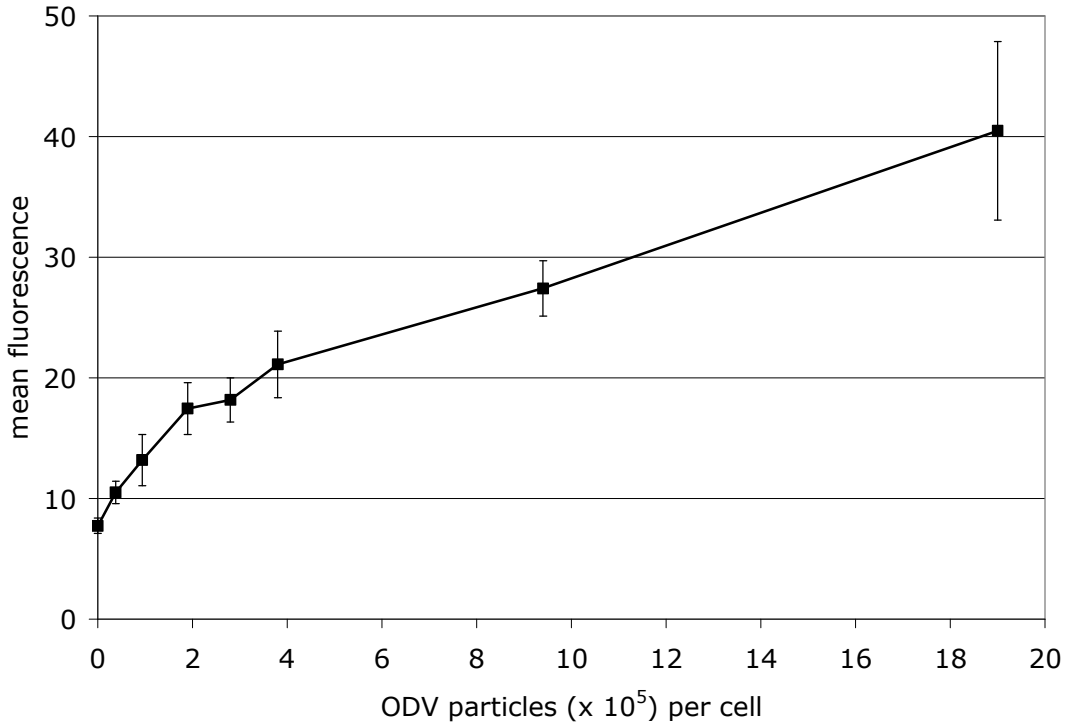

Figure 4.14 Quantification of *Ac*WT ODV binding to HepG2 cells. *Ac*WT ODV binding to HepG2 cells was quantified by flow cytometry using increasing ODV concentrations (3.8×10^4, 9.4×10^4, 1.9×10^5, 2.8×10^5, 3.8×10^5, 9.4×10^5, and 1.9×10^6 ODV particles per cell). The ODVs were allowed to bind to HepG2 cells for 1.5 h at 4 °C, and subsequently detected by labeling with a monoclonal p74 antibody followed by secondary antibody Alexa Fluor® 488. Two parallel samples of each concentration were used, and three runs per sample were performed (1×10^4 cells/run). The experiment was repeated three times, and one representative set of results is illustrated here. Mean fluorescence values for each concentration ± standard deviations of dublicate samples are indicated.

*Transmission electron microscopy of AcWT ODV binding to HepG2 cells. Ac*WT ODV binding to HepG2 cells was studied by transmission electron microscopy. ODVs (1.9×10^5 particles per cell) were incubated with HepG2 cells for 1 h and subsequently prepared for

transmission electron microscopy. ODVs were observed bound to the surface of HepG2 cells (Fig. 4.15). However, only a scarce amount of ODVs were detected in total.

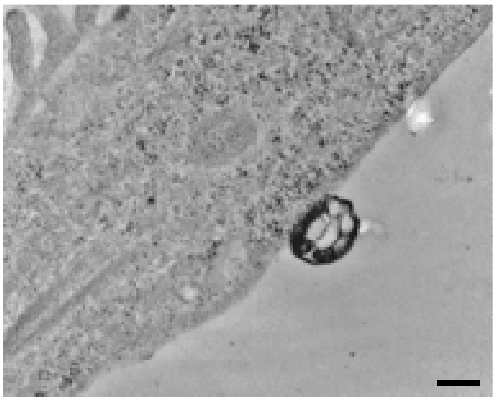

Figure 4.15 *Ac*WT ODV binding to HepG2 cells. Transmission electron microscopy image of *Ac*WT ODV bound to the surface of HepG2 cell. Scale bar 200 nm.

4.3.2 Transduction of HepG2 cells

The ability of *Ac*WT BV and ODV as well as *Ac*ZZp74 BV to transduce HepG2 cells were qualitatively studied by monitoring EGFP expression by confocal microscopy. HepG2 cells were incubated either with *Ac*WT ODV or with *Ac*WT or *Ac*ZZp74 BVs, followed by detection of EGFP expression at 24 h p.t. The EGFP reporter gene of *Ac*WT was demonstrated to be functional, since intense green fluorescence was detected both in the nucleus and in the cytoplasm in approximately 30% of the treated HepG2 cells (Fig. 4.16). On the contrary, no green fluorescence was detected in the HepG2 cells transduced with either *Ac*WT ODV or *Ac*ZZp74 BV (Fig. 4.16). The transduction abilities and efficiency of *Ac*WT or *Ac*ZZp74 BVs were also assessed by monitoring the enzymatic activity of luciferase, the protein product of the reporter gene *luciferase*, in transduced HepG2 cells. Transduction of HepG2 cells was performed with 400 pfu/cell of *Ac*WT or *Ac*ZZp74 BVs, and at 24 h p.t., the amount of emitted light was measured immediately after the addition of D-luciferin, the substrate of luciferase. *Ac*WT BV was again shown to efficiently transduce HepG2 cells, since intense light emission was detected (approximately 2 x 10^4 counts/s for 1 x 10^5 cells). In *Ac*ZZp74 BV-transduced HepG2 cells, no light emission could be detected.

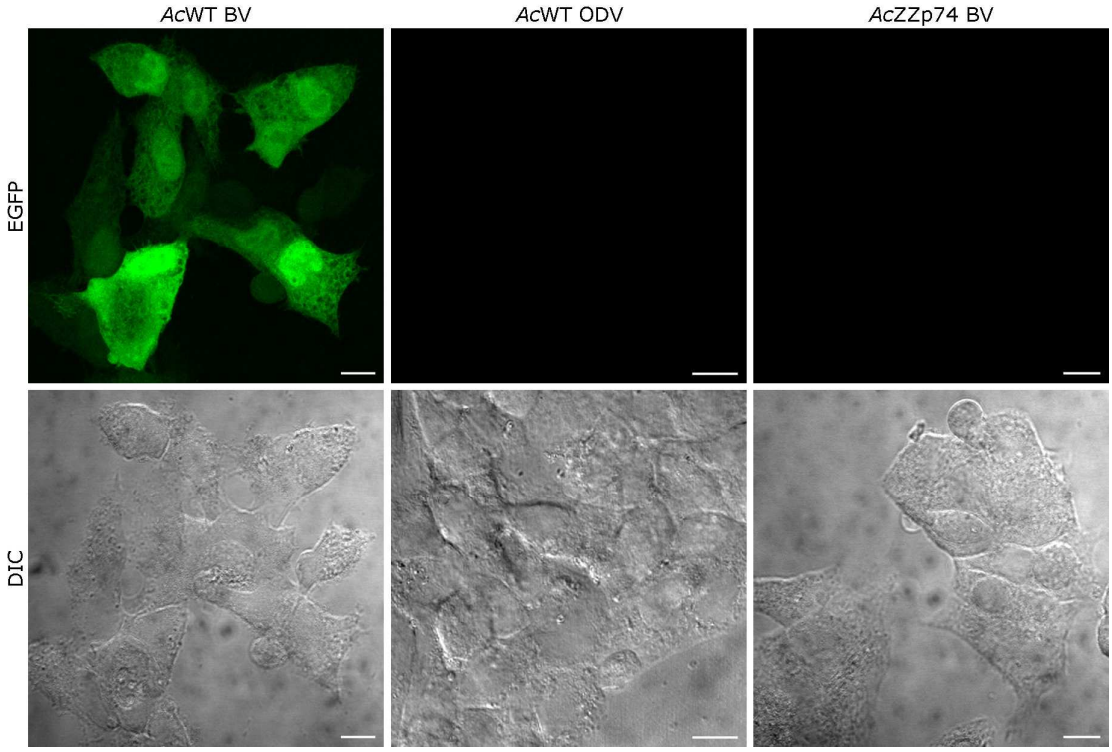

Figure 4.16 EGFP expression in transduced HepG2 cells. *Ac*WT ODV (7.1 x 10^6 ODV particles per cell) or 200 pfu/cell of *Ac*WT or *Ac*ZZp74 BVs were allowed to bind to HepG2 cells for 1 h at 4 °C, followed by incubation at 37 °C. At 24 h p.t., EGFP expression was detected by confocal microscopy. The EGFP images represent 3D projections of z-stacks. Scale bar 10 µm. DIC, differential interference contrast; EGFP, enhanced green fluorescent protein.

*Ac*WT ODV transduction of HepG2 cells was also attempted by using different pH values (7.4, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0), due to the preference of ODV to enter its natural target cells at alkaline conditions (Horton and Burand, 1993). However, no EGFP expression was detected at 44 h p.t. (data not shown). A preliminary study with several liposomes to aid the *Ac*WT ODV transduction of HepG2 cells was also conducted. No viral transduction could be detected this time either (data not shown).

4.3.3 *Ac*ZZp74 BV entry into human cancer cells

AcZZp74 BV entry into HepG2 and A549 cells. To gain further insights into the mechanisms underlying the reduced transduction ability of *Ac*ZZp74 BV in HepG2 and human lung carcinoma A549 cells, a time series was performed with 0 h, 4 h, 8 h, and 24 h time points p.t. *Ac*-luc BV (Mäkelä et al., 2006) was used as a positive control to confirm the success of the labeling protocol (not shown). The viruses were allowed to bind to

HepG2 or A549 cells for 1 h on ice. *Ac*ZZp74 BV was observed to enter both HepG2 and A549 cells in low quantities at the 4 h, 8 h, and 24 h time points (Fig. 4.17 for HepG2 and Fig. 4.18 for A549; the 8 h time points are not shown). However, most of the virus remained on the cell surface, and from the 4 h time point on, large aggregates were characteristic for the *Ac*ZZp74 BV, especially in HepG2 cells. The entry of *Ac*ZZp74 BV into the two cell lines was not quantified, although it would have been useful to assess the differencies in the amounts of internalized virus as compared to the control virus. Nevertheless, these results indicate that *Ac*ZZp74 BV does not enter HepG2 and A549 cells as efficiently as wt BV, and even the few *Ac*ZZp74 BVs internalizing are not able to mediate transgene expression (as was detected in section 4.3.2), suggesting lack of nuclear localization of the internalized virus particles.

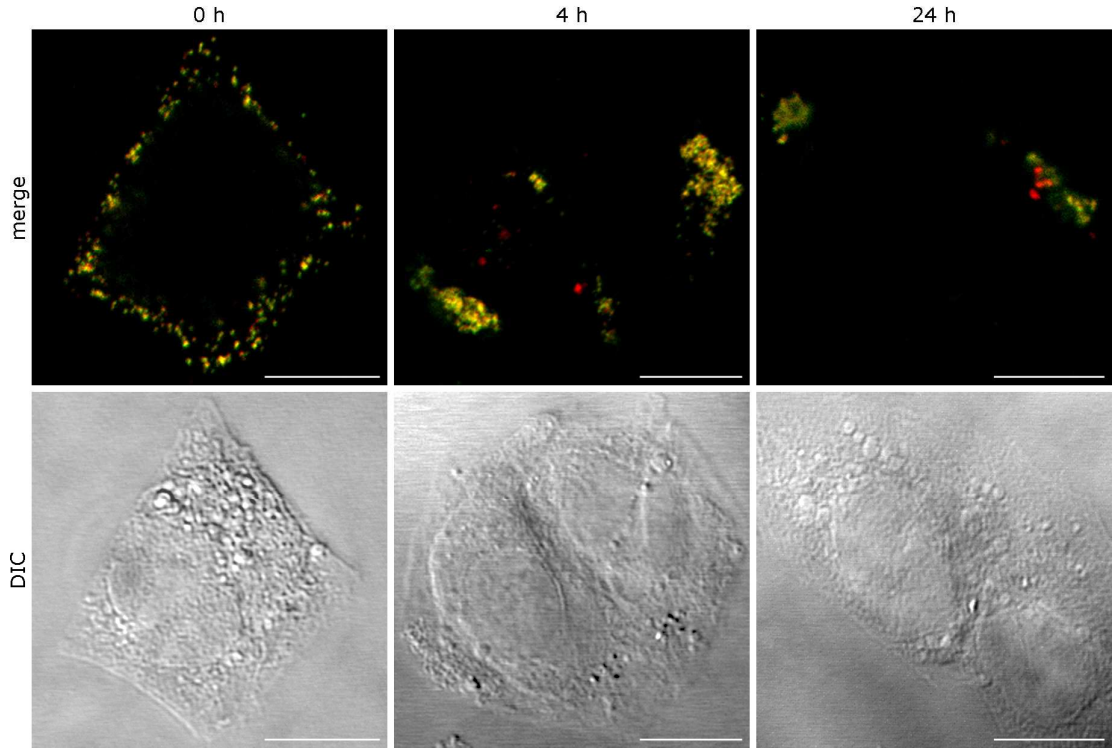

Figure 4.17 *Ac*ZZp74 BV entry into HepG2 cells. A time series of *Ac*ZZp74 BV entry into HepG2 cells was performed using time points 0 h, 4 h, 8 h (not shown), and 24 h. *Ac*-luc BV (Mäkelä et al., 2006) was used as a positive control (not shown). The viruses (200 pfu/cell) were allowed to bind to HepG2 cells for 1 h on ice, after which the cells were fixed either immediately (0 h time point) or after further incubation at 37 °C. The virus particles were first labeled with a monoclonal vp39 antibody and secondary antibody Alexa Fluor® 488 (green), followed by permeablization and a new labeling with anti-vp39, this time followed by a secondary antibody Alexa Fluor® 555 (red). With this labeling protocol, the virus inside the cell should be seen as red and the virus on the cell surface as yellow in the merge image. The images are 3D projections of 3-5 individual slices of z-stacks in the middle of the cell. Scale bar 10 µm. DIC, differential interference contrast.

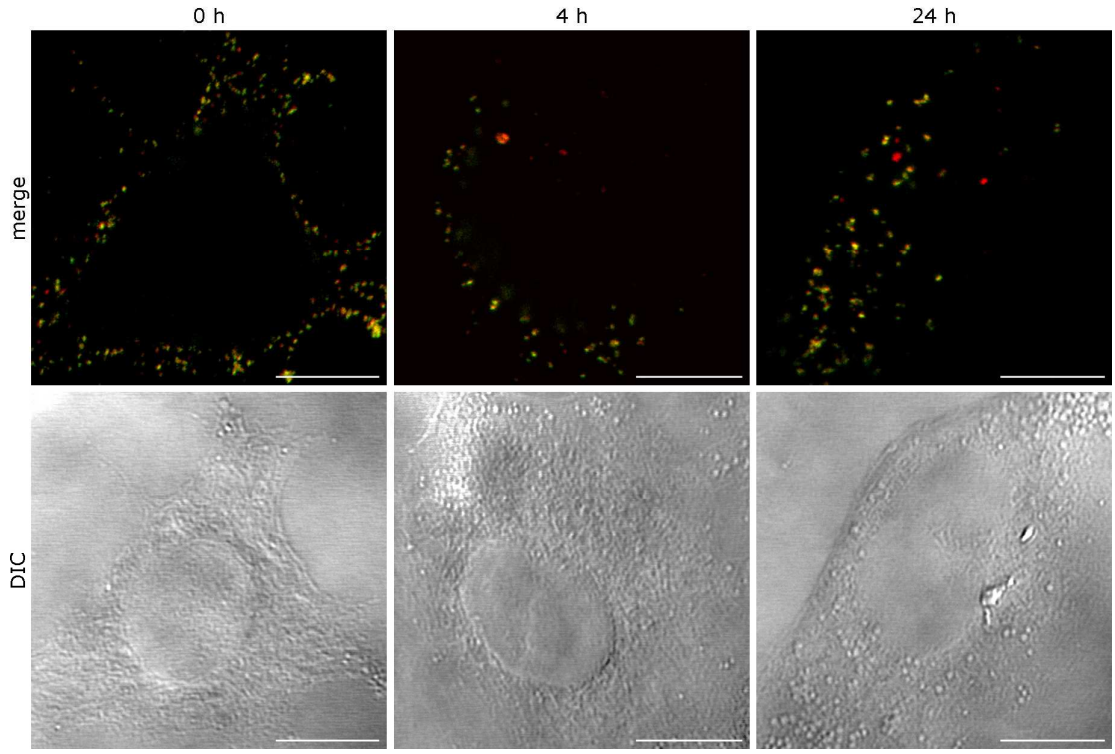

Figure 4.18 *Ac*ZZp74 BV entry into A549 cells. A time series of *Ac*ZZp74 BV entry into A549 cells was performed using time points 0 h, 4 h, 8 h (not shown), and 24 h. *Ac*-luc BV (Mäkelä et al., 2006) was used as a positive control (not shown). The viruses (200 pfu/cell) were allowed to bind to A549 cells for 1 h on ice, after which the cells were fixed either immediately (0 h time point) or after further incubation at 37 °C. The virus particles were first labeled with a monoclonal vp39 antibody and secondary antibody Alexa Fluor® 488 (green), followed by permeablization and a new labeling with anti-vp39, this time followed by a secondary antibody Alexa Fluor® 555 (red). With this labeling protocol, the virus inside the cell should be seen as red and the virus on the cell surface as yellow in the merge image. The images are 3D projections of approximately 3-5 individual slices of z-stacks in the middle of the cell. Scale bar 10 µm. DIC, differential interference contrast.

AcZZp74 BV entry into early endosomes of HepG2 cells. To examine the lack of transduction by *Ac*ZZp74 BV, the entry of BV into early endosomes was studied by performing a time series with 0 h, 2 h, 6 h, and 24 h time points. *Ac*WT was used as a positive control (not shown). Matilainen and collagues (2005) have shown that the antibody against early endosome antigen 1 (EEA-1), which was also used in this study, colocalizes with *Ac*MNPV BV 15 to 45 min p.t. Accordingly, Kukkonen and coworkers (2003) have detected colocalization of EEA-1 and vp39 30 min p.t. In our study, no colocalization could be detected between *Ac*ZZp74 BV and EEA-1 marker in any of the time points (Fig. 4.19; the 6 h and 24 h time points are not shown). This indicates that *Ac*ZZp74 BVs possibly have never reached the early endosomes. From the 2 h time point on, large aggregates were characteristic for the *Ac*ZZp74 BV.

AcZZp74 BV entry into late endosomes and lysosomes of HepG2 cells. To further ensure that *Ac*ZZp74 BVs are not confined in the endosomes and thus in lysosomes, a time series was performed with 0 h, 2 h, 6 h, and 24 h time points, using a late endosome/lysosome marker LAMP-2. *Ac*WT was used as a positive control (not shown). A previous study has shown that a different late endosome marker, CI-MPR, partially colocalizes with *Ac*MNPV first at 45 min p.t. and maximumly at 1.5 h p.t. (Matilainen et al., 2005). Moreover, in an experiment with a late endosome/lysosome marker CD63, *Ac*MNPV has been observed in CD63 positive vesicles 2 to 4 h p.t. by electron microscopy (Matilainen et al., 2005). In our study, *Ac*ZZp74 BV and LAMP-2 did not show significant colocalization; only at 6 h p.t. an exiguous amount of colocalization was detected (Fig. 4.20; data not shown for the 24 h time point). Again, from the 2 h time point on, large aggregates were again characteristic for the *Ac*ZZp74 BV.

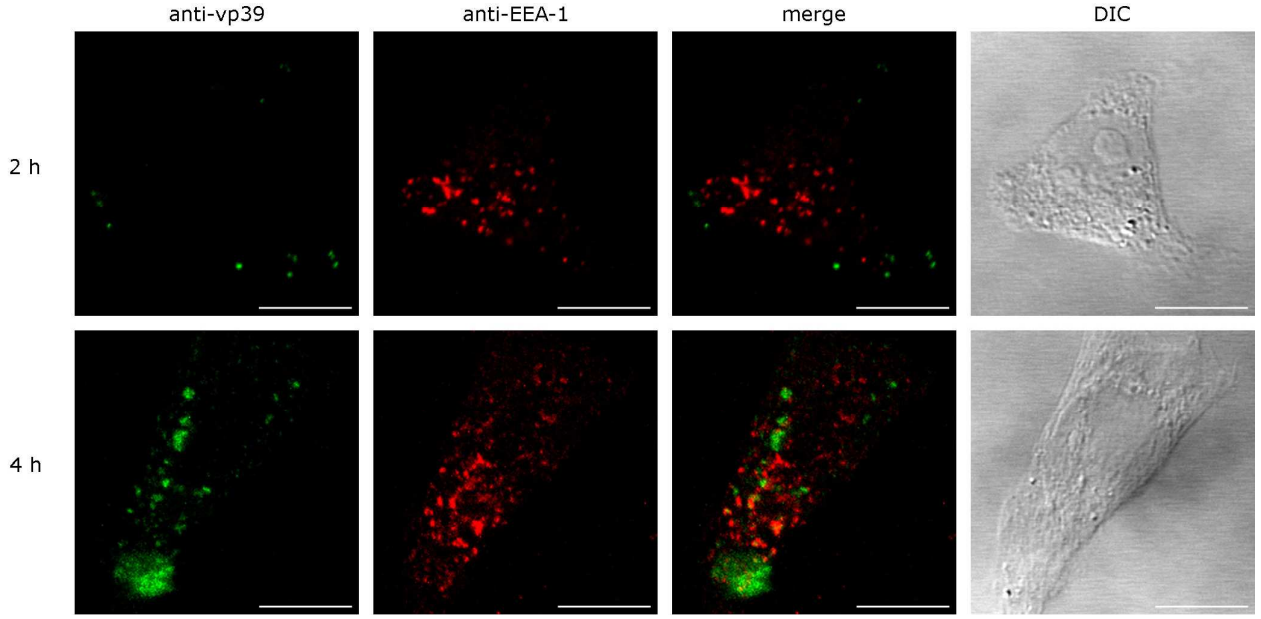

Figure 4.19 *Ac*ZZp74 BV entry into the early endosomes of HepG2 cells. A time series of *Ac*ZZp74 BV entry into the early endosomes of HepG2 cells was performed using time points 0 h, 2 h, 6 h, and 24 h (data not shown for time points 6 h and 24 h). *Ac*WT BV was used as a positive control (not shown). The viruses (1000 pfu/cell) were allowed to bind to HepG2 cells at 4 °C, after which the cells were fixed either immediately (0 h time point) or after further incubation at 37 °C. The virus particles were labeled with a monoclonal anti-vp39 antibody and secondary antibody Alexa Fluor® 488 (green) and early endosomes with monoclonal anti-EEA-1 (early endosome antigen 1) antibody and secondary antibody Alexa Fluor® 555 (red). The images are 3D projections of 3 individual slices of z-stacks in the middle of the cell. Scale bar 10 µm. DIC, differential interference contrast.

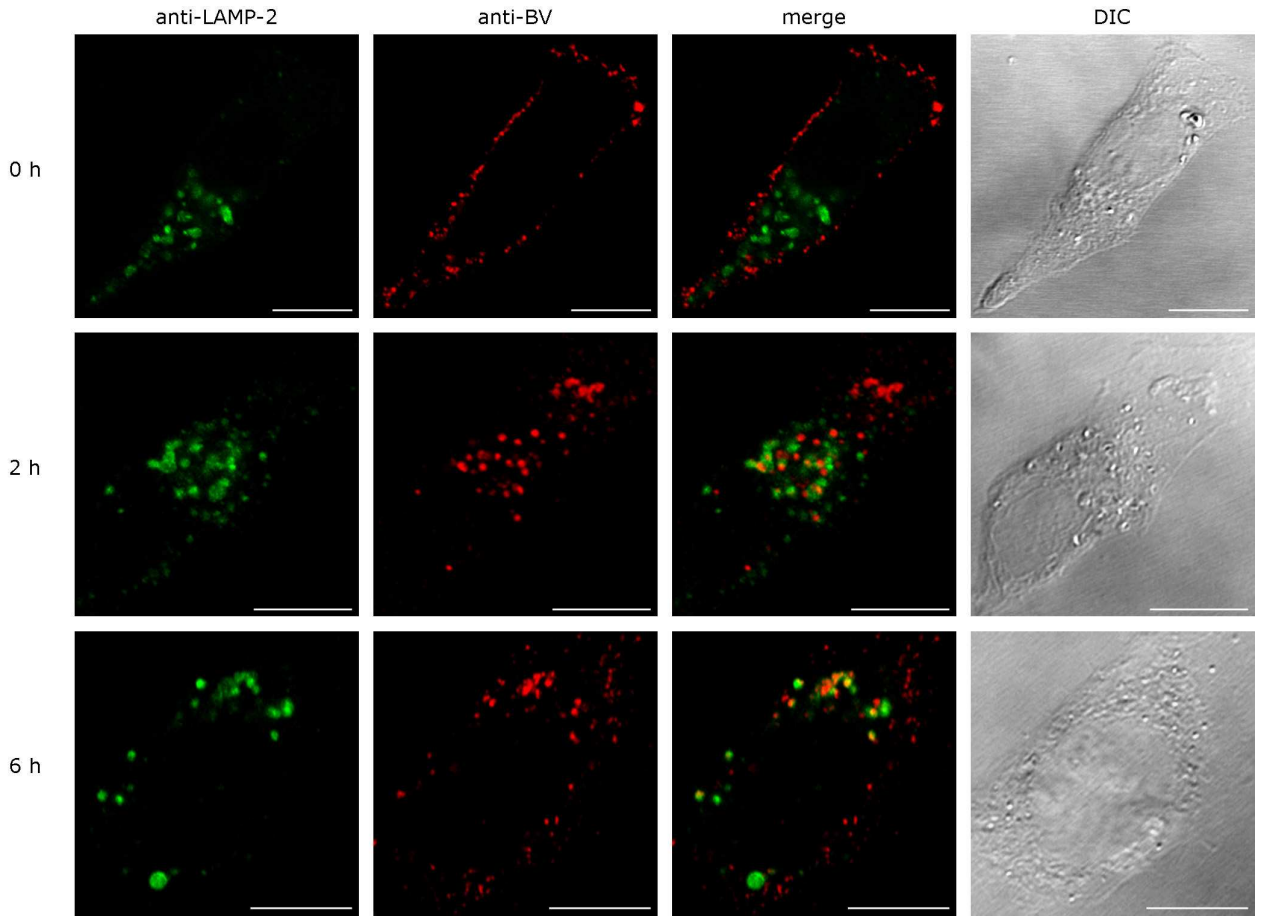

Figure 4.20 *Ac*ZZp74 BV entry into the late endosomes and lysosomes of HepG2 cells. A time series of *Ac*ZZp74 BV entry into the late endosomes and lysosomes of HepG2 cells was performed using time points 0 h, 2 h, 6 h, and 24 h (data not shown for the 24 h time point). *Ac*WT BV was used as a positive control (not shown). The viruses (1000 pfu/cell) were allowed to bind to HepG2 cells at 4 °C, after which the cells were fixed either immediately (0 h time point) or after further incubation at 37 °C. The virus particles were labeled with a polyclonal anti-BV antibody and secondary antibody Alexa Fluor® 555 (red) and late endosomes and lysosomes with monoclonal anti-LAMP-2 (lysosome associated membrane protein 2) antibody and secondary antibody Alexa Fluor® 488 (green). The images are 3D projections of 3 individual slices of z-stacks in the middle of the cell. Scale bar 10 μm. DIC, differential interference contrast.

5 DISCUSSION

The baculovirus, *Ac*MNPV, is a rather new and attractive candidate for *in vivo* applications, especially for gene delivery and vaccination purposes. The more studied and applied phenotype of *Ac*MNPV in this field is the BV, while the potential of the other phenotype, ODV, in the same context, has been studied to a lesser degree. The inherent inability of baculovirus to replicate in mammalian cells and lack of cytotoxic effects render it a safe gene delivery vector candidate (Carbonell and Miller, 1987; Gröner et al., 1984; Hofmann et al., 1995; Kenoutis et al., 2006; Sandig et al., 1996; Shoji et al., 1997; Tjia et al., 1983; Volkman and Goldsmith, 1983). BV has been used to transiently and stably transduce a wide range of mammalian cells *in vitro* and *in vivo* with the aid of mammalian cell-active promoters (for review see Hu, 2006; Kost and Condreay, 2002; Kost et al., 2005). The transduction ability of BV has been further improved by employing baculovirus display technology, which has been applied to a variety of other purposes as well, such as cell- and tissue-specific targeting of BV vectors and antibody production against a displayed immunogen (for review see Mäkelä and Oker-Blom, 2006; Oker-Blom et al., 2003). The transduction potential of ODV in mammalian cells has been scarcely studied. In 1983, Volkman and Goldsmith detected that ODV appears to enter various human and nonhuman vertebrate cell lines, although no nucleocapsids were detected to enter the nucleus. The entry route of ODV in mammalian cells has not been identified to date. In principle, ODV could have several advantages over BV as a gene delivery vector, since ODV is easy to purify, possesses no natural tropism towards mammalian cells, and can simultaneously transport several genetic payloads into target cells. The aim of the present study was to investigate the entry and gene delivery of ODV to human hepatocarcinoma HepG2 cells as well as to apply the baculovirus display technology to include ODV surface display.

5.1 Binding, entry, and transgene delivery of ODV to HepG2 cells

ODV enters its natural target cells in the host midgut by direct membrane fusion (Granados, 1978; Granados and Lawler, 1981; Horton and Burand, 1993; Kawanishi et al., 1972; Tanada et al., 1975), although the precise mechanism has not been completely

characterized. Also, the entry of ODV to mammalian cells has been poorly studied, and the route is currently unknown. In 1983, ODV was observed to enter the cytoplasm of various human and nonhuman vertebrate cell lines, whereas no nucleocapsids were observed in the nuclei of the target cells (Volkman and Goldsmith, 1983). In the ODV-inoculated cells, nucleocapsids were detected by electron microscopy in cytoplasmic vacuoles, or, in some cell lines, in cytoplasmic projections at the cell surface as enveloped virions or in the cytoplasm as unenveloped nucleocapsids (Volkman and Goldsmith, 1983). Moreover, although no virus was detected in the nucleus, aberrant bodies, resembling commonly observed structures in the baculovirus-infected lepidopteran cell nuclei, were detected (Volkman and Goldsmith, 1983). In this study, the binding of ODV to human hepatocarcinoma HepG2 cells was examined. Detected by confocal microscopy, *Ac*WT ODV was observed to efficiently bind to the surface of HepG2 cells (Fig. 4.12). Flow cytometry was used to quantify the binding of ODV to HepG2 cells by using increasing concentrations of ODV. The binding appeared to be concentration-dependent, although a clear saturation point for viral attachment to target cells was not observed (Fig. 4.14). In 1993, Horton and Burand conducted similar binding experiments with *Lymantria dispar* MNPV ODV and its natural target cells and observed maximal ODV binding to occur 4 to 5 h p.i. at 4 °C. This might partially explain the lack of saturation in our study, the binding time being only 1.5 h, during which an equilibrium between the bound and unbound virus might not be achieved. The amounts of ODV particles per cell were similar both in this study and in the experiments conducted by Horton and Burand with insect cells. ODV is known to bind to specific receptors on its target cells (Haas-Stapleton et al., 2004; Horton and Burand, 1993), whereas ODV binding to HepG2 cells might not be receptor-dependent, but could be mediated by non-specific interactions with ubiquitous cell surface components, such as heparan sulfate proteoglycans or phospholipids.

ODV has been observed to enter its natural host cells by direct membrane fusion besides at 27 °C but also at 4 °C, although with decreased efficiency as compared to warmer temperatures (Horton and Burand, 1993). In this study, ODV was detected to enter into the cytoplasm of HepG2 cells (Fig. 4.13). Interestingly, ODV was detected to enter the cells already during viral binding at 4 °C, suggesting that ODV might use direct membrane fusion to gain entry into mammalian cells. Three proteins, p74, pif-1, and, pif-2, are known

to be involved in the binding of ODV to midgut cells (Faulkner et al., 1997; Haas-Stapleton et al., 2004; Kikhno et al., 2002; Kuzio et al., 1989; Ohkawa et al., 2005; Pijlman et al., 2003). The components involved in the fusion process of ODV envelope with cell membrane are still unknown. It also remains undetermined whether the same viral and cellular proteins are involved in the entry of ODV to both insect and mammalian cells. ODV might even use some alternative route to enter mammalian cells, if, for example, specific components required in direct membrane fusion were absent from the surface of mammalian cells. Ohkawa and collagues (2005) have contemplated the possibility of integrins being involved in ODV entry into insect cells (see section 1.2.4.1). Integrins are reasonable candidates for the cellular receptors of ODV binding, although they are very scarcely studied in lepidopteran larvae.

Volkman and Goldsmith (1983) have demonstrated the uptake of ODV to several vertebrate cell lines, although no nucleocapsids could be detected in the nuclei of the ODV-inoculated cells. Accordingly, in the present study, no transduction, i.e. transgene (EGFP) delivery and expression, was observed in the ODV-exposed HepG2 cells (Fig. 4.12 and 4.16). Moreover, preliminary transduction experiments were also conducted with different pH values or liposomes in order to enhance or enable transduction. Horton and Burand (1993) have shown that ODV prefers alkaline conditions for the entry to its target cells, which is logical since alkaline conditions prevail in the larval midgut. However, no transduction occurred even though pH values ranging from 7.4 to 11.0 were applied. Moreover, cationic liposomes are known to enhance viral gene delivery (Fukuhara et al., 2003; Hodgson and Solaiman, 1996; Porter et al., 1998; Swaney et al., 1997), and in addition, they can be used to broaden the range of cells susceptible to transduction or for targeting of the virus vector (Innes et al., 1990; Porter, 2002; Price et al., 2005). Accordingly, preliminary transduction experiments were conducted with several different liposomes, but again transduction was not detected. Together, these studies suggest that ODV is not capable of transducing HepG2 cells, although HepG2 cell line is highly susceptible to BV transduction (Boyce and Bucher, 1996; Hofmann et al., 1995; Matilainen et al., 2005; Tani et al., 2001).

ODVs were detected in the cytoplasm of mammalian cells, but not in the nucleus, both in this study and by Volkman and Goldsmith in 1983. The cause to the absence of ODV-derived nucleocapsids from the nucleus might be due to problems in the release of the nucleocapsids to the cytoplasm or in their nuclear transport. If ODV used direct membrane fusion to gain entry to mammalian cells, the release of nucleocapsids into the cytoplasm would be supposed to be followed by nuclear transport, probably with the aid of actin filaments (Charlton and Volkman, 1993; Goley et al., 2006; Lanier and Volkman, 1998; Roncarati and Knebel-Mörsdorf, 1997), since identical BV-derived nucleocapsids are transported into the nuclei of transduced mammalian cells as evidenced by expression of reporter genes (Boyce and Bucher, 1996; Hofmann et al., 1995; Matilainen et al., 2005; van Loo et al., 2001). Moreover, purified nucleocapsids microinjected into the cytoplasm of HepG2 cells have been shown to be transported into the nucleus, although some remain in the cytoplasm (Salminen et al., 2005). This result also suggests that no structural alterations affecting the nuclear transport of BV-derived nucleocapsids occur in the endosomes (Salminen et al., 2005). However, ODV nucleocapsids might remain very near the cell surface after their release, and thus possibly have problems in the transport process, since, for example, BV nucleocapsids are released from endosomes nearer the nucleus and thus have shorter distance to be transported. Interestingly, ODV has a structural glycoprotein, gp41, residing between its nucleocapsid and envelope (Whitford and Faulkner, 1992a; Whitford and Faulkner, 1992b). The function of this protein has not been determined, but due to its localization it is possible that it could have a role in the entry and/or nuclear transport of the released nucleocapsids following membrane fusion. Thus, gp41 might require a specific binding partner which is absent from mammalian cells. It is also possible that ODV might use some other entry route than direct membrane fusion to enter mammalian cells. Volkman and Goldsmith (1983) detected ODV-inoculated mammalian cells to contain enveloped virions in the cytoplasmic projections at the cell surface as well as partially enveloped nucleocapsids entering the cells and in cytoplasmic vacuoles. Thus, ODV entry might occur without loss of the ODV envelope, which could disturb nuclear transport of the nucleocapsids. ODV has also been observed in phagocytic vacuoles (Volkman and Goldsmith, 1983), and hence might have difficulties to be released from the vacuoles. However, Volkman and Goldsmith (1983) also observed unenveloped nucleocapsids in the cytoplasm of the ODV-inoculated cells, and in the present study,

ODV was observed to enter HepG2 cells at 4 °C, a temperature at which phagocytosis and other endocytosis is strongly reduced (for review see Silverstein et al., 1977), suggesting that direct membrane fusion might occur. It is also possible that ODV could enter mammalian cells via several routes, direct membrane fusion, for example, being the most prevalent. In conclusion, the entry route of ODV to mammalian cells remains to be determined as well as the block to ODV entry.

5.2 Expression of the ZZp74 fusion protein in cellular and viral membranes

Baculovirus display technology has been applied to target or enhance BV-mediated transduction (see section 1.4.1; for review see Mäkelä and Oker-Blom, 2006), one approach of targeting being the display of IgG-binding Z domains, derived from *Staphylococcus aureus* protein A, on the surface of BV (Mottershead et al., 2000; Ojala et al., 2004; Ojala et al., 2001). In principle, the Z domain should enable rather universal targeting, since it can mediate binding to any cell surface antigen for which an antibody exists. To enable viral targeting, Mottershead and coworkers (2000) and Ojala and collagues (2001) displayed the Z/ZZ domains on the surface of BV by fusion to the N-terminus of gp64. The viruses were indeed observed to be specific in binding (Mottershead et al., 2000; Ojala et al., 2001), and targeted transduction of baby hamster kidney cells via an IgG antibody against a cell surface antigen, α5β1 integrin, was achieved, although the efficiency was not improved (Ojala et al., 2001). More recently, fusion of the ZZ domains to the transmembrane anchor of VSVg, instead of gp64, was shown to increase the amount and provide uniform distribution of the displayed fusion protein on the viral surface as well as to enhance the binding of the display virus to IgG antibodies, whereas no improvement in transduction efficiency was gained (Ojala et al., 2004).

In the present study, the ZZ display strategy was applied to ODV by fusing the ZZ domains to the N-terminus of the ODV envelope protein p74, the N-terminus of which resides on the surface of ODV (Faulkner et al., 1997; Slack et al., 2001). The display of foreign proteins or peptides on the ODV envelope has not been previously reported, whereas polyhedra surface display has been performed. Accordingly, influenza

hemagglutinin epitope has been inserted into polyhedrin protein and thus displayed on the polyhedron surface (McLinden et al., 1992), and in addition, polyhedral surface display of polyhedrin-GFP fusion protein with (Chang et al., 2003; Seo et al., 2005) or without (Je et al., 2003) *Bacillus thuringiensis* insect toxin has also been reported. Although ODV display as such has not been conducted, foreign genes have been fused to complete or truncated ODV envelope proteins (see also section 1.4.2). The resulting fusion proteins are not considered as displayed proteins if the foreign fusion partner is not exposed on the virion surface. One of the ODV envelope fusion proteins constructed to date was generated from the ODV envelope protein E56, and the resulting E56-β-gal fusion protein was subsequently observed to be incorporated into the viral nucleocapsids instead of ODV envelope (Braunagel et al., 1996). Moreover, GFP has been fused to full-length and N-terminally truncated forms of the ODV envelope protein p74, although the fusions were conducted to the C-terminus of p74 (Slack et al., 2001), which is not exposed on the viral surface. However, the full-length p74-GFP fusion protein was confirmed to be incorporated into polyhedra (Slack et al., 2001). Similarly, GFP and/or β-gal have been fused to the C-termini of the N-terminal sequences of ODV envelope proteins E66 and E25 (Braunagel et al., 2004; Hong et al., 1997), the C-termini of which are suggested not to reside on the viral surface (Slack and Arif, 2007). Moreover, the fusion protein containing 23 N-terminal amino acids from the E66 fused to GFP was observed to be normally incorporated into polyhedra (Hong et al., 1997). In conclusion, there are no reports of ODV envelope fusion proteins, in which the foreign fusion partner would have been exposed on the virion surface. Together, these studies suggest that ODV display is possible, since some of the ODV envelope fusion proteins have localized intracellularly in a normal manner and also been incorporated into polyhedra. The presence of the fusion proteins in polyhedra does not ensure that the fusion proteins are correctly localized on the ODV envelope, but it is highly possible. It is also of utmost importance to retain the original function of the ODV envelope protein to which foreign proteins or peptides are fused.

Slack and collagues (2001) have constructed a p74 fusion protein by fusing GFP to the C-terminus of a second copy of p74. The p74-GFP fusion protein was observed to localize in the intranuclear ring zone of the infected cells (Slack et al., 2001), where the assembly of

ODVs is known to occur (Summers and Volkman, 1976). In this study, the fusion of the ZZ domains to the N-terminus of p74 was also performed to a second copy of p74, and Western blotting was used to confirm the presence of the ZZp74 fusion protein in *Ac*ZZp74 ODV and polyhedra (Fig. 4.6). However, in addition to ODV and polyhedra, the fusion protein appeared to be incorporated in the *Ac*ZZp74 BV as well (Fig. 4.6). This is very interesting, since p74 is an ODV-specific protein and totally absent from BVs (Faulkner et al., 1997; Haas-Stapleton et al., 2004). More recently, the *Ac*ZZp74 BV was shown to be purified by immunoprecipitation with protein A sepharose conjugated with rabbit IgG, which further demonstrates the presence of the ZZp74 fusion protein in the BVs (Mäkelä et al., manuscript). The efficiencies of p74 and VSVg to incorporate ZZ domains on the viral envelope were also compared, VSVg being approximately three to four times more efficient (Mäkelä et al., manuscript). Despite its abnormal localization to BV, the ZZp74 was detected to localize on nuclear membranes in virus-infected *Sf*9 cells similarly to wt p74, although the corresponding labels did not completely colocalize (Fig. 4.9 B). The partial colocalization might result from the ZZp74 residing under the control of the *p10* promoter, which is a very strong promoter, unlike the *p74* promoter. Moreover, the cells were examined at a rather late time point (48 h p.i.), at which time the *p10* promoter is still active while the *p74* promoter is not (Kuzio et al., 1989; Smith et al., 1983b). In addition to detecting that p74-GFP localizes in the intranuclear ring zone of infected cells, Slack and coworkers (2001) also observed the changes in the nuclear localization of p74-GFP occurring over time. Accordingly, p74-GFP was detected to accumulate within vesicle-like structures in the intranuclear ring zone at 24 h p.i. and later (72 h p.i.) in the centre of the nucleus (Slack et al., 2001). The observed p74-GFP localization was similar than previously reported for the fusion of GFP and the N-terminal sequence of ODV envelope prtotein E66 (Hong et al., 1997). More recent studies performed to investigate the temporal localization of ZZp74 in infected *Sf*9 cells show that the protein is located similarly than was observed for the p74-GFP fusion protein (Mäkelä et al., manuscript).

Braunagel and coworkers (1996) have constructed a fusion protein consisting of the ODV envelope protein E56 with its C-terminal portion replaced with β-galactosidase. Interestingly, it was detected that the fusion protein was incorporated into viral nucleocapsids instead of ODV envelope or intranuclear microvesicles (Braunagel et al.,

1996). Thus, it is possible that the ZZp74 fusion protein is also incorporated into viral nucleocapsids, since then, as was detected (Fig. 4.6), it would inevitably be incorporated into both BVs and ODVs. Moreover, as the ZZp74 was not detected on the plasma membrane of the *Ac*ZZp74-infected *Sf*9 cells (Fig. 4.9), it is rather improbable that the fusion protein would be incorporated to the plasma membrane-derived envelope of BV. The localization of the ZZp74 in nucleocapsids would also indicate that the fusion protein is most likely not displayed on the ODV surface, which was also the case with E56-β-gal. Braunagel and collagues (1996) also used electron microscopy to compare the localizations of the E56-β-gal fusion protein and the major capsid protein vp39 in insect cells (Braunagel et al., 1996). Vp39 was observed to associate with elongated empty capsid structures and viral nucleocapsids unlike the E56-β-gal fusion protein. E56-β-gal localized in the densely staining regions and vp39 in the clear areas of the virogenic stroma, whereas wt E56 was not observed in the virogenic stroma at all (Braunagel et al., 1996). These results indicate that the E56-β-gal fusion protein has a different route of incorporation into the nucleocapsids than vp39 (Braunagel et al., 1996). Since both E56 and E56-β-gal were observed to localize similarly in the cytoplasm and on the outer and inner nuclear membranes, it was suggested that the initial nuclear transport of both proteins is membrane-mediated, and that the nuclear localization signal is in the N-terminal portion of E56 present in the fusion protein (Braunagel et al., 1996). Based on the observed E56-β-gal localization, it was also suggested that some transport and/or retention signal responsible for the intranuclear localization of E56 resides in the deleted C-terminal portion (Braunagel et al., 1996). The ZZp74 fusion protein appears to behave similarly to E56-β-gal, since it also first localizes on the nuclear membrane, and then most likely is incorporated into the nucleocapsids. However, since ZZp74 appears to localize into the intranuclear membranes as well, it is possible that ZZp74 and E56-β-gal do not use entirely identical routes for incorporation into nucleocapsids. The N-terminus of p74 is supposed to function in the nuclear import of the protein, and the C-terminus in intranuclear localization and as a membrane insertion sequence (Slack et al., 2001). Rather similar functions were suggested for the corresponding termini of E56 (Braunagel et al., 1996), and hence also wt p74 appears to behave quite similarly than wt E56. A difference between the fusion proteins ZZp74 and E56-β-gal is the terminus the fusion resides at. No deletions were performed to p74 before fusion, but since the three-dimensional structure of p74 is

unknown, the ZZ domains might disturb the normal conformation of p74 virtually in any region of the protein. If the C-terminus of p74 indeed functions in membrane insertion, it operates normally in ZZp74, since ZZp74 was observed to reside on nuclear membrane, whereas the ZZ domains might disturb the intranuclear localization function of the C-terminus.

*Ac*ZZp74 BV appeared to be normally infective in *Sf*9 cells (Fig. 4.9), whereas its transduction ability of HepG2 cells, detected by monitoring expression of the reporter genes, was observed to be completely abolished compared to the *Ac*WT BV (Fig. 4.16). This is most intriguing, since BV is supposed to use a similar endocytic route for entry into both insect and mammalian cells (Boyce and Bucher, 1996; Hofmann et al., 1995; Long et al., 2006; Matilainen et al., 2005; Pieroni et al., 2001; van Loo et al., 2001; Volkman and Goldsmith, 1985; Volkman et al., 1984), and our results suggest that there must be some differencies in the entry processes. Most likely, incorporation of the ZZp74 fusion protein into the *Ac*ZZp74 BV somehow disturbs the uptake of the virus into mammalian cells or inhibits later steps in the transduction process. The BV major envelope glycoprotein gp64 is necessary and sufficient for the low pH-induced fusion of BV envelope with the membrane of an endosome in insect cells (Blissard and Wenz, 1992; Chernomordik et al., 1995; Leikina et al., 1992; Markovic et al., 1998; Monsma and Blissard, 1995), and it has also been suggested to have a similar role in the entry of BV into mammalian cells, since anti-gp64 antibodies that prevent infection of insect cells also inhibit transduction of mammalian cells (Hefferon et al., 1999; Hofmann et al., 1998; van Loo et al., 2001; Volkman and Goldsmith, 1985). It is likely that the function of gp64 is not affected in the present study, since budding and entry of BV in insect cells appeared to be normal. Prior to virus entry, gp64 also functions as a viral host cell receptor-binding protein (Hefferon et al., 1999). This function also appeared to be unaffected in the *Ac*ZZp74 BV, since it was observed to effectively bind to both HepG2 and A549 cells (Fig. 4.17 and 4.18, respectively). Moreover, the *Ac*ZZp74 BV was detected to enter the cytoplasm of both HepG2 and A549 cells 4 h to 24 h p.t. in small amounts with most of the virus remaining at the cell surface (Fig. 4.17 and 4.18). Further indication of the abnormal behaviour of the virus was gained as the virus was detected to form crescent-shaped aggregates, especially around HepG2 cells (Fig. 4.17, 4.19, and 4.20).

The block to *Ac*ZZp74 BV transduction did not appear to be due to the BVs confined in early or late endosomes or lysosomes (Fig. 4.19 and 4.20). Although the chosen time points were not ideal, the absence of colocalization between *Ac*ZZp74 BV and an early endosome marker (Fig. 4.19) suggest that the BVs probably have not even entered the endosomes. Colocalization of BV with a late endosome/lysosome marker LAMP-2 was detected only minimally 6 h p.t. (Fig. 4.20). However, the colocalization might as well result from the virus and the late endosomes or lysosomes merely residing near each other, even if the virus was not in the endosomes or lysosomes. Halftime for the endosomal escape of *Ac*MNPV BV nucleocapsid in mammalian has been determined to be 50 min in LLC-Pk1 cells (van Loo et al., 2001), and the release has been suggested to occur during the early stages of endocytic pathway (Salminen et al., 2005), although not before 30 min p.t. (Kukkonen et al., 2003). Moreover, a late endosome marker CI-MPR has been observed to colocalize with *Ac*MNPV first at 45 min p.t. and maximumly at 1.5 h p.t., and in addition, electron microscopic studies have shown *Ac*MNPV in vesicles containing a late endosome/lysosome marker CD63 at 2 to 4 h p.t. (Matilainen et al., 2005). Together, these results indicate that in this study, in the 2 h time point, the *Ac*ZZp74 BV virus was already released from the endosomes, if it had entered them in the first place, or otherwise colocalization should have been observed between the virus and LAMP-2. It has also been suggested that some virus is transported to lysosomes for degradation (Matilainen et al., 2005), but that probably is not the case here, since colocalization was not detected already earlier. If the ZZp74 fusion protein was assembled into the nucleocapsids as discussed above, it would be logical to assume that the fusion protein affects BV transduction during or after the endosomal release of the nucleocapsids into the cytoplasm. However, it appears that already the entry of *Ac*ZZp74 BV to mammalian cells is diminished, and thus it is likely that the ZZp74 fusion protein causes some disturbances already during viral uptake.

BV is known to exiguously enter insect cells at 4 °C by direct fusion of its envelope with the plasma membrane, although it is not known whether this entry process is followed by infection (Volkman et al., 1986). Moreover, baculovirus has been suggested to also use macropinocytosis (Matilainen et al., 2005) for the entry into mammalian cells, whereas clathrin-mediated endocytosis has been suggested to be the main route of entry into both insect and mammalian cells (Long et al., 2006; Matilainen et al., 2005). Thus, if BV

normally uses several pathways to enter mammalian cells, *Ac*ZZp74 BV might be restricted to use only some minor entry pathway(s), if the major endocytosis route was inhibited. However, this would be supposed to lead to viral transduction and transgene expression, unless the fusion protein also affects intracellular trafficking and/or nuclear transport of the nucleocapsids.

Detected by confocal microscopy and Western blotting, the production of polyhedra by *Ac*ZZp74 was detected to be minimal or totally absent (Fig. 4.9; Mäkelä et al., manuscript). The virus might have attained genomic deletions during passaging, which has been observed to occur rapidly during serial passaging of the virus in insect cells (Pijlman et al., 2001). However, another option for the lack of polyhedra is the presence of a few polyhedra (FP) phenotype, which is characterized by significantly reduced polyhedrin expression and diminution of its nuclear localization (Harrison et al., 1996). FP phenotype viruses produce fewer polyhedra containing fewer ODV compared to wt virus, and furthermore, the FP ODVs predominantly contain only single enveloped nucleocapsids (Fraser and Hink, 1982; Hink and Vail, 1973; MacKinnon et al., 1974; Potter et al., 1976; Ramoska and Hink, 1974). Moreover, the extent of the FP phenotype characteristics can vary (Beames and Summers, 1988; Pedrini et al., 2005; Slavicek et al., 1998). Thus, if the *polyhedrin* gene was present in the *Ac*ZZp74 genome, the lack of polyhedra could be due to FP phenotype. The polyhedra production of the *Ac*WT virus appeared also to be rather low (Fig. 4.7), lower than with genuine wt *Ac*MNPV, and the *Ac*WT ODVs were observed to contain predominantly only single nucleocapsids (Fig. 4.10), which imply that *Ac*WT might as well represent the FP phenotype. While wt viruses produce several dozens of polyhedra per infected cell, FP mutant viruses typically produce fewer than 10 polyhedra per cell (Potter et al., 1976), as was also the case with *Ac*WT (Fig. 4.7). The FP phenotype viruses have also higher BV titer than wt viruses (Fraser and Hink, 1982; Harrison and Summers, 1995b; Potter et al., 1978). The titers of *Ac*WT and *Ac*ZZp74 were observed to be at a normal level. The FP phenotype can result from mutations occurring in several regions of the *Ac*MNPV genome (see also section 1.2.2; Beames and Summers, 1988; Beames and Summers, 1990; Fraser et al., 1985; Fraser et al., 1983; Kumar and Miller, 1987), and is known to be rapidly produced by serial passaging of baculoviruses in cell culture (Fraser and Hink, 1982; Kumar and Miller, 1987; Lua et al., 2002; MacKinnon et

al., 1974; Potter et al., 1976).. Transposable elements of the host integrating into the virus genome are one known cause for the FP phenotype (Beames and Summers, 1988; Beames and Summers, 1990; Fraser et al., 1985; Fraser et al., 1983), and relevant to this study, this is also known to occur during serial passaging of viruses in *Spodoptera frugiperda* cells (Beames and Summers, 1988; Beames and Summers, 1990). Thus, the FP phenotype could have emerged during serial passaging of the *Ac*WT and/or *Ac*ZZp74 in *Sf*9 cells. However, the presence of several of the characteristics of the FP phenotype were not investigated in the viruses, and further experiments might have revealed evidence for or against the FP phenotype hypothesis.

In this study, ODV was shown to bind to human hepatocarcinoma HepG2 cells in a concentration dependent-manner. Moreover, ODV entry into the cytoplasm of the cells was demonstrated to occur at 4 °C and in warmer temperatures, although ODV was not detected to enter the nuclei of the cells, and consequently no transgene expression was observed. ODV enters insect cells by direct membrane fusion, and our results suggest that ODV might use direct membrane fusion also to enter mammalian cells. The baculovirus surface display strategy was also applied to ODV. However, the ZZp74 fusion protein was most likely not incorporated on the ODV envelope but to the nucleocapsids of both BV and ODV. Although we were unable to successfully produce an ODV displaying a heterologous targeting peptide, the ODV display technology is promising and should be readily applicable for several purposes, which could also include gene therapy, providing that the block to ODV transduction is first bypassed. An interesting possibility would be to pseudotype ODV with (membrane) proteins from other viruses, such as VSVg, which has proven to supplement BV with several beneficial characteristics, including wider host range and increased transduction efficiency. Interestingly, the ZZp74 fusion protein-containing BV was observed to be infective in insect cells but had lost its ability to transduce mammalian cells. To date, BV has been suggested to use a similar endocytic route for the entry into both insect and mammalian cells, whereas our results suggest that there are some differencies in the entry processes. The intriguing characteristics of the ZZp74-containing BV could provide a feasible tool for future studies of BV entry mechanism into mammalian cells.

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APPENDICES

Appendix I: Primers

The sequences of the primers used in PCR reactions and sequencing. All the primers are presented in 5' → 3' direction and the restriction enzyme sites are notified in parentheses and underlined in the sequences.

polyhedrin primers (Oligomer, Helsinki, Finland):
*Polh*Forw (*Eco*RI): AAAAGAATTCATGCCGGATTATTCATACCGTC
*Polh*Rev (*Pst*I): AAAACTGCAGTTAATACGCCGGACCAGTGAA

ZZ primers (Oligomer, Helsinki, Finland):
*ZZ*Forw(*Xba*I): ATATCTCTAGAATGGTAGACAACAAATTCAACAAAGAAC
*ZZ*Rev (*Pst*I): AAAAACTGCAGCGCGTCTACTTTCGGCG

p74 primers (Oligomer, Helsinki, Finland):
*p74*Forw(*Pst*I): AAAAACTGCAGATGGCGGTTTTAACAGCCG
*p74*Rev (*Hind*III): AAAAAAAGCTTTTAAAATAACAAATCAATTGTTTTATAATATTCG

ZZp74 primers (Thermo Electron Corporation, Ulm, Germany):
*ZZp74*Forw (*Sma*I): ATATCCCCGGGATGGTAGACAACAAATTCAACAAAGAAC
SV40polyARev (*Sma*I): ATATCCCCGGGGATCCAGACATGATAAGATACATTGATG

Primers for sequencing the inserts under the *polyhedrin* promoter (TAG Copenhagen A/S, Copenhagen, Denmark):
PolhIRD-700: AATGATAACCATCTCGCA
PolhIRD-800: CTACAAATGTGGTATGGCTG

Primers for sequencing the inserts under the *p10* promoter (Oligomer, Helsinki, Finland):
p10IRD-700: CATTTTATTTACAATCACTCGACG
p10IRD-800: GGTATTGTCTCCTTCCGTG

Appendix II: Nucleic acid and amino acid sequences of polyhedrin and ZZp74

Polyhedrin (738 bp; 245 amino acids; 28 591 Da). Start and stop codons are underlined.

```
1          ATGCCGGATTATTCATACCGTCCCACCATCGGGCGTACCTACGTGTACGACAACAAGTAC
1           M  P  D  Y  S  Y  R  P  T  I  G  R  T  Y  V  Y  D  N  K  Y

61         TACAAAAATTTAGGTGCCGTTATCAAGAACGCTAAGCGCAAGAAGCACTTCGCCGAACAT
21          Y  K  N  L  G  A  V  I  K  N  A  K  R  K  K  H  F  A  E  H

121        GAGATCGAAGAGGCTACCCTCGACCCCCTAGACAACTACCTAGTGGCTGAGGATCCTTTC
41          E  I  E  E  A  T  L  D  P  L  D  N  Y  L  V  A  E  D  P  F

181        CTGGGACCCGGCAAGAACCAAAAACTCACTCTCTTCAAGGAAATCCGTAATGTTAAACCC
61          L  G  P  G  K  N  Q  K  L  T  L  F  K  E  I  R  N  V  K  P

241        GACACGATGAAGCTTGTCGTTGGATGGAAAGGAAAAGAGTTCTACAGGGAAACTTGGACC
81          D  T  M  K  L  V  V  G  W  K  G  K  E  F  Y  R  E  T  W  T

301        CGCTTCATGGAAGACAGCTTCCCCATTGTTAACGACCAAGAAGTGATGGATGTTTTCCTT
101         R  F  M  E  D  S  F  P  I  V  N  D  Q  E  V  M  D  V  F  L

361        GTTGTCAACATGCGTCCCACTAGACCCAACCGTTGTTACAAATTCCTGGCCCAACACGCT
121         V  V  N  M  R  P  T  R  P  N  R  C  Y  K  F  L  A  Q  H  A

421        CTGCGTTGCGACCCCGACTATGTACCTCATGACGTGATTAGGATCGTCGAGCCTTCATGG
141         L  R  C  D  P  D  Y  V  P  H  D  V  I  R  I  V  E  P  S  W

481        GTGGGCAGCAACAACGAGTACCGCATCAGCCTGGCTAAGAAGGGCGGCGGCTGCCCAATA
161         V  G  S  N  N  E  Y  R  I  S  L  A  K  K  G  G  G  C  P  I

541        ATGAACCTTCACTCTGAGTACACCAACTCGTTCGAACAGTTCATCGATCGTGTCATCTGG
181         M  N  L  H  S  E  Y  T  N  S  F  E  Q  F  I  D  R  V  I  W

601        GAGAACTTCTACAAGCCCATCGTTTACATCGGTACCGACTCTGCTGAAGAGGAGGAAATT
201         E  N  F  Y  K  P  I  V  Y  I  G  T  D  S  A  E  E  E  E  I

661        CTCCTTGAAGTTTCCCTGGTGTTCAAAGTAAAGGAGTTTGCACCAGACGCACCTCTGTTC
221         L  L  E  V  S  L  V  F  K  V  K  E  F  A  P  D  A  P  L  F

721        ACTGGTCCGGCGTATTAA
241         T  G  P  A  Y  *
```

ZZp74 (2 304 bp; 767 amino acids; 87 670 Da). Start and stop codons are underlined (also the start codon of p74 is illustrated).

```
1          ATGGTAGACAACAAATTCAACAAAGAACAACAAAACGCGTTCTATGAGATCTTACATTTA
1           M  V  D  N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L  H  L

61         CCTAACTTAAACGAAGAACAACGAAACGCCTTCATCCAAAGTTTAAAAGATGACCCAAGC
21          P  N  L  N  E  E  Q  R  N  A  F  I  Q  S  L  K  D  D  P  S
```

```
121       CAAAGCGCTAACCTTTTAGCAGAAGCTAAAAAGCTAAATGATGCTCAGGCGCCGAAAGTA
41         Q  S  A  N  L  L  A  E  A  K  K  L  N  D  A  Q  A  P  K  V

181       GACAACAAATTCAACAAAGAACAACAAAACGCGTTCTATGAGATCTTACATTTACCTAAC
61         D  N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N

241       TTAAACGAAGAACAACGAAACGCCTTCATCCAAAGTTTAAAAGATGACCCAAGCCAAAGC
81         L  N  E  E  Q  R  N  A  F  I  Q  S  L  K  D  D  P  S  Q  S

301       GCTAACCTTTTAGCAGAAGCTAAAAAGCTAAATGATGCTCAGGCGCCGAAAGTAGACGCG
101        A  N  L  L  A  E  A  K  K  L  N  D  A  Q  A  P  K  V  D  A

361       CTGCAGATGGCGGTTTTAACAGCCGTCGATTTAACTAATGCCAGTAGGTATGCCATACAT
121        L  Q  M  A  V  L  T  A  V  D  L  T  N  A  S  R  Y  A  I  H

421       ATGCATCGTCTCGAGTTTATTTCGAGATGGCGCACCAGGTTTCCGCATATACTAATCGAC
141        M  H  R  L  E  F  I  S  R  W  R  T  R  F  P  H  I  L  I  D

481       TACACGCTGCGTCCCGCGTCAAGTGACGACGATTATTATGTGCCGCCGAAATTGGCCGAC
161        Y  T  L  R  P  A  S  S  D  D  D  Y  Y  V  P  P  K  L  A  D

541       AAAGCGCTGGCCGTCAAACTGGCTTTCAGCAAGCGCGGGTGTGTAAGCATGAGCTGCTAT
181        K  A  L  A  V  K  L  A  F  S  K  R  G  C  V  S  M  S  C  Y

601       CCGTTTCACGAAACCGGCGTCGTGTCCAACACGACGCCGTTCATGTACATGCAGACCTCC
201        P  F  H  E  T  G  V  V  S  N  T  T  P  F  M  Y  M  Q  T  S

661       GAGACTAGCGTGGGTTACGCGCAGCCCGCGTGCTACCACCTGGACAGGGCGGCGGCCATG
221        E  T  S  V  G  Y  A  Q  P  A  C  Y  H  L  D  R  A  A  A  M

721       CGTGAAGGCGCCGAAACCCAAGTTCAGTCTGCCGAGTTTAGATACACGCTCGACAACAAA
241        R  E  G  A  E  T  Q  V  Q  S  A  E  F  R  Y  T  L  D  N  K

781       TGCATCTTAGTGGACTCGCTGTCCAAAATGTACTTTAACAGCCCATACTTGCGCACCGAA
261        C  I  L  V  D  S  L  S  K  M  Y  F  N  S  P  Y  L  R  T  E

841       GAACACACGATCATGGGCGTGGACGATGTGCCGGCTTTTAACGTTCGGCCCGATCCCGAC
281        E  H  T  I  M  G  V  D  D  V  P  A  F  N  V  R  P  D  P  D

901       CCCTTGTTTCCCGAACGCTTTAAAGGCGAATTCAACGAAGCCTACTGTCGACGTTTCGGC
301        P  L  F  P  E  R  F  K  G  E  F  N  E  A  Y  C  R  R  F  G

961       AGAGAGCTATTCAACGGCGGTTGTTCGTTTAGATGGTGGGAATCGCTCATCGGATTCGTG
321        R  E  L  F  N  G  G  C  S  F  R  W  W  E  S  L  I  G  F  V

1021      CTGGGCGACACGATCTTTGTCACTTTCAAAATGCTGGCCAACAACATTTTTAGCGAACTG
341        L  G  D  T  I  F  V  T  F  K  M  L  A  N  N  I  F  S  E  L

1081      CGCGATTTTGATTACAAAGCGCCTTCCAGCATACTACCGCCACGACCCAACGTGGACTCC
361        R  D  F  D  Y  K  A  P  S  S  I  L  P  P  R  P  N  V  D  S

1141      AACGCAATACTAGCGCAATGGCGCAGCGTGCGCGATAACGCCACAGACTTGGAATTTGAA
381        N  A  I  L  A  Q  W  R  S  V  R  D  N  A  T  D  L  E  F  E

1201      AAACTTTTTAACAAAAATCCAACACTAAACGATTTGGGCATGATCGTCAACGGGTCGCCC
401        K  L  F  N  K  N  P  T  L  N  D  L  G  M  I  V  N  G  S  P

1261      GTCCAGATCACGTACACGGCCGAAACCGGCTTTACTAAAACTCCGATCGCATACAATTAC
421        V  Q  I  T  Y  T  A  E  T  G  F  T  K  T  P  I  A  Y  N  Y
```

```
1321      CGCGGTAACGAACGTGCACGTGTCGAACATTTCGAGGCGCTCGACCGTTCAATCAGCGAC
441        R  G  N  E  R  A  R  V  E  H  F  E  A  L  D  R  S  I  S  D

1381      CAAGACTTGGAGTCAATTATAACATCGTTTTTGGAAGATTACGCGCTCGTTTTCGGTATT
461        Q  D  L  E  S  I  I  T  S  F  L  E  D  Y  A  L  V  F  G  I

1441      GCTACGGACATTGGCTTTGATATGCTAATGTCCGGGTTTAAAAGCATGTTGAAAAAAATT
481        A  T  D  I  G  F  D  M  L  M  S  G  F  K  S  M  L  K  K  I

1501      AACACTTCTCTCATTCCGGCCATGAAACACATGTTGCTTAGCACGACTCGGCGCGTGACC
501        N  T  S  L  I  P  A  M  K  H  M  L  L  S  T  T  R  R  V  T

1561      GTGCGCATGTTGGGAGAGACTTACAAAGCCGCGTTAGTGCACTCGCTCAACGTGATCGCC
521        V  R  M  L  G  E  T  Y  K  A  A  L  V  H  S  L  N  V  I  A

1621      ATCAAAACGCTGACCGTGACTGCCAAAGCGTTAACTCGAATCGCTATCCAAGCCAGCTCC
541        I  K  T  L  T  V  T  A  K  A  L  T  R  I  A  I  Q  A  S  S

1681      ATTGTCGGCATCGTGCTCATTCTATTGACGCTGGCAGATTTGGTTTTGGCGCTATGGGAC
561        I  V  G  I  V  L  I  L  L  T  L  A  D  L  V  L  A  L  W  D

1741      CCGTTCGGTTACAACAACATGTTTCCGCGCGAGTTTCCCGACGACATGTCGCGCACGTTC
581        P  F  G  Y  N  N  M  F  P  R  E  F  P  D  D  M  S  R  T  F

1801      CTGACTGCGTACTTTGAGAGTTTCGACAACACCACGTCCAGAGAAATCATAGAGTTTATG
601        L  T  A  Y  F  E  S  F  D  N  T  T  S  R  E  I  I  E  F  M

1861      CCCGAGTTCTTTTCGGAAATGGTCGAAACGGACGATGACGCCACGTTTGAATCTCTATTT
621        P  E  F  F  S  E  M  V  E  T  D  D  D  A  T  F  E  S  L  F

1921      CATTTATTAGATTATGTGGCATCTTTAGAAGTTAATTCCGACGGCCAAATGTTAAACTTG
641        H  L  L  D  Y  V  A  S  L  E  V  N  S  D  G  Q  M  L  N  L

1981      GAGGAGGGTGATGAAATTGAGGATTTTGACGAATCTACTTTGGTGGGGCAAGCGTTAGCC
661        E  E  G  D  E  I  E  D  F  D  E  S  T  L  V  G  Q  A  L  A

2041      ACTAGCTCGCTATACACTCGCATGGAGTTTATGCAGTACACGTTTAGGCAAAACACACTA
681        T  S  S  L  Y  T  R  M  E  F  M  Q  Y  T  F  R  Q  N  T  L

2101      TTGTCTATGAACAAAGAAAACAACAATTTTAATCAAATAATACTGGGTTTATTTGCAACA
701        L  S  M  N  K  E  N  N  N  F  N  Q  I  I  L  G  L  F  A  T

2161      AACACAATTGTGGCGTTTACAGCATTTGTTATACACACAGAACTCATATTTTTTATATTT
721        N  T  I  V  A  F  T  A  F  V  I  H  T  E  L  I  F  F  I  F

2221      TTCGTAATCTTCCTAATGATCACATTTTATTACATAATCAAAGAATCGTACGAATATTAT
741        F  V  I  F  L  M  I  T  F  Y  Y  I  I  K  E  S  Y  E  Y  Y

2281      AAAACAATTGATTTGTTATTTTAA
761        K  T  I  D  L  L  F  *
```

Appendix III: Primary and secondary antibodies

The primary and secondary antibodies used in immunolabeling are listed below. The antibodies were diluted as indicated here unless otherwise stated in the text.

Primary antibodies

Rabbit IgG (1 mg/ml); Sigma, Saint Louis, MO; 1:200.

Mouse monoclonal anti-vp39 (p10 C 6 α-capsid)*; reference: Whitt and Manning, 1988; 1:100.

Mouse monoclonal anti-gp64 (B12D5)*; reference: Keddie et al., 1989; 1:100.

Rabbit polyclonal anti-polyhedrin*; reference: Volkman, 1983; 1:100.

Mouse monoclonal anti-p74 (N25-8C)*; reference: Faulkner et al., 1997; 1:50.

Rabbit polyclonal anti-BV**; 1:500.

Rabbit monoclonal anti-EEA-1; BD Transduction Laboratories, Lexington, KY; 1:500.

Mouse monoclonal anti-LAMP-2 (0.1 mg/ml); Southern Biotechnology Associates, Inc., Birmingham, AL; 1:50.

* kindly provided by Dr. Loy Volkman from the University of California, Berkeley, CA
** kindly provided by Dr. Max Summers and Dr. Sharon Braunagel from the University of Texas A&M College Station, TX

Secondary antibodies

Alkaline phosphatase-conjugated goat anti-mouse IgG.

Alkaline phosphatase-conjugated goat anti-rabbit IgG.

Both: 1 mg/ml; Promega, Madison, WI; 1:5 000.

Alexa Fluor® 488 goat anti-mouse IgG conjugate (green).

Alexa Fluor® 633 goat anti-rabbit IgG conjugate (purple).

Alexa Fluor® 555 goat anti-rabbit IgG conjugate (red).

Alexa Fluor® 555 goat anti-mouse IgG conjugate (red).

All: 2 mg/ml; Invitrogen Molecular Probes; 1:200.