Functional analysis of a novel baculovirus envelope fusion protein

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ABBREVIATIONS

General

a.a.	Amino acid
Ac23	Translation product of the AcMNPV ORF 23 (F protein)
Acgp64	AcMNPV envelope glycoprotein
bp	Basepair
BV	Budded virus
CTD	Cytoplasmic tail domain
Da	Dalton
EFP	Envelope fusion protein
Env	Envelope glycoprotein
ER	Endoplasmatic reticulum
F	Group II NPV envelope fusion protein
F_0	Primary translation product of F
F_1	C-terminal cleavage fragment of F
F_2	N-terminal cleavage fragment of F
FCS	Furin cleavage site
FP	Fusion peptide
GV	Granulovirus
H.I.	Hydrophobic index
HA	Influenza virus hemagglutinin
HaF	HaSNPV F protein
HR	Heptad repeat
Ld130	Translation product of the LdMNPV ORF 130 (F protein)
LdF	LdMNPV F protein
m.o.i.	Multiplicity of infection
Mab	Monoclonal antibody
MNPV	Multiple Nucleopolyhedrovirus
NC	Nucleocapsid
NMR	Nuclear Magnetic Resonance
NPV	Nucleopolyhedrovirus
nt	Nucleotide
OB	Occlusion body
ODV	Occlusion derived virus
Op21	Translation product of the OpMNPV ORF 21 (F protein)
ORF	Open reading frame
p.i.	Postinfection
p.t.	Posttransfection
Pab	Polyclonal antibody
PxF	PlxyGV F protein
Se8	Translation product of the SeMNPV ORF 8 (F protein)
SeF	SeMNPV F protein
SNPV	Single Nucleopolyhedrovirus
SP	Signal peptide
$TCID_{50}$	Tissue culture infectious dose of 50 %
Thogp75	Thogoto virus envelope glycoprotein
TM	Transmembrane domain
UTR	Untranslated region

Viruses

AcMNPV	*Autographa californica* MNPV
AdhoNPV	*Adoxophyes honmai* NPV
AdorGV	*Adoxophyes orana* GV
APV	Avian pneumovirus
BmNPV	*Bombyx mori* NPV
BRSV	Bovine respiratory syncytial virus
CcaYoV	*Ceratitis capitata* yoyo virus
CDV	Canine distemper virus
CfMNPV	*Choristoneura fumiferana* MNPV
CpGV	*Cydia pomonella* GV
CrleGV	*Cryptophlebia leucotreta* GV
CuniNPV	*Culex nigripalpus* NPV
DanTomV	*Drosophila ananassae* Tom virus
Dme176V	*Drosophila melanogaster* 17.6 virus
Dme297V	*Drosophila melanogaster* 297 virus
DmeGypV	*Drosophila melanogaster* gypsy virus
DmeIdeV	*Drosophila melanogaster* Idefix virus
DmeTirV	*Drosophila melanogaster* Tirant virus
DmeZamV	*Drosophila melanogaster* ZAM virus
DsuGypV	*Drosophila sobobscura* gypsy virus
DviGypV	*Drosophila virilis* gypsy virus
DviTv1V	*Drosophila virilis* Tv1 virus
EppoNPV	*Epiphyas postvittana* NPV
HaSNPV	*Helicoverpa amigera S*NPV
HIV	Human immunodeficiency virus
HRSV	Human respiratory syncytial virus
HzSNPV	*Helicoverpa zea S*NPV
LdMNPV	*Lymantria dispar* MNPV
LyxyNPV	*Lymantria xylina* NPV
MacoNPV	*Mamestra configurata* NPV
MHV	Mouse hepatitis virus
MV	Measles virus
NDV	Newcastle disease virus
NeleNPV	*Neodiprion lecontei* NPV
OpSNPV	*Orgyia pseudotsugata S*NPV
OpMNPV	*Orgyia pseudotsugata M*NPV
PhopGV	*Phthorimaea operculella* GV
PlxyGV	*Plutella xylostella* GV
PVM	Pneumovirus of mice
RoMNPV	*Rachiplusia ou* MNPV
SeMNPV	*Spodoptera exigua* MNPV
SpltMNPV	*Spodoptera litura* MNPV
SV5	Simian virus 5
TniTedV	*Trichoplusia ni* TED virus
VSV	Vesicular stomatitis virus
XecnGV	*Xestia c-nigrum* GV

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Baculoviridae

The *Baculoviridae* are a family of large, enveloped viruses, with circular double-stranded DNA genomes varying in sizes between 80 and 180 kilobasepairs (kbp). They are exclusively pathogenic to arthropods, predominantly insects in the order *Lepidoptera* (butterflies and moths*).* Some baculoviruses have also been identified in other insect orders such as *Diptera* (flies, mosquitos) and *Hymenoptera* (sawflies), as well as in crustaceans, order *Decapoda* (shrimps). So far baculoviruses have been identified in over 800 different insect species inhabiting fields, forests, rivers and households. Most baculoviruses have limited host ranges and infect only a limited set of closely related insects from a single order, some being even restricted to a single host (Adams and McClintock, 1991; Federici, 1997; Federici and Maddox, 1996; Summers, 1977). Their enormous species diversity proved irresistibly the attractiveness of these viruses to control pest insect species, in particular moths and butterflies. The larvae of these insects often cause severe feeding damage to agriculturally important crops, ornamental plants or to forest trees. Unfortunately, the baculoviruses infecting these larvae are often unable to compete with classical chemicals mainly due to their relatively slow speed of action (Moscardi, 1999). However, insects have a strong ability to develop resistance to chemical insecticides and therefore there is an urgent demand for pest control alternatives. Several biotechnological approaches, e.g. genetic modification of viruses have been used to increase the speed of kill or to reduce the feeding damage (Black *et al*., 1997; van Beek and Hughes, 1998), but none of the recombinant viruses have reached the market yet.

Besides their potential application as biological control agents baculoviruses are widely used as gene expression vectors to produce large quantities of foreign proteins in insects cells for biotechnological or pharmaceutical applications (Luckow, 1991). To date, the baculovirus insect cell expression system is one of best tools available to obtain large amounts of near authentic recombinant proteins derived from a variety of (vertebrate or invertebrate) sources in an eukaryotic host. Most posttranslational modifications (e.g. phosphorylation, acetylation, amidation, etc) occur in insect cells in a same way as in mammalian cell systems. Only the protein glycosylation pathway in insect cells is not necessarily identical to that in vertebrate cells. This problem can be overcome by generating transgenic cell lines expressing genes that are characteristic of the mammalian glycosylation pathway (Jarvis, 2003). A third potential application of baculoviruses is as delivery system for gene therapy in vertebrates (Ghosh *et al*., 2002; Pieroni and La Monica, 2001). Baculoviral genomes can carry up to 38 kb of heterologous DNA (Cheshenko *et al*., 2001) and are able to deliver this in various types of mammalian cells *in vitro* and *in vivo* (Kost and Condreay, 2002). The virus does not

replicate in mammalian cells nor does it cause a cytopathic effect, but it does express foreign genes with levels of expression that are dependent on the strength of the promoter used.

To improve the exploitation of baculoviruses as bioinsecticides, eukaryotic expression vector or as gene delivery system for gene therapy, in-depth studies of their infection cycle and in virus-host cell interaction are required. Baculoviruses are excellent models to study the biology of large enveloped dsDNA viruses, which include also the family *Poxviridae*, *Herpesviridae*, and *Nimaviridae* (Marks *et al.,* 2003; van Regenmortel, 2002). Most of the current fundamental insights about baculoviruses have been gathered by research on the type species *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). Nowadays a lot more baculoviruses are studied because of their unique features and their potential use as biological insecticide. This will ultimately led to new discoveries with respect to baculoviral properties and applications. The research in this thesis focuses on the process of infection of baculoviruses, in particular the steps of virus attachment, entry and budding and the role of viral protein(s) involved there in. Published data about these processes available at the onset of this thesis will be presented in the following sections.

Baculovirus taxonomy and structure

The family *Baculoviridae* can be taxonomically divided into two genera, based upon occlusion body (OB) morphology (Blissard *et al*., 2000). Viruses of the genus *Nucleopolyhedrovirus* (NPV) produce large (0.13-15 μm) proteinaceous polyhedron-shaped OBs, also called polyhedra that contain multiple virions within each polyhedron. Viruses of the genus *Granulovirus* (GV) are characterized by smaller (0.3 x 0.5 μm) ovicylindrical-shaped OBs, called granules that contain only a single virion. Based upon sequences of the *polyhedrin/granulin* (Zanotto *et al*., 1993), *ecdysteroid UDP-glycosyltransferase* (Chen *et al.*, 1997), *late essential factor-2* (Chen *et al.*, 1999) and *DNA polymerase* (Bulach *et al.*, 1999) genes the division is also phylogenetically supported. On the basis of the same analysis a further subdivision of the NPVs into group I and group II NPV has been proposed. To date, complete genome sequences of 22 baculoviruses have become available (Table 1.1). The genome sequence of *Culex nigripalpus* NPV suggests a further extension of the lepidopteran NPV with a dipteran NPV group (Alfonso *et al*., 2001; Herniou *et al*., 2001; Herniou *et al*., 2003). Species parameters are not well defined, because for most baculoviruses detailed comparative data are lacking. Therefore the species demarcations are based on host range and specificity, DNA restriction profiles, DNA sequences from various regions of the genome, and predicted sequence similarities of some proteins (Blissard *et al*., 2000).

The virions of the *Baculoviridae* consist of one or more rod-shaped (30-60 x 250-300 nm) nucleocapsids (NC), with a claw-like structure at the bottom and a ring-like structure at the top, which are enclosed by a lipid bilayer (Blissard, 1996). The enveloped virions derived from OBs are named occlusion derived viruses (ODV). For NPVs those ODVs consists of only a single (S) or multiple (M) NCs and are therefore designated as SNPV or MNPV, respectively. The S and the M characters of NPV are not phylogenetically supported (Herniou *et al*., 2003). Characteristic for baculoviruses is that

they produce a second, non-occluded phenotype during the infection cycle, named the budded virus (BV). While apparently both phenotypes consist of the same NC, they differ in the lipid and protein composition of their envelopes (Braunagel and Summers, 1994) and have distinctive roles in the infection cycle (Fig. 1.1). ODVs are highly infectious only to midgut columnar epithelial cells and are responsible for the horizontal transmission of the virus from insect-to-insect through OBs. BVs are highly infectious to almost all insect cells and are responsible for the transmission of the virus from cell to cell *in vivo* as well as in cell culture (Keddie and Volkman, 1985; Volkman and Summers, 1977).

Table 1.1 Baculoviruses of which complete genome sequences have become available

genus	subgroup	species	Abbreviation	reference[a]
Nucleopolyhedrovirus	ledidopteran group I	*Autographa californica* MNPV	AcMNPV	Ayres *et al*., 1994
		Orgyia pseudotsugata MNPV	OpMNPV	Ahrens *et al*., 1997
		Bombyx mori NPV	BmNPV	Gomi *et al*., 1999
		Epiphyas postvittana NPV	EppoNPV	Hyink *et al.*, 2002
		Choristoneura fumiferana MNPV	CfMNPV	[a]De Jong *et al*., 2002
		Rachiplusia ou MNPV	RoMNPV	Harrison and Bonning, 2003
	lepidopteran group II	*Lymantria dispar* MNPV	LdMNPV	Kuzio *et al*., 1999
		Spodoptera exigua MNPV	SeMNPV	IJkel *et al*, 1999
		*Helicoverpa amigera S*NPV	HaSNPV	[a]Zhang and Jin, 2000
		Spodoptera litura MNPV	SpltMNPV	Pang *et al*., 2001
		*Helicoverpa amigera S*NPV G4	HaSNPV G4	Chen *et al*., 2001
		*Helicoverpa zea S*NPV	HzSNPV	Chen *et al*., 2002
		Mamestra configurata NPV	MacoNPV A	Li *et al*., 2002b
		Mamestra configurata NPV	MacoNPV B	Li *et al*., 2002a
		Adoxophyes honmai NPV	AdhoNPV	Nakai *et al*., 2003
	dipteran	*Culex nigripalpus* NPV	CuniNPV	Afonso *et al*., 2001
Granulovirus		*Xestia c-nigrum* GV	XecnGV	Hayakawa *et al*., 1999
		Plutella xylostella GV	PlxyGV	Hashimoto *et al*., 2000
		Phthorimaea operculella GV	PhopGV	[a]Croizier *et al*., 2002
		Cydia pomonella GV	CpGV	Luque *et al*., 2001
		Adoxophyes orana GV	AdorGV	Wormleaton *et al*., 2003
		Cryptophlebia leucotreta GV	CrleGV	Lange and Jehle, 2003

[a]Sequences have not been published but are available in GenBank

Baculovirus infection cycle

OBs are commonly found on plant surfaces in the environment and are taken up by insect larvae that are feeding on those surfaces (Fig. 1.2). The OBs disintegrate upon exposure to the alkalinity in the midgut of the insects releasing the ODVs, which then pass the peritrophic membrane and bind to the microvilli of the columnar midgut epithelial cells. Here the NCs are released into the cytoplasma by direct membrane fusion (Granados, 1978; Granados and Lawler, 1981; Horton and Burand 1993). The NCs migrate to the nucleus by a cytoskeleton-dependent transport mechanism (Charlton

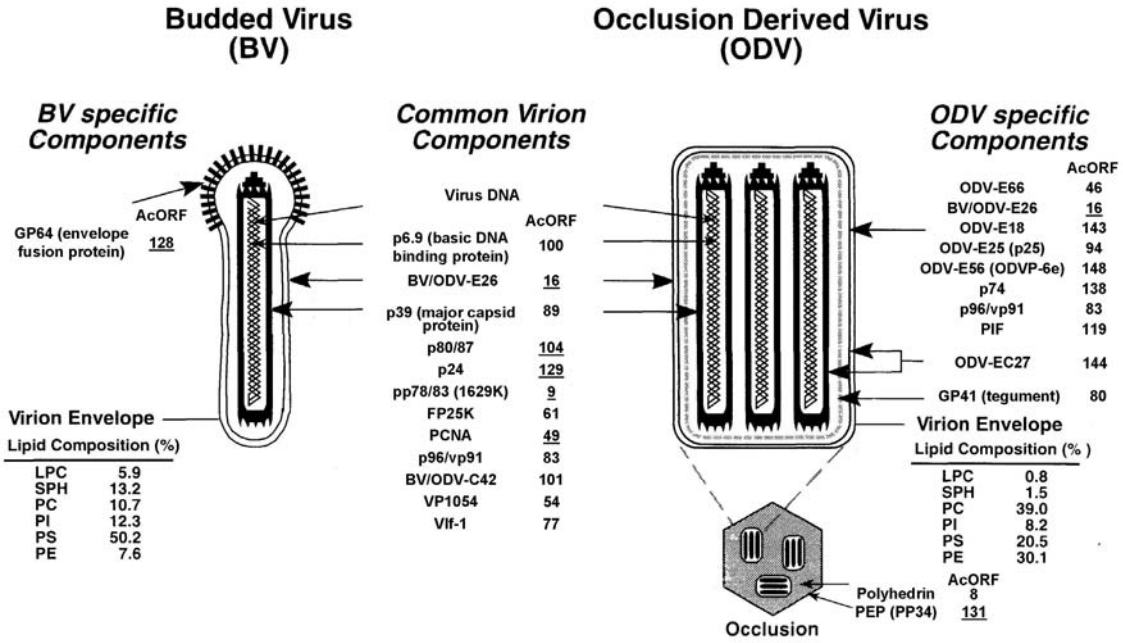

Figure 1.1 Structural composition of the budded virus (BV) en occlusion derived virus (ODV) (adapted from Blissard, 1996 and Braunagel *et al*., 2003). In this figure the ODV structure represent the multiple nucleopolyhedrovirus (MNPV) group. BV and ODV specific proteins are indicated at the left and the right, respectively. Proteins common for both phenotypes are indicated in the center. The corresponding AcMNPV ORF numbers are indicated (Ayres *et al*., 1994), those underlined are not baculovirus conserved genes (Herniou *et al*., 2003). Lipid composition of the BVs and the ODVs are derived from AcMNPV-infected Sf9 cells (Braunagel and Summers, 1994). LPC (lysophoshatidylcholine), SPH (spingomyelin), PC (phosphatidylcholine), PI (phosphatidylinositol), PS (phosphatidylserine), PE (phosphatidylethanolamine).

and Volkman**,** 1993**;** Lanier and Volkman**,** 1998) and gain excess via the nuclear pore to the nucleus (Nishimura *et al.,* 1994). In contrast to other viruses with large spherical capsids, such as Herpes simplex viruses and adenoviruses, the NC disassembles after nuclear transport (Granados and Lawler, 1981; Whittaker and Helenius, 1998). Within the nucleus transcription of viral genes, replication of the viral genome and NC assembly take place.

Baculovirus gene expression is regulated at the transcriptional level and can be separated in three phases, early, late and very late. In the early phase of infection genes are transcribed by the host's RNA polymerase II (Friesen, 1997). In the late phase, which starts after the onset of DNA replication, the transcription is taken over by a viral encoded α-amanitin-resistant RNA polymerase (Guarino *et al*., 1998; Huh and Weaver, 1990). When sufficient structural proteins are produced and viral DNA is replicated newly NCs are assembled and transported through the nuclear pore (Nishimura *et al*., 1994) and along the cytoskeleton (Lanier and Volkman, 1998; Ohkawa *et al*., 2002) to plasma membrane (Hess and Falcon, 1977) where they line up at sites were GP64, the major envelope glycoprotein of the BV, has accumulated (Blissard and Rohrmann, 1989; Oomens *et al*., 1995; Volkman, 1986; Volkman and Goldsmith, 1984; Volkman *et al*., 1984). Here they bud from the cell thereby acquiring a loosely fitting envelope, hence

the name BV phenotype (Tanada *et al*., 1975). The GP64 proteins are indispensable in this budding process (Oomens *et al*., 1995).

The BV phenotype is responsible for the spread of the virus beyond the initially infected midgut epithelium. They can be demonstrated in the hemolymph in the early phase, thus before the onset of DNA replication (Granados and Lawler, 1981). It is believed that a portion of the incoming NCs bypasses the nucleus and is directly transported to the plasma membrane. This seems to be the case for MNPVs, but not for SNPVs (Washburn *et al*., 2003). This biological difference may be related to the fact that primary target cells infected with the S phenotype receive only one NC, whereas those infected with the M phenotype receive multiple NCs, of which only a portion of the incoming NCs are transported to the nucleus producing essential proteins for the budding of NCs which are directly transported to the plasma membrane.

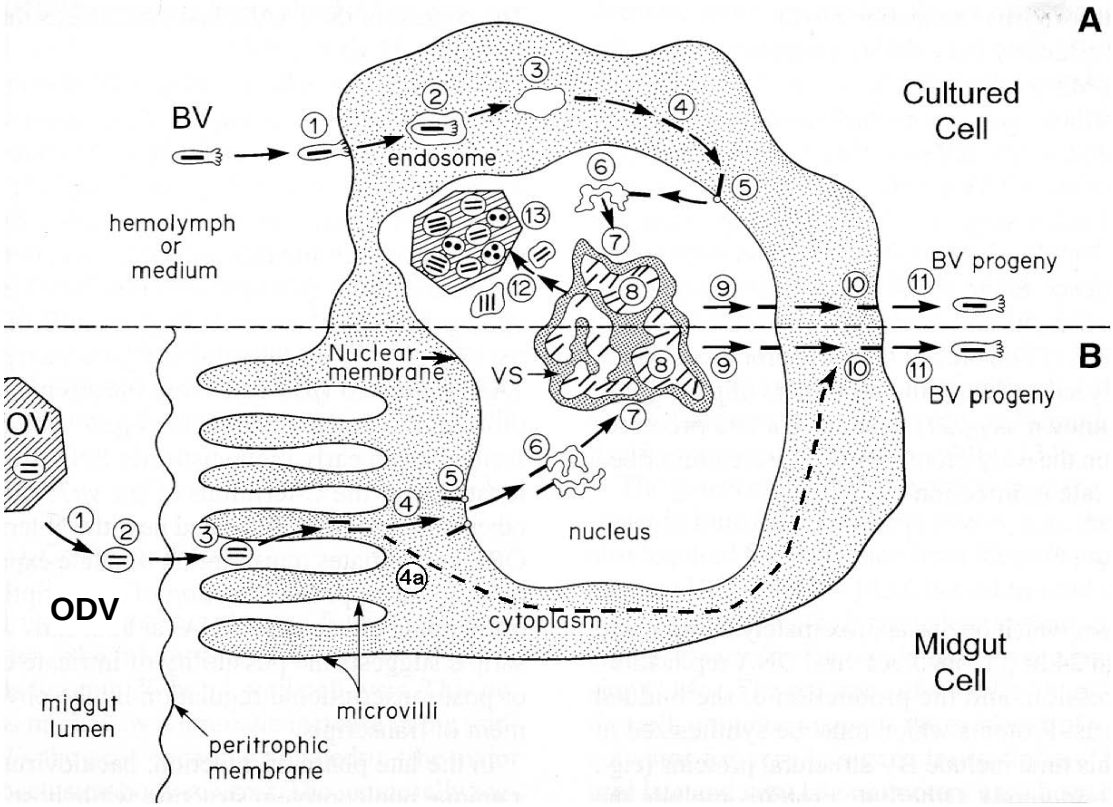

Figure 1.2 The baculovirus infection cycle (adapted from Miller, 1996). A) In the first step of the budded virus (BV) attaches to the cell surface of cultured cells *in vitro* or cells other then midgut epithelial cells *in vivo* (1), and enters by endocytosis (2). The endosomal and viral membrane fuse upon protonation of the endosome, releasing the nucleocapsid (NC) into cytoplasm (3). The NC moves to the nucleus (4), and interacts with the nuclear pore (5). After entering the nucleus the core is released (6), and the viral DNA is transcribed (7), replicated and packaged into newly formed NCs (8) within the virogenetic stroma (VS). During the late phase of infection the newly formed NCs leave the nucleus (9) and travel to the plasma membrane (10) were they bud from the cell (11). During the very late phase, the NCs are enveloped within the nucleus (12), and packaged in a polyhedron matrix to form occlusion bodies (OBs) (13). B) Infection of the midgut epithelial cells starts with the uptake of OBs (1), which dissolve due to the alkaline environment of the midgut lumen, in order to release the occlusion derived viruses (ODVs). The ODVs pass the peritrophic membrane (2) and attach to the microvilli of the midgut epithelial cells (3). The NCs enter the cytoplasma when the cell membrane and the viral membrane fuse (4). The remaining events are similar then that of the BV infection described above, except that little polyhedra are formed in the columnar cells of the midgut epithelium. Further it is believed that a portion of the incoming NCs May bypass the nucleus and migrate directly to the basolaterale membrane to bud from the cell (4a).

During the secondary infection the progeny BVs attach by their GP64 protein to a yet unidentified cellular receptor on cells other than midgut epithelium cells (Hefferon, 1999; Wickham *et al*., 1992). Upon uptake of the BVs in an endosome the acidification of the endosome triggers the GP64-mediated fusion of the viral and endosomal membrane (Blissard and Wenz, 1992; Kingsley *et al*., 1999; Plonsky *et al*., 1999; Volkman and Goldsmith, 1985), whereby the NC is released in the cytoplasm. Although the mode of entry of ODVs and BVs are quite different, once NCs are released from the virions into the cytoplasm, the final steps of the infection appear to be similar. The progeny NCs in the nucleus can either be transported to the cell membrane for the formation of BVs or in the very late phase of infection they can become enveloped in the nucleus *de novo* to form ODV particles, which later on are embedded in polyhedrin to form OBs (Lu and Miller, 1997). Upon death and liquefaction of the insect host, OBs are released into the environment, where they are able to initiate a new round of infection. The very late phase is rarely observed during the primary infection of midgut cells. Most of the infected cells are sloughed off into the gut and replaced by regenerating epithelial cells before the onset of the very late phase could have occurred (Flipsen *et al*., 1995). This allows the larvae to continue feeding while being infected.

Early steps in baculovirus entering

The first step a virus encounters by entering a host cell is the attachment of the virus to a cell surface receptor. Identified receptors include (glyco)proteins, (glyco)lipids, and even recognizable motifs on biomolecules. The specific interaction between the virus and those receptors determines in general host en tissue tropism (Schneider-Schaulies, 2000).

During normal infection of insect larvae the ODVs attach to the microvilli membranes of the midgut epithelium cells. But they are also able to attach to other insect cells *in vivo* as well as *in vitro* when they are liberated from the OBs *in vitro* (Horton and Burand, 1993). This attachment occurs in a saturable and competitive manner, indicating that a specific cellular receptor is involved. Enzymatic treatment of cells and of microvillar membranes has revealed that this receptor might be a protein. For LdEIta insect cells it has even been estimated that each cell contains about 1×10^6 receptors for *Lymantria dispar* (Ld)MNPV ODVs (Horton and Burand, 1993). It is further believed that the ODV-specific envelope protein p74 is involved in the binding to this receptor. Deletion of the *p74* gene results in the normal production of ODVs, but they lacked infectivity when they were fed to larvae. ODVs were still infectious when injected into the hemocoel of larvae (Faulkner *et al*., 1997; Kuzio *et al*., 1989). The *p74* gene is found conserved in all baculoviruses sequenced to date.

After attachment the ODV envelope fuses with the cellular plasma membrane. This fusion process is most efficient under the alkaline conditions of the midgut (Horton and Burand 1993), although the detailed mechanism behind this fusion process is not well understood. The ODV envelope protein p74 may also be involved in the fusion process. In an *in vivo* binding assay it was demonstrated that ODVs, which lack p74 had significantly lower binding and fusion levels (Haas-Stapleton *et al*., 2002). Recently, two other conserved genes, *pif* and *pif-2*, are also required for the infectivity of OBs (Kikhno

et al., 2002; Pijlman *et al*., 2003). Deletions of these genes also abolish the ability of OBs to orally infect insect larvae, but the virions are still infectious when injected into the hemocoel of insect larvae. At least for *pif* it has also been shown that it is a structural protein of the ODV envelope. So it is well possible that p74, PIF and PIF-2 may interact to achieve ODV entry into cells, either by direct contact or by acting in a cascade fashion.

The BV phenotype attaches just as ODVs do to insect cells in a saturable and competitive manner. For AcMNPV it has been reported that insect cells have numbers of receptors ranging from 900 for Hz1075 cells to 13.700 for TnF cells. AcMNPV binds to those receptors with a rather high but equal avidity of about 2 x 10^{10} M^{-1} (Wickham *et al*., 1992). Enzymatic treatment of insect cells suggested that a protein receptor might be involved in the attachment. Furthermore, treatment of cells with tunicamycin as inhibitor of N-glycosylation indicated that the receptor could be a glycoprotein. However, enzymatic trimming of these oligosaccharides had no effect on binding, suggesting that a saccharide motif is not involved (Wang *et al.,* 1997b; Wickham *et al*., 1992). AcMNPV can be concentrated by cation-exchange chromatography suggesting that BVs might also bind by means of electrostatic interactions (Barsoum *et al*., 1994). Enzymatic treatment of mammalian cells with heparinases showed that cellular heparan sulfate is possibly involved in the electrostatic interaction (Duisit *et al.*, 1994). In contrast to mammalian cells electrostatic interactions seem not involved in the attachment of BVs to insect cells (Wang *et al*., 1997b; Westenberg, unpublished data). These findings suggest that baculoviruses use distinct receptors for mammalian and insect cells.

Infectivity of BVs is dependent on the major envelope glycoprotein GP64, which is concentrated on one end of the virion in the form of peplomers, consisting of phosphorylated, acetylated and glycosylated disulfide-linked GP64 homotrimers (Carstens *et al*., 1979; Goldstein and McIntosh, 1980; Oomens *et al*., 1995; Volkman *et al*., 1984). Originally it was believed that GP64 was involved in the attachment, because a monoclonal antibody AcV1 directed against this protein was able to neutralize virus infection (Hohmann and Faulkner, 1983). Later it was shown that BVs probed with the monoclonal antibody AcV1 were still able to bind to insect cells indicating that the antibody inhibits a process other than binding during virus entry (Volkman and Goldsmith, 1985). Direct evidence that GP64 is the receptor binding protein was shown when a soluble form of GP64 was able to compete with AcMNPV BVs for binding sites on insect cells (Hefferon *et al.*, 1999). The glycosylation state of GP64 plays an important role in the attachment. Mutant viruses lacking one or more N-glycans are impaired in their binding capacity to insect cells (Jarvis, 1998)

In contrast to ODVs, BVs are taken up by endocytosis after attachment. This was revealed by the use of lipophilic amines, which prevent the acidification of the endosome and thereby result in a drastic reduction of the infection (Volkman and Goldsmith, 1985). The uptake in the endosome occurs between 10 and 20 min after binding (Hefferon *et al*., 1999). This entry mechanism is not restricted to insect cells, as AcMNPV also enters mammalian cells by endocytosis (Boyce and Bucher, 1996; Hofmann *et al*., 1995; van Loo *et al*., 2001). Blissard and Wenz (1992) showed that GP64, besides being the receptor binding protein, also has fusiogenic properties. Insect cells expressing GP64 on

their surface form syncytia when the pH of medium drops below 5.5. The existence of a fusion domain on GP64 has been studied by mutagenesis studies of two small hydrophobic domains (Monsma and Blissard, 1995). One of the domains is involved in the pH-dependent membrane fusion. Nonconservative substitutions of two leucines in this domain completely abolished the fusion activity of GP64. The second hydrophobic domain possesses characteristics of a leucine zipper. Substitutions of multiple leucines for alanines or prolines revealed that this domain is involved in the oligomerization of the protein. The majority of the latter mutant proteins was found as dimers and monomers rather than trimers and did not show any fusion activity. Reduction of the intersubunit disulfide bridges by dithiothreitol also shows an inhibition of fusion indicating that GP64-mediated fusion involves the formation of stable trimers (Fig. 1.3) (Markovic *et al*., 1998). For baculoviruses it is estimated that at least five to ten GP64 trimers are needed to form the initial fusion pore (Plonsky and Zimmerberg, 1996; Markovic *et al*., 1998). After the formation of the fusion pore the NCs can be released in the cytoplasm, which occurs between 15 and 30 min after cell attachment by the BV (Hefferon *et al*., 1999)

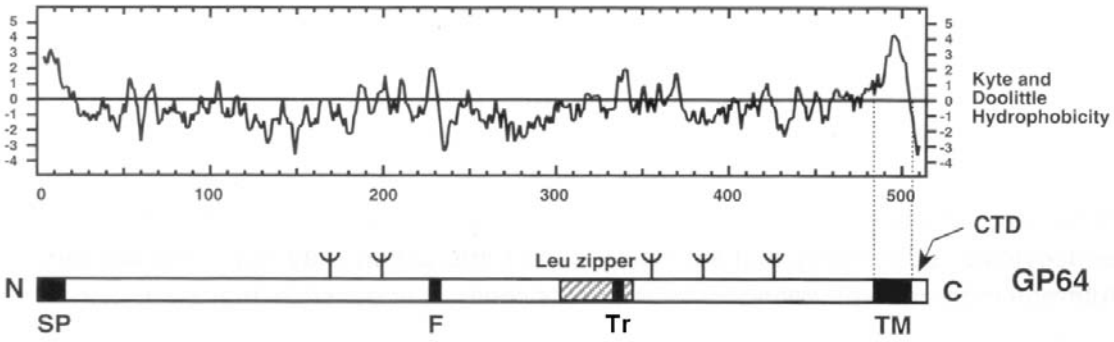

Figure 1.3 Features of AcMNPV GP64 (Adapted from Oomens, 1999b). Various domains and hydrophobicity profile of AcMNPV GP64 are shown: N (N-terminus), C (C-terminus), SP (signal peptide), TM (transmembrane domain), CTD (cytoplasmic tail domain), leu zipper (heptad repeat of leucines, predicting an amphiphatic helix), F (domain involved in fusion), Tr (domain involved in trimerization), ψ (N-linked glycosylation site). The hydrophobicity profile is based on Kyte and Doolittle (1982); The vertical axis indicates Kyte-Doolittle hydrophobicity values (+ hydrophibic; - hydrophilic).

Scope of the thesis

Until the start of this thesis work it was generally believed that baculoviruses employ only one type of cell entry mechanism. Potential differences in the entry mechanism has remained unnoticed for long time, as most of the research on this aspect focussed on AcMNPV and *Orgyia pseudotsugata* (Op)MNPV, which are both group I NPVs and share GP64 homologs as their envelope fusion protein. Competition studies showed that AcMNPV and OpMNPV GP64 proteins use the same insect cell receptor for the attachment (Hefferon *et al.,* 1999). However, it was demonstrated that LdMNPV and AcMNPV are not able to compete in receptor binding (Wickham *et al.*, 1992). This suggested at the time that LdMNPV either did not have a *gp64* gene or that this gene had evolved in such way that it supported the interaction with a different receptor. Electronmicrograph images of LdMNPV BVs showed that they possess the same peplomer structure as AcMNPV on one apical side of the virion (Blissard and Rohrmann, 1990), suggesting that they did contain a GP64-like BV envelope protein.

This however appeared not to be the case when the genome sequence of two group II NPVs, LdMNPV and *Spodoptera exigua* (Se)MNPV, became available (IJkel *et al*., 1999; Kuzio *et al*., 1999). Neither LdMNPV nor SeMNPV contained a *gp64*-like gene. The absence of a *gp64*–like gene suggested that the group II NPVs, or at least LdMNPV and SeMNPV, possess another BV envelope (glyco)protein with a function analogous to GP64.

To obtain more insight in the infection pathway of baculoviruses lacking a *gp64*-like gene, the attachment to and entry in insect cells of the BV phenotype of these viruses and the viral proteins involved in these processes are studied in this thesis using SeMNPV as a model. In **Chapter 2** a novel type of BV envelope fusion protein is identified. This so-called F protein seemed to be rather different to GP64 and has remarkable similarities with several envelope fusion proteins of vertebrate viruses.

The presence of a C-terminal protein fragment in the BVs suggested that the F protein is posttranslational cleaved. Such a cleavage is a general mechanism of several vertebrate viruses to activate their fusion proteins (Klenk and Garter, 1994). Therefore in **Chapter 3** it is investigated whether or not the cleavage of the SeMNPV F protein is necessary for its fusiogenic activity and which (cellular) protease is responsible for the cleavage. Furthermore, it is examined whether or not the cleavage products stays associated with each other after the cleavage.

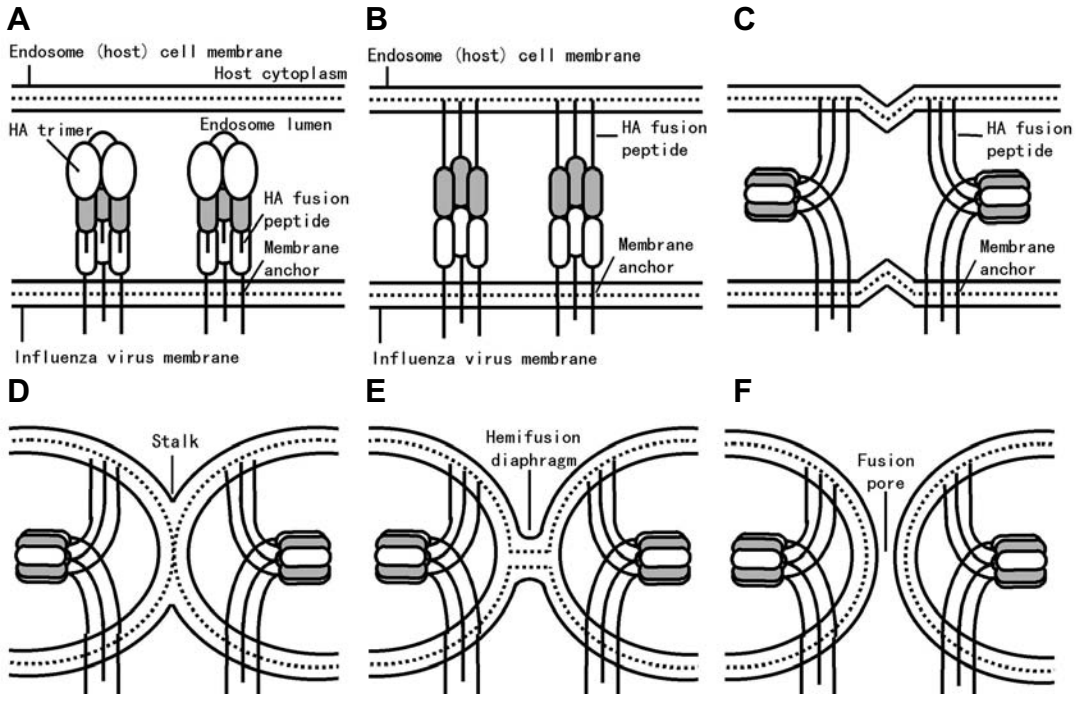

Figure 1.4 Membrane fusion model mediated by influenza haemagglutinin (HA) as proposed by cross *et al.,* 2002a. The influenza virus is internalized into an endosome (A). Acidification of the endosome causes conformational changes in HA that result in fusion peptide insertion into the target endosomal membrane and close proximity of host endosomal and viral membranes (B). Upon further conformational changes of the membrane-anchored domain of HA and clustering of HA trimers leads to bending of the two membranes towards each other (C). The outer lipid bilayer leaflets of the two membranes are pulled together to form a highly bent stalk structure, leading to the hemifusion intermediate structure (D). In the hemifusion intermediate, the distal leaflets are pulled towards each other to form a dimple (E). The hemifusion diaphragm continues to expand as a result of the bending tension of the HA molecules and eventually breaks. This results in the formation of a fusion pore, allowing the mixing of the contents of the virus interior with the cytoplasm.

Besides its function in BVs as a fusion protein in group I NPVs GP64 is also involved in receptor binding and efficient budding. Whether or not the F protein also contains these functions in SeMNPV is investigated in **Chapter 4** and **Chapter 5**. It had been shown before that AcMNPV with a deletion of the *gp64* gene could be pseudotyped with the vesicular stomatitis virus G protein (Mangor *et al*., 2001). In **Chapter 4** it is investigated whether GP64 can be substituted in AcMNPV for the F proteins of SeMNPV and LdMNPV as well as for the F homolog of the granulovirus *Plutella xylostella* (Plxy)GV. The latter virus also lacks a GP64 homolog. The reverse is investigated in **Chapter 5** where the SeMNPV *f* gene is replaced by AcMNPV *gp64*.

The AcMNPV pseudotyping system is in **Chapter 6** further used to investigate the functional significance of a putative domain of the SeMNPV F protein responsible for fusion. Cleavage of viral fusion proteins usually occurs in front of a hydrophobic sequence, the “fusion peptide”. Upon cleavage, such peptides are able to conformationally translocate to the ‘top’ of the protein and interact with the target membrane, which is triggered by receptor binding or pH changes. Upon further conformational changes, the fusion peptide, which stays anchored in the membrane, is brought in close proximity of its transmembrane domain, facilitating apposition of the viral en cellular membrane (Fig. 1.4) (Singh *et al.,* 1999). By mutational analysis of the putative N-terminal fusion peptide of the SeMNPV F protein it is investigated what the role of this domain in the infection is and what the contribution of individual amino acids in the fusion process is.

In **Chapter 7** the experimental data obtained during this thesis research are discussed in the context of recent literature and current insights on baculovirus entry, evolution and taxonomy.

Chapter 2

A NOVEL BACULOVIRUS ENVELOPE FUSION PROTEIN

SUMMARY

The entry mechanism of *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV), a group II NPV, in cultured cells was examined. SeMNPV budded virus (BV) enters by endocytosis as do the BVs of the group I NPVs, *Autographa californica* (Ac)MNPV and *Orgyia pseudotsugata* (Op)MNPV. In group I NPVs, upon infection acidification of the endosome triggers fusion of the viral and endosomal membrane, which is mediated by the BV envelope glycoprotein GP64. However, the SeMNPV genome lacks a homolog of GP64 envelope fusion protein (EFP). A functional analog of the OpMNPV GP64 EFP was identified in SeMNPV ORF8 (Se8; 76 kDa) and appeared to be the major BV envelope protein. Surprisingly, a 60-kDa cleavage product of this protein is present in the BV envelope. A furin-like proprotein convertase cleavage site (R-X-K/R-R) was identified immediately upstream of the N-terminus of the mature Se8 protein and this site was also conserved in the *Lymantria dispar* (Ld)MNPV homolog (Ld130) of Se8. Syncytium formation assays showed that Se8 and Ld130 alone were sufficient to mediate membrane fusion upon acidification of the medium. Furthermore, C-terminal GFP-fusion proteins of Se8 and Ld130 were primarily localized in the plasma membrane of insect cells. This is consistent with their fusogenic activity and supports the conclusion that the Se8 gene product is a functional analog of the GP64 EFP.

This chapter is a slightly modified version of

Wilfred F. J. IJkel, Marcel Westenberg, Rob W. Goldbach, Gary W. Blissard, Just M. Vlak and Douwe Zuidema. 2000. A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. Virology **275:**30-41.

INTRODUCTION

The *Baculoviridae* are a family of large, enveloped double-stranded DNA (80 to 180 kbp) viruses that almost exclusively infect insects (Adams and McClintock, 1991). The family is taxonomically subdivided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV), distinguished by occlusion body (OB) morphology (Volkman *et al*., 1995). The NPVs produce large polyhedron-shaped OBs called polyhedra that contain many virions, whereas the GVs have smaller OBs called granules that normally contain a single virion. The NPVs are designated as single (S) or multiple (M), depending on the potential number of nucleocapsids (NC) packaged in a virion. A more recent subdivision, based on the phylogenetics of polyhedrin/granulin (Zanotto *et al*., 1993), EGT (Chen *et al*., 1997), LEF-2 (Chen *et al*., 1999) and DNA polymerase (Bulach *et al*., 1999) proteins, has been proposed that distinguishes lepidopteran NPVs into two distinct groups, named group I and II. The baculoviruses AcMNPV (Ayres *et al*., 1994), *Bombyx mori* (Bm)NPV (Gomi *et al*., 1999) and OpMNPV (Ahrens *et al*., 1997) are members of group I, whereas SeMNPV (IJkel *et al*., 1999) and LdMNPV (Kuzio *et al*., 1999) belong to group II.

A NPV infection starts with the uptake of polyhedra by the insect larvae. Upon ingestion the OBs dissolve in the alkaline environment of the larval midgut liberating numerous infectious virions, which are termed ‘occlusion derived virus’ (ODV). After direct membrane fusion of ODVs with the midgut columnar epithelial cells (Granados, 1978; Granados and Lawler, 1981; Horton and Burand, 1993), the virions are uncoated and transported to the nucleus, where gene expression, DNA replication and assembly of progeny NCs occur. Newly assembled NCs then migrate from the nucleus towards the plasma membrane. During infection of group I NPVs, a viral encoded major envelope glycoprotein, GP64, is synthesized and transported to the plasma membrane (Blissard and Rohrmann, 1989; Oomens *et al*., 1995; Volkman and Goldsmith, 1984; Volkman *et al*., 1984). When the NCs bud from the basal side of the epithelial cells into the hemocoel, they acquire a loosely adhering plasma membrane envelope containing the GP64 protein. This protein is required for efficient budding (Monsma *et al*., 1996; Oomens and Blissard, 1999). This second virion phenotype is termed ‘budded virus’ (BV) and believed to be essential for systemic infection, mediating movement of the virus from midgut to other tissues and propagating the infection from cell to cell within the infected animal (Flipsen, 1995; Granados and Lawler, 1981; Keddie *et al*., 1989). BVs of AcMNPV and OpMNPV belonging to group I NPVs, infect insect cells, other than midgut epithelial cells, through the endocytotic pathway (Volkman and Goldsmith, 1985). After BV binding to the cell membrane and uptake into an endosome (Hefferon *et al*., 1999) the acidification of the endosome triggers GP64-mediated fusion of the viral and endosomal membrane (Blissard and Wenz, 1992; Kingsley *et al*., 1999; Plonsky *et al*., 1999). Then the nucleocapsids are released into the cytoplasm and are transported to the nucleus, where viral transcription and DNA replication occurs.

Recently, the complete genome of SeMNPV has been sequenced and its genome analyzed (IJkel *et al.*, 1999). Surprisingly, SeMNPV lacks a homolog of the AcMNPV *gp64* BV envelope fusion protein (EFP) gene. A similar situation exists for

LdMNPV (Kuzio *et al*., 1999) and *Helicoverpa armigera* (Ha)SNPV (Chen *et al*., 2001). Since, the GP64 protein of AcMNPV and OpMNPV plays an essential role in spreading the infection in the insect and is required for efficient virus budding, it is questioned whether SeMNPV infects insects by endocytosis and has a functional GP64 analog. In the current study, the mechanism of entry of SeMNPV, a group II NPV, is examined in its target cells. A functional analog of the OpMNPV GP64 EFP is identified in the SeMNPV ORF8 (Se8) protein, and was shown to be the major BV envelope protein. In addition, evidence is provided to demonstrate that the Se8 protein is present in the BV envelope as a cleavage product.

MATERIALS AND METHODS

Cells, Insects and Viruses

The *Spodoptera frugiperda* cell-line IPLB-SF-21 (Vaughn *et al*., 1977) and the *Spodoptera exigua* cell-line Se301 (Hara *et al*., 1995) were cultured in plastic tissue culture flasks (Nalge Nunc International, Naperville, IL) in Grace's insect medium, pH 5.9-6.1, (GibcoBRL, Gaitherburg, MD), supplemented with 10% fetal bovine serum (FBS). A culture of *Spodoptera exigua* insects was maintained according to Smits and Vlak (1988). The SeMNPV isolate (Gelernter and Federici, 1986) was originally obtained from B. A. Federici (Department of Entomology, University of California, Riverside, CA) and was called SeMNPV-US1 (Muñoz *et al*., 1998). The AcMNPV-E2 strain (Smith and Summers, 1978) was originally obtained from M. D. Summers (Texas A&M University, College Station, TX).

Endocytosis assay

Se301 and Sf21 cells were incubated 30 min prior to infection in Grace's insect medium with 10% FBS containing 50 mM ammonium chloride as lysosomotrophic reagent to inhibit acidification of endosomes (Lenard and Miller, 1982), or without the latter compound (control). AcMNPV and SeMNPV (m.o.i of 5 $TCID_{50}$ units/cells) were incubated with cells for 2 h at 4°C in Grace's medium containing 50 mM ammonium chloride to synchronize the binding. Subsequently, the cells were washed gently twice in 2 ml Grace's medium with 10% FBS with or without (control) 50 mM ammonium chloride, and then incubated in the presence or absence (control) of ammonium chloride throughout the infection at 27°C for 72 h. The number of infected cells compared to the control (without ammonium chloride) was determined.

Purification of SeMNPV BV and ODV

S. exigua fourth-instar larvae were infected by contamination of artificial diet with polyhedra (10 x LD_{99}) (Smits and Vlak, 1988). To purify BVs, 5 ml hemolymph was collected 3 days post infection (p.i.) in 0.5 ml 0.1 x TE (TE is 10 mM Tris, pH 7.5, 1.0 mM EDTA) containing 5 mM phenylthiocarbamide to inhibit prophenoloxidase activity. Hemolymph was clarified at 2,000 x *g* for 10 min at 4°C. The supernatant was filtered (0.45 μm filter) and the filtrate overlaid onto a 35 ml 25-56% (w/w) continuous sucrose

gradient in 0.1 x TE. Gradients were centrifuged at 100,000 x *g* for 90 min at 4°C (Beckman SW28, 24,000 rpm). The BV band was collected and dialyzed overnight at 4°C against 0.1 x TE. The virus suspension was concentrated by overlaying onto a 1.5 ml 25-56% discontinuous sucrose gradient. Gradients were centrifuged at 100,000 x *g* for 90 min at 4°C (Beckman SW55, 30,000 rpm). Bands were collected and dialyzed overnight at 4°C against 0.1 x TE.

Polyhedra were purified from larvae using the method of Braunagel and Summers (1994). ODVs were purified from polyhedra using a combination of the methods described by Caballero *et al.* (1992) and Braunagel and Summers (1994). Briefly, ODVs were liberated from polyhedra (40 mg/ml) by incubating at RT for 15 min in 0.1 M Na_2CO_3, 166 mM NaCl, 10 mM EDTA, pH 10.5. Undissolved polyhedra were removed by low-speed centrifugation for 5 min (500 x *g*). The supernatant (5 ml) was layered onto a 35 ml, 25-56% (w/w) continuous sucrose gradient in 10 mM Tris-HCl, pH 7.5 and centrifuged at 100,000 x *g* for 90 min at 4°C (Beckman SW28, 24,000 rpm). The multiple virus bands were collected, washed by dilution in 0.1 x TE, concentrated by centrifugation at 55,000 x *g* for 60 min at 4°C (Beckman SW41, 18,000 rpm) and resuspended in 0.1 x TE. The purity and integrity of BVs and ODVs were checked by electron microscopy.

Fractionating of virions into envelope and nucleocapsids

BVs were incubated in 0.1 x TE containing 1% NP-40, at RT for 30 min with gentle agitation. NCs were sedimented by centrifugation at 150,000 x *g* for 60 min at 4°C (Beckman SW55, 35,000 rpm). The pellet was resuspended in 0.1 x TE. The envelope proteins in the supernatant were acetone-precipitated, concentrated by centrifugation (4,000 x *g*, 30 min) and the pellet dissolved in 0.1 x TE.

SDS-PAGE and protein sequencing

Purified SeMNPV BV proteins were analyzed in a 12 % SDS-PAGE gel, according to Laemmli (1970), and stained with Coomassie Brilliant Blue. Semi-dry blotting was performed onto a polyvinyl difluoride (PVDF) membrane (Bio-Rad) using CAPS buffer (10 mM CAPS, 10 % (v/v) methanol, pH 11). Proteins were visualized on the PVDF membrane using Coomassie Brilliant Blue. The major protein band from SeMNPV BV was N-terminally sequenced (Protein Research Facility Amsterdam, The Netherlands).

Computer-assisted analysis of fusion proteins

The Se8 (IJkel *et al*., 1999) and the Ld130 proteins were analyzed using software of the Predict Protein server (Rost, 1996) for the prediction of transmembrane domains, N-terminal signal sequences, leucine zippers, coiled coil structures and the pI of the cytoplasmic tail domain (CTD). Motif searches were done against the Prosite release 14 database (Bairoch *et al*., 1997; Fabian *et al*., 1997). DNA and protein comparisons with entries in the updated GenBank/EMBL, SWISS-PROT and PIR databases were performed with FASTA and Psi-BLAST programs (Altschul *et al*., 1997; Pearson, 1990). Multiple sequence alignments were performed with the GCG PileUp and Gap computer

programs with gap creation and extension penalty set to 8 and 2, respectively (Devereux *et al*., 1984). Alignment editing was performed with Genedoc Software.

Plasmid constructions

To construct plasmids that allow expression in insect cells upon transfection, a plasmid vector, p166BRNX-AcV5 that contains the OpMNPV *gp64* early promoter plus a multiple cloning site and an AcV5 epitope tag (provided by G. Lin), was used to construct vectors containing Se8 and Ld130 encoding putative viral fusion proteins. Primers containing 5' *Bam*HI and 3' *Eco*RI restriction sites were designed for the directional PCR-cloning of Se8 and Ld130. These ORFs were amplified by high fidelity "Expand" long template PCR (Boehringer Mannheim) using the plasmids pSe *Bgl*II-H (IJkel *et al*., 1999) and genomic LdMNPV DNA (Riegel *et al*., 1994), respectively, as template DNA. The PCR products were ligated into the *Bam*HI and *Eco*RI sites of p166BRNX-AcV5. The plasmids were named p166AcV5-Se8 and p166AcV5-Ld130, respectively, and tested in syncytium formation assays. The complete OpMNPV *gp64* gene used as a positive control in transfection experiments was from plasmid p64-166 and was described previously (Blissard and Wenz, 1992).

To determine the localization of the putative fusion proteins in insect cells, GFP-fusion constructs were made. The red-shifted GFP ORF (Davis and Vierstra, 1996) was amplified by high fidelity "Expand" long template PCR (Boehringer Mannheim) using primers containing 5' *Eco*RI and 3' *Xba*I restriction sites and cloned in frame as an *Eco*RI/*Xba*I fragment into the plasmids p166AcV5-Se8 and p166AcV5-Ld130 to give C-terminal fusions with the putative BV envelope fusion proteins. Plasmid DNA was purified using Jetstar columns according to manufacturer's protocol (ITK Diagnostics) and used for transfections. All constructs were sequenced to confirm the in frame cloning of the AcV5 tag or GFP with the putative fusion ORFs. Sequencing was carried out using an Applied Biosystems automated DNA sequencer (Eurogentec, Belgium).

Syncytium formation assay

Syncytium formation (Sf21-Sf21 fusion or Se301-Se301 fusion) assays were performed by either transfection of 5.0 x 10^5 Sf21 or Se301 cells, with 5 μg of plasmid DNA using Cellfectin (GibcoBRL) or infection with SeMNPV or AcMNPV BVs (m.o.i. of 10 $TCID_{50}$ units/cell). The empty p166BRNX-AcV5 plasmid vector was used as a negative control for the syncytium formation assay. Forty eight h after transfection, cells were washed three times with 2 ml Grace's medium (pH 6.1) without FBS, and afterwards cells were treated for 2 min in 1 ml acidic Grace's medium at pH 5.0. The acidic medium was removed and replaced with 2 ml Grace's (pH 6.1) with 10% FBS. Syncytium formation was scored and observed by light microscopy 4 h after treatment with the acidic medium. Syncytium formation was recorded when at least 4 nuclei were present in each syncytial mass.

Fluorescence microscopy

Sf21 cells (1.0 x 10^5) were grown on glass cover slips and transfected with 5 μg of plasmid DNA. At 48 h post transfection the cells were examined with a Zeiss LSM510

(confocal) laser scanning microscope for fluorescence using an excitation wave length of 488 nm and an emission band pass filter of 505 - 530 nm.

RESULTS

Budded virions of SeMNPV enter insect cells by endocytosis

BVs of AcMNPV and OpMNPV, group I NPVs, enter host cells by endocytosis (Hefferon *et al*., 1999; Volkman and Goldsmith, 1985; Wang *et al*., 1997a). To examine the mechanism of entry by BVs of SeMNPV, a member of the group II NPVs, the lysosomotrophic reagent, ammonium chloride, was used to demonstrate that SeMNPV BV enters cells by endocytosis. This lipophilic amine buffers the endosomal pH and inhibits acid-triggered membrane fusion in the endosome during viral entry by endocytosis (Helenius *et al*., 1982; Lenard and Miller, 1982).

Se301 cells were infected with SeMNPV BVs in the presence of a final concentration of 50 mM ammonium chloride. As positive control, Sf21 and Se301 cells were infected with AcMNPV BVs. Polyhedra production was used as an indicator of infectivity. The number of cells that produced polyhedra was counted at 72 h p.i. (Table 2.1). When cells were infected in the presence of ammonium chloride, a severe reduction (>95 %) in the number of cells containing polyhedra, was observed. Control experiments performed in parallel showed that ammonium chloride did not affect polyhedra formation when ammonium chloride was added up to 6 h post infection. These results provide strong evidence that BVs of SeMNPV enter insect cells primarily via endocytosis, like the BVs of group I NPVs.

Table 2.1 Effects of ammonium chloride as endosome acidification preventive on the percentage of infected cells as determined by polyhedra production. The experiments were performed in triplicate with 200 cells per analysis.

	SeMNPV		AcMNPV	
	0 mM	50 mM	0 mM	50 mM
Se301	75	1	78	1
Sf21	0	0	98	2

Membrane fusion of SeMNPV BV is triggered by acidification

Previous studies of AcMNPV and OpMNPV infection showed that acidification triggers fusion of the viral and cellular membranes, and that GP64 is the acid-triggered membrane fusion protein (Blissard and Wenz, 1992; Volkman and Goldsmith, 1985). To determine whether fusion of the SeMNPV BV envelope is triggered by acidification, Sf21 and Se301 cells were infected with SeMNPV and AcMNPV and syncytium formation assays were used as a direct measure of the acid-triggered fusogenic activity of the BVs.

Cell-to-cell fusion of both Sf21 and Se301 cells infected with either SeMNPV or AcMNPV was observed only after the pH of the tissue culture medium was lowered to pH 5.0 (Fig. 2.1). If the acidic treatment was excluded from the assay, no cell-to-cell

fusion was observed (results not shown). Cell-to-cell fusion of Se301 or Sf21 cells induced upon infection with AcMNPV BVs showed no detectable differences from those infected with SeMNPV (Fig. 2.1). SeMNPV BVs were thus capable of inducing pH-dependent membrane fusion in susceptible insect cells.

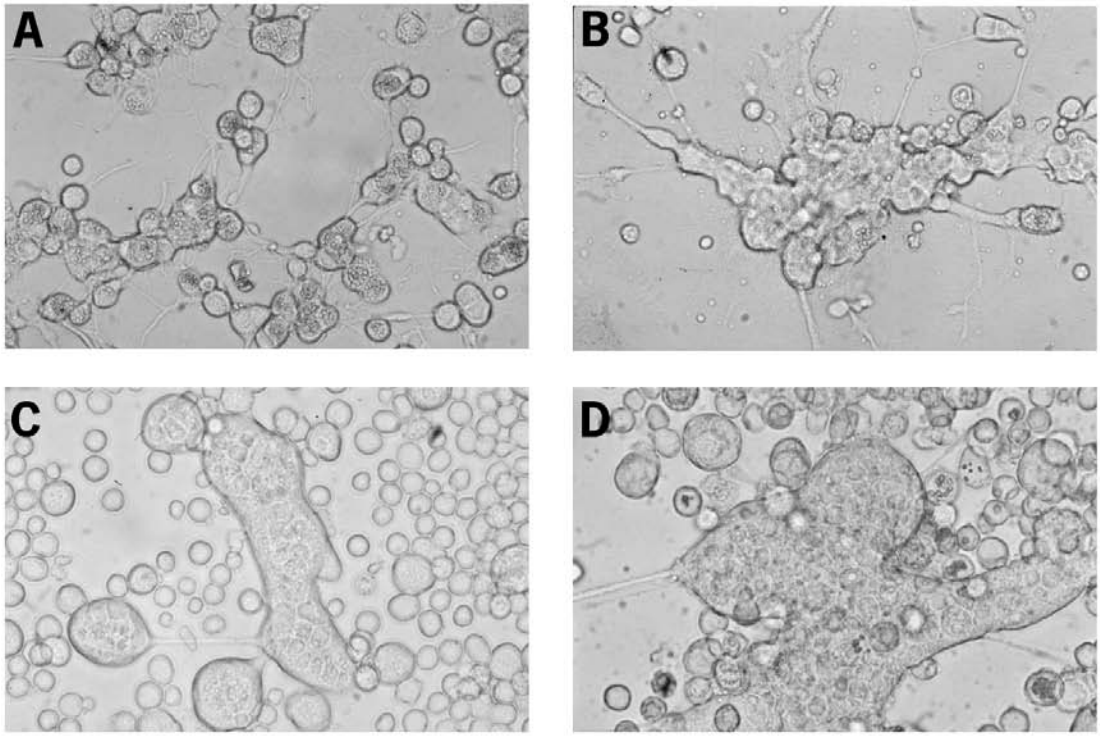

Figure 2.1 Baculovirus-mediated pH-dependent membrane fusion of insect cells. Se301 (A, B) and Sf21 cells (C, D) were infected with 10 $TCID_{50}$ units/cells with either SeMNPV (A, C) or AcMNPV (B, D). Forty-eight hours after infection, cells were treated for 2 min with Grace's medium pH 5.0. Syncytium formation was scored 4 h after dropping the pH by phase-contrast microscopy.

Identification of the SeMNPV major envelope protein

The previous experiments demonstrated that representative viruses of group I and group II NPVs enter host cells by endocytosis, and that acidification triggers membrane fusion. In AcMNPV and OpMNPV, fusion is mediated by the viral encoded GP64/67 protein, which is the major envelope protein of these BVs (Blissard and Wenz, 1992; Carstens *et al*., 1979; Hohmann and Faulkner, 1983). However, SeMNPV lacks a GP64 homolog (IJkel *et al*., 1999). To identify the envelope proteins of SeMNPV, BVs were isolated from hemolymph derived from SeMNPV-infected fourth-instar *S. exigua* larvae. Sucrose gradient purified BVs were separated on SDS-PAGE and the proteins were visualized by Coomassie Brilliant Blue (Fig. 2.2). A number of proteins were detected in BV preparations (Fig. 2.2 lane 2), which were not present in a sample from mock-infected insects (Fig. 2.2 lane 1), and are therefore related to proteins of the SeMNPV virions. Proteins appearing in the mock-infected sample are probably derived from the insect hemolymph and copurified with the BVs.

The major protein band present in the SeMNPV BV preparation had a size of

approximately 60 kDa. This is approximately 4-7 kDa smaller in size than the AcMNPV and OpMNPV major envelope proteins, respectively (Blissard and Rohrmann, 1989; Carstens *et al*., 1979; Whitford *et al*., 1989). To investigate whether this protein is present in the envelope or the NC, purified BVs were treated with a non-ionic detergent (NP40), fractionated and analyzed by SDS-PAGE. A 60 kDa protein was the major component of the SeMNPV BV envelope fraction (Fig. 2.2 lane 3). A 60 kDa protein was also present in the NC fraction (Fig. 2.2 lane 4) and this probably resulted from incomplete separation of the envelope and the NCs.

To determine whether the major 60 kDa protein of SeMNPV BV was also present in ODV, ODVs were purified from isolated polyhedra and its proteins separated by SDS-PAGE. A major protein band of 60 kDa was not detected in ODV (Fig. 2.2 lane 5) although conclusive evidence of the possible presence of lower quantities of this protein in ODV will require analysis with an antibody directed against the 60 kDa protein. However, it was concluded that SeMNPV BV contains a major envelope protein with an apparent molecular weight of 60 kDa that does not appear to be present in the ODV.

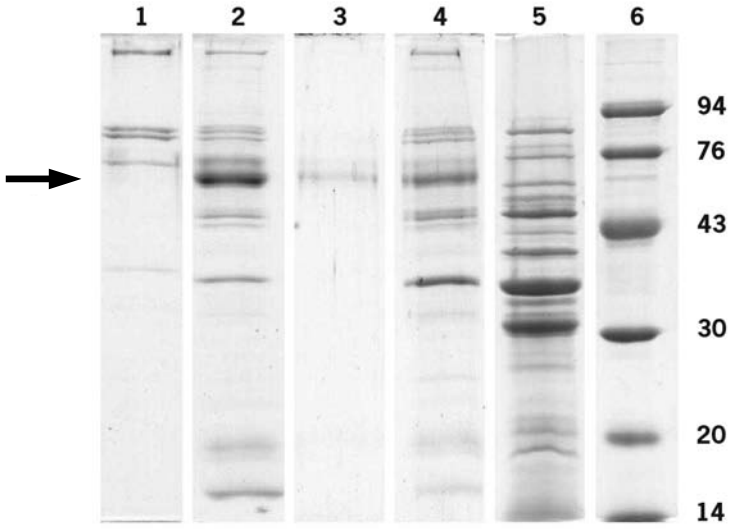

Figure 2.2 Coomassie Brilliant Blue stained SDS-PAGE gel (12 %) of purified SeMNPV. Lane 1: Mock purification of BVs from hemolymph of uninfected *S. exigua* larvae. Lane 2: SeMNPV BVs. Lane 3: SeMNPV BV envelopes. Lane 4: SeMNPV BV nucleocapsids. Lane 5: SeMNPV ODVs. Lane 6: Low molecular weight marker. The major BV envelope protein (60 kDa) is indicated with an arrow.

The BV major envelope protein is a proprotein convertase cleavage product

To determine the SeMNPV ORF encoding the BV major envelope protein, BVs were purified and its proteins were separated by SDS-PAGE. The proteins were blotted onto a PVDF membrane and the major 60 kDa envelope protein was N-terminally sequenced. The sequence obtained, GLFNFMG, matched that derived from translation of the Se8 ORF but was located 150 amino acids downstream of the putative N-terminus (Fig. 2.3). This strongly suggested that the product of Se8 was present in the form of a cleavage product in BVs. The C-terminal cleavage product has a predicted molecular weight of 59 kDa, which is in close agreement with the observed size of the BV major envelope protein in SDS-PAGE. The uncleaved Se8 protein has a predicted molecular weight of 76 kDa (74 kDa minus the signal peptide) (IJkel *et al*., 1999) and contains a furin-like proprotein convertase cleavage site (Arg-Xaa-(Arg/Lys)-Arg↓) (Hosaka *et al*., 1991; Nakayama, 1997) located at the amino acids 146 to 149. This cleavage site in the Se8 product is therefore likely to be responsible for the occurrence of the 59 kDa protein in BVs.

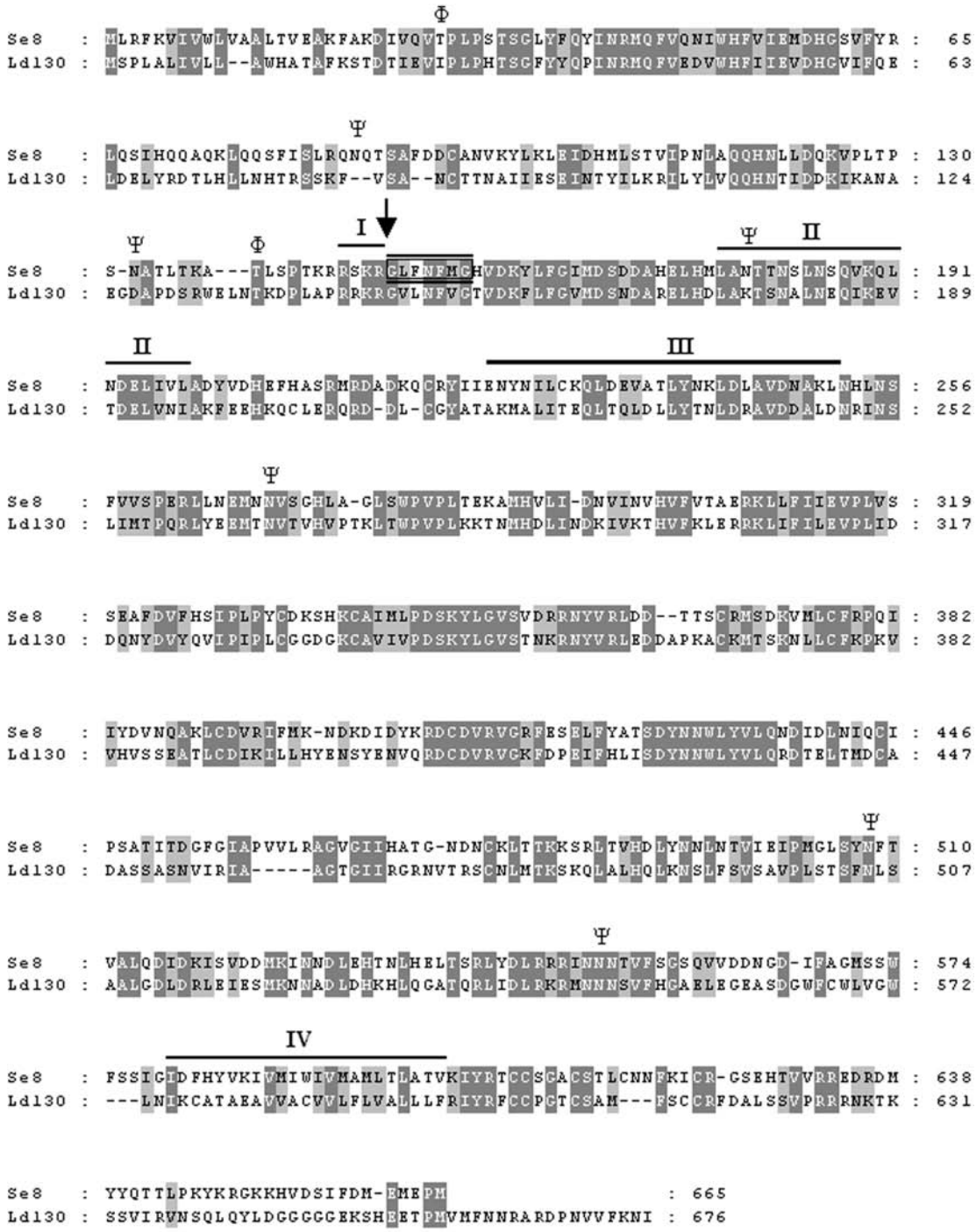

Figure 2.3 Alignment of the predicted amino acid sequences of the Se8 and Ld130 proteins. Gaps introduced to optimize the alignment are indicated with dashes. Shading is used to indicate the occurrence of identical (dark grey) or substitutional (light grey) amino acids. The box represents the obtained N-terminal amino acid sequence of the 60 kDa BV envelope protein; the vertical arrow indicates the putative endoprotease cleavage site. Line I indicates the furin-like cleavage consensus sequence. Line II, III and IV represent the predicted leucine zipper, the coiled-coil domain and the C-terminal transmembrane domain, respectively. Computer predicted consensus N- and O-linked glycosylation sites are indicated by the symbols Ψ and Φ, respectively.

Computer analysis of Se8 predicted the occurrence of a C-terminal transmembrane domain (amino acids 580-602), a coiled coil structure (amino acids 222-249), a leucine zipper (amino acids 177-198) and a positively charged cytoplasmic tail domain (CTD) (Fig. 2.3). In the AcMNPV and OpMNPV major envelope fusion proteins

similar domains were identified. A predicted amphiphatic alpha helix and a small hydrophobic domain have been identified as necessary for membrane fusion by GP64 (Kingsley *et al*., 1999; Monsma and Blissard, 1995). While GP64 proteins have a small charged cytoplasmic tail domain, it was not essential for efficient budding of AcMNPV BVs (Oomens and Blissard, 1999).

Previously, Se8 was identified as a homolog to the Ld130 protein encoded by ORF 130 of LdMNPV, with 41% amino acid identity and 60% similarity (IJkel *et al*., 1999). The furin-like proprotein convertase consensus cleavage sequence was also present in Ld130 (amino acids 144-147) at an analogous position compared to Se8 (Fig. 2.3). In conclusion, Se8 encoded the major envelope protein of SeMNPV BV and the Se8 protein appears to be present as a cleavage product in the BV. Se8 contained several protein domains similar to those identified in the AcMNPV and OpMNPV EFPs.

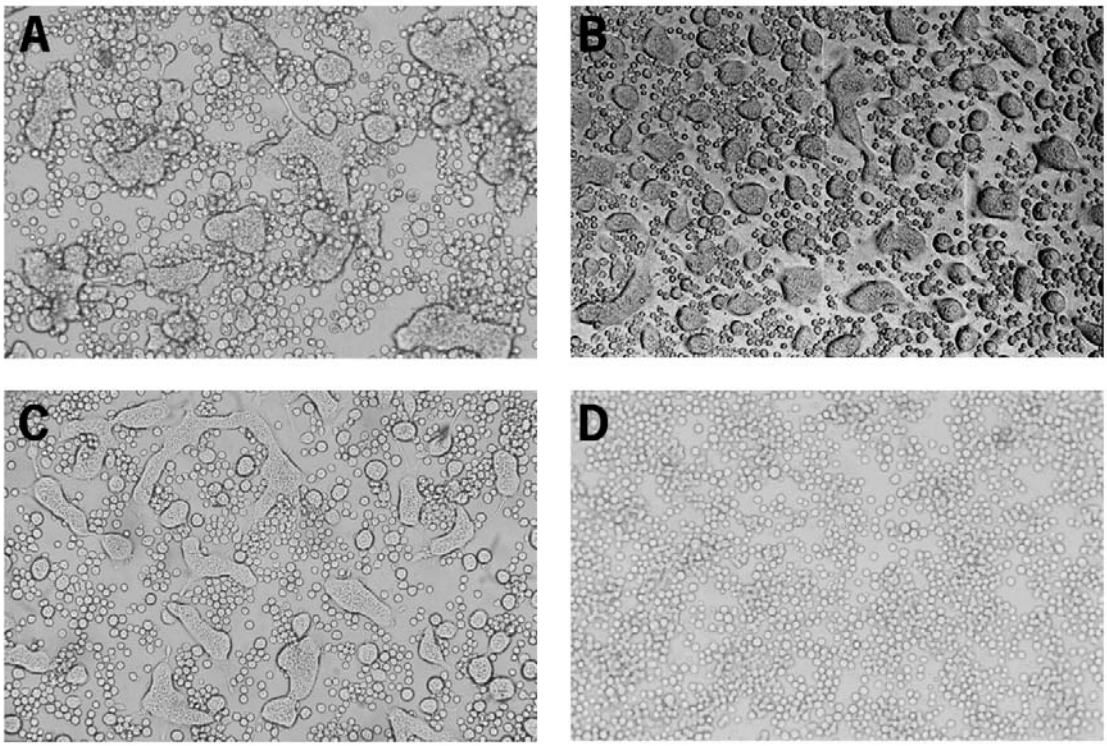

Figure 2.4 Cell to cell fusion of Sf21 cells transfected with putative baculovirus EFP. Cells (5.0×10^5) were transfected with 5 ug of plasmid p166AcV5-Se8 (panel A), p166AcV5-Ld130 (panel B), p166-OpGP64 (panel C) or a control p166BRNX-AcV5 plasmid (panel D). At 48 h after transfection, cells were treated for 2 min with Grace's medium pH 5.0. Syncytium formation was scored 4 h after dropping the pH by phase contrast microscopy.

The Se8 encoded protein mediates pH-dependent membrane fusion

To determine if Se8 and its LdMNPV homolog (Ld130) could mediate pH-dependent membrane fusion, plasmids were constructed in which these ORFs were cloned under the control of an optimized OpMNPV early *gp64* promoter, to facilitate expression in insect cells (Blissard and Rohrmann, 1991). An OpMNPV *gp64* gene, which contained the OpMNPV *gp64* ORF behind the same *gp64* promoter was used as a positive control. The empty plasmid vector (p166BRNX-AcV5) was used as a negative control. Sf21 cells were transfected with these constructs and syncytium formation assays were performed. Because Se301 cells are difficult to efficiently transfect (in

contrast to Sf21 cells) and because cell-to-cell fusion was difficult to monitor in Se301 cells (multimorphic cell phenotypes are found in this line), transfected Sf21 cells were used to examine cell-to-cell fusion. The examination of cell-to-cell fusion of SeMNPV infected Sf21 cells (Fig. 2.1C), indicated that this cell line was suitable for studies of membrane fusion mediated by SeMNPV proteins. Cells transfected with the Se8, the Ld130 or the OpMNPV *gp64* construct clearly showed low pH-dependent cell-to-cell fusion (Fig. 2.4). Although some membrane fusion was observed at earlier times after the pH drop, a substantial degree of fusion occurred by 4 h after shifting the pH to 5.0. In cells transfected with the empty p166BRNX-AcV5 construct, no cell-to-cell fusion was detectable upon lowering the pH to 5 (Fig. 2.4). Thus, the Se8 protein or the LdMNPV homolog (Ld130) protein alone was sufficient to mediate pH-dependent membrane fusion in Sf21 cells.

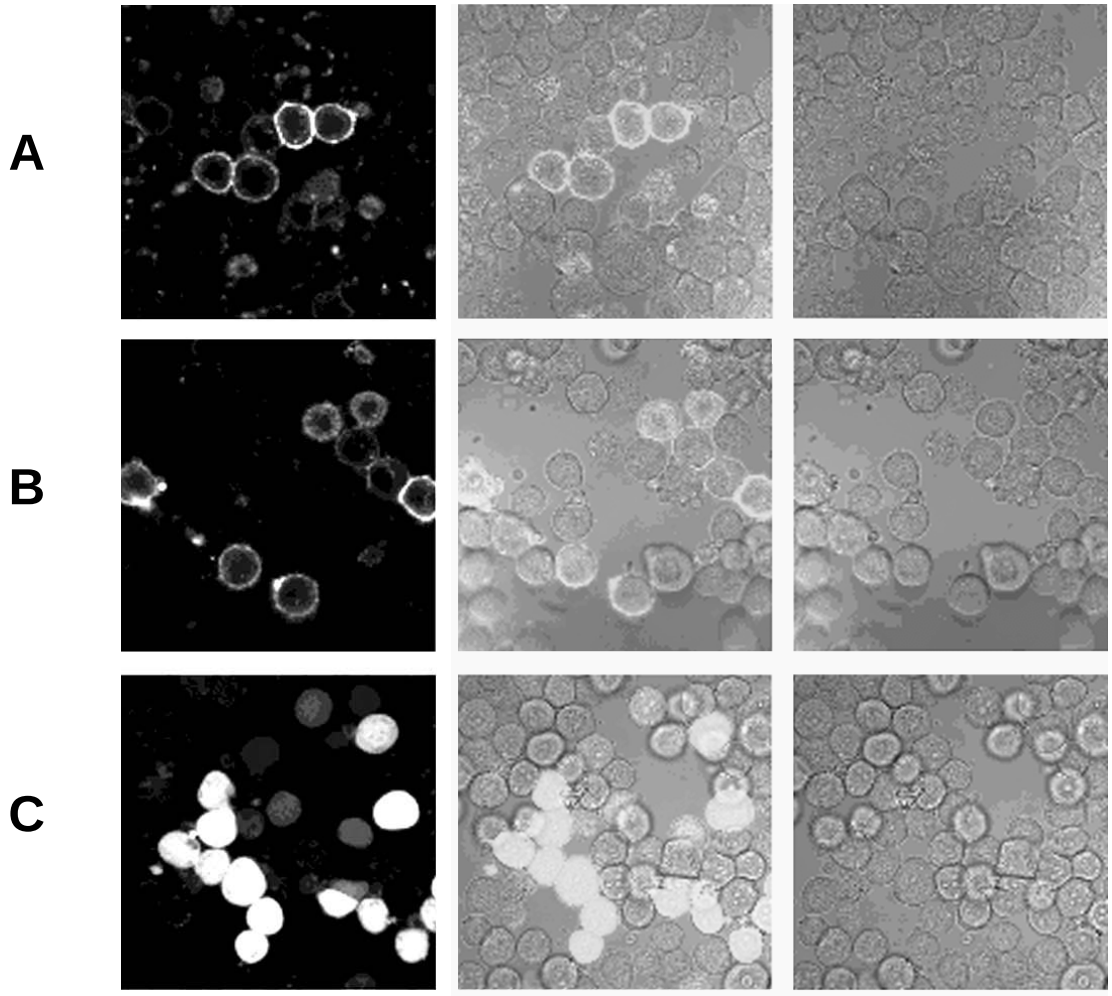

Figure 2.5 Localization of the Se8 and Ld130 GFP-fusion proteins in Sf21 cells. Sf21 cells (1.0×10^5) were transfected with 5 μg of plasmid p166AcV5-Se8GFP (A), p166AcV5-Ld130GFP (B) or with the control plasmid p166AcV5-GFP (C). At 48 h after transfection the cells were examined by confocal laser scanning microscopy for fluorescence. Fluorescence (left), phase-contrast (right) and overlay the fluorescence and phase-contrast micrographs are shown.

The Se8 protein is localized in the plasma membrane

To investigate the cellular localization of the Se8 and the Ld130 proteins, C-terminal GFP-fusion constructs were made in plasmid p166BRNX-AcV5. As a negative

control, GFP alone was cloned in the same vector. Sf21 cells were transfected with 5 μg plasmid DNA, incubated for 48 h and examined for fluorescence by confocal laser scanning microscopy. The Se8 and the Ld130 GFP-fusion proteins were primarily localized in the plasma membrane (Fig. 2.5). Diffuse patches of fluorescence were observed in the cytoplasm (Fig. 2.5). No fluorescence was observed in the nucleus of transfected insect cells. This is in agreement with the previously observed localization of the OpMNPV GP64 in the plasma membrane (Blissard and Rohrmann, 1989). The GFP protein when expressed alone showed homogeneous fluorescence in the cytoplasm and nucleus (Fig. 2.5). Similar results were obtained when Se301 cells were transfected with the GFP-fusion constructs. Thus, the Se8 and Ld130 GFP-fusion proteins were primarily localized in the plasma membrane of Sf21 and Se301 cells and this localization of Se8 and Ld130 is consistent with their fusogenic activity.

DISCUSSION

The major envelope glycoprotein (GP64) of the AcMNPV BV is involved in host receptor binding and fusion with the host cell membrane during viral entry. Deletion of the AcMNPV *gp64* gene resulted in a virus unable to move from cell to cell, non-lethal to infected larvae and incapable of efficient budding (Oomens and Blissard, 1999; Monsma *et al*., 1996). Homologs of the AcMNPV *gp64* gene (Whitford *et al*., 1989) have been found in *Anagrapha falcifera* NPV (Federici and Hice, 1997), *Anticarsia gemmatalis* MNPV (Oomens, 1999a), BmNPV (Gomi *et al*., 1999), *Choristoneura fumiferana* MNPV (Hill and Faulkner, 1994), *Epiphyas postvittana* NPV (AF061579), *Galleria mellonella* NPV (Blinov *et al*., 1984) *Hyphantria cunea* NPV (AF190124) and OpMNPV (Blissard and Rohrmann, 1989). These viruses are all phylogenetically placed within subgroup I of the NPVs (Zanotto *et al*., 1993). No *gp64* homolog has been identified so far in group II NPVs and GVs. In this study, evidence is provided that group II NPVs enter host cells in a manner similar to that of group I NPVs, and a functional analog of the group I GP64 protein from the group II virus SeMNPV has been identified.

The BV entry mechanism of SeMNPV was examined, using ammonium chloride as an inhibitor of the endocytotic pathway. The SeMNPV infection of Sf21 cells did not lead to a productive infection as evidenced by the absence of polyhedra. However, the observation that SeMNPV infection of Sf21 cells can result in cell-to-cell fusion (Fig. 2.1C), suggests that some viral proteins are expressed from either an early or late SeMNPV promoter. This observation further suggests that the abortive infection of Sf21 cells by SeMNPV may not be due to inhibition of early gene expression but is likely due to a block in later events, since BV and polyhedra were not produced from such infections (Shirata *et al*., 1999). When Se301 cells were infected with SeMNPV in the presence of ammonium chloride a •95% reduction in polyhedra production was observed. Similar reduction rates were observed when Se301 or Sf21 cells were infected with AcMNPV in the presence of ammonium chloride and these values are in agreement with those reported for AcMNPV in Sf21 and Sf9 cells (Hefferon *et al*., 1999; Volkman and Goldsmith, 1985). The data suggest that the entry mechanisms of

AcMNPV and SeMNPV BVs are similar in this regard for Se301, Sf21 and Sf9 cells. Thus, BVs from both group I and II NPVs enter cells by endocytosis although the group II NPVs lack a GP64 protein.

The SeMNPV structural proteins of BVs and ODVs were isolated and examined by SDS-PAGE. The major BV envelope protein of SeMNPV had an apparent molecular weight of 60 kDa and a major protein of similar size was not detected in ODVs. Sequencing of the N-terminus of the 60 kDa BV major envelope protein from SeMNPV revealed that this protein was encoded by Se8 and was present in BVs as a truncated product. The amino acid sequence (RRSKR) that immediately precedes the N-terminal amino acid (G) of the 60 kDa protein resembles a furin-like proprotein convertase cleavage site (R-X-R/K-R). Therefore it is hypothesized that the N-terminal 150 amino acids are cleaved from the full length Se8 protein during synthesis and processing. This processing would likely result in cleavage products with predicted molecular weights of approximately 15 kDa (the N-terminal fragment minus the signal peptide) and 59 kDa (the C-terminal fragment). The size of the 59 kDa cleavage product is in close agreement with the estimated 60 kDa of the major envelope protein isolated from SeMNPV BVs. The presence of the 60 kDa major envelope protein in the BV envelope is in accordance with the predicted C-terminal transmembrane domain of the Se8 protein. The 59 kDa product possesses a number of putative phosphorylation, N-glycosylation, myristoylation and amidation sites. It is not known whether these modifications actually occur but the close similarity between measured and predicted size would suggest that the C-terminal fragment is not heavily modified. Several protein bands around 15-17 kDa were detected in SeMNPV BVs (Fig. 2.2), of which one may be the 15 kDa cleavage product of the Se8 encoded protein.

To determine whether the major envelope protein from SeMNPV and LdMNPV BV could mediate pH-dependent membrane fusion, a previously described syncytium formation assay of *Lymantria dispar* cells was adopted for Sf21 cells (Blissard and Wenz, 1992). The low pH value (5.0) was selected because similar values have been reported for eucaryotic endosomes after acidification (Helenius *et al*., 1982) and because this pH was shown to be sufficient to trigger AcMNPV, OpMNPV and AgMNPV *gp64*-mediated membrane fusion (Blissard and Wenz, 1992; Oomens, 1999a). The syncytium formation assays with cells expressing Se8 and the LdMNPV homolog (Ld130) both showed membrane fusion and syncytium formation upon acidification of the medium. This is in agreement with the observed pH-dependent cell to cell fusion observed in Sf21 and Se301 cells infected with SeMNPV BVs. To mediate cell-to-cell fusion, the Se8 and Ld130 proteins must be localized at the plasma membrane. GFP-fusion constructs of Se8 and Ld130 proteins were observed at the periphery of transfected cells using confocal fluorescence microscopy (Fig. 2.5). Because Se8 and Ld130 both contain a predicted hydrophobic signal peptide at the N-terminus and a predicted C-terminal transmembrane domain, it is likely that both proteins are found at the surface of infected cells. Thus, the SeMNPV major BV envelope protein (Se8) and the homolog from LdMNPV (Ld130) are each able to independently mediate low pH-triggered membrane fusion, and represent therefore functional analogs of the OpMNPV GP64 protein. It is likely to believe that all group II NPVs has the Se8 homolog as their

BV envelope fusion protein. For this reason the common name F (fusion) protein will be introduced for the envelope fusion protein of the group II NPVs.

The SeMNPV and LdMNPV F proteins are 41% identical and 60% similar on amino acid level (IJkel *et al*., 1999). Both ORFs also showed very low amino acid identity (~22%) to AcMNPV ORF23 (Ac23), BmNPV ORF14 and OpMNPV ORF21 (IJkel *et al*., 1999). The latter ORFs also lacked a proprotein convertase consensus cleavage sequence in their predicted amino acid sequences. Also, in preliminary syncytium formation experiments, the Ac23 protein was unable mediated membrane fusion (W.F.J. IJkel and J. Mangor, unpublished results). The higher degree of relatedness among the group I NPV *gp64* genes, combined with a much larger degree of divergence among the group II NPVs and GVs and their Se8 homologs, suggest that *gp64* may have been acquired more recently, perhaps resulting in the branching off of the group I NPVs from the group II NPVs and the GVs. Like GP64 proteins, the F proteins appear to be type I membrane glycoproteins. In the AcMNPV and OpMNPV GP64 proteins, 4-3 heptad repeats of leucines and/or methionine residues have been identified. These repeats are predicted to form amphipathic α-helices (Monsma and Blissard, 1995). Disruption of the OpMNPV heptad repeat resulted in defective trimerization (Monsma and Blissard, 1995). Mutational analysis of the AcMNPV heptad repeat revealed that it was essential at a stage after the initiation of the individual trimer conformational change and before the functioning of the putative fusion complex (Kingsley *et al*., 1999). Hydrophobic 4-3 heptad repeats and a leucine zipper motif (LANTTNSLNSQVKQLNDELIVL at amino acids 177-198 of SeMNPV F) have also been identified in the SeMNPV and LdMNPV F proteins (Fig. 2.3). Coiled coil regions are predicted for amino acids 222 – 249 of the SeMNPV F protein and amino acids 165-247 of the LdMNPV F protein. It is thus possible that these predicted coiled coil regions may play a similar role in the membrane fusion process.

Cleavage of viral envelope glycoproteins seems to be a general mechanism used by viruses to activate envelope fusion proteins. A number of viral envelope proteins are cleaved by furin and these include the human immunodeficiency virus gp160 (Hallenberger *et al*., 1992), human cytomegalovirus glycoprotein B (Vey *et al*., 1995), Mouse mammary tumor virus-7 superantigen (Park *et al*., 1995), Measles virus F_0 (Watanabe *et al*., 1995), Newcastle disease virus F_0 (Gotoh *et al*., 1992), Sindis virus gpE2 (Gotoh *et al*., 1992), Human parainfluenza virus type 3 F_0 (Ortmann *et al*., 1994) and Avian Influenza virus hemagglutinin A (HA) (Stieneke-Grober *et al*., 1992). Furin is a member of a family of subtilisin-like endoproteases called "proprotein convertases" that function in proteolytic processing of a large variety of precursor proteins. Recently, furin was identified and characterized from *S. frugiperda* cells (Sf9) (Cieplik *et al*., 1998). Sf9 cells are a clonal isolate of Sf21 cells and it is likely that Se301 cells also contain a subtilisin-like endoprotease like Sf furin. The Sf furin was localized mainly in the *trans*-Golgi network (TGN) but was also present at the plasma membrane. The cellular localization of Sf furin in the TGN and the presence of the Sf furin recognition motif (R-X-K/R-R) at the SeMNPV F cleavage site and at a similar site in LdMNPV F strongly suggests that both proteins are cleaved by an Sf furin-like endoprotease. Although the possibility that SeMNPV F may be processed by a viral gene product cannot yet be

excluded.

The avian influenza HA protein most closely resembles the SeMNPV F protein, when the amino acid sequence of its cleavage site and the size of the protein are considered. HA is synthesized as a precursor (HA_0) of 75 kDa. The precursor, HA_0, is post-translationally cleaved at a conserved arginine residue into two subunits. The two subunits, HA_1 and HA_2, are linked by a single disulfide bond. The X-ray crystal structure of HA_0 revealed that its cleavage site (amino acids 323-341 of HA_0) can be seen as a prominent surface loop that protrudes out into solution and is accessible to proteases (Steinhauer, 1999). The amino acid sequence (PTKRRSKR↓GLFNFM) of the putative cleavage site of SeMNPV F has 71% similarity to the HA_0 protein cleavage site (PQRKRKKR↓GLFGAI) and the 5 amino acids at the cleavage site (KR↓GLF) are identical. Cleavage of HA_0 is necessary for virus infectivity (Klenk *et al*., 1975; Lazarowitz and Choppin, 1975), because it activates the membrane fusion potential of the HA (Maeda and Ohnishi, 1980; Steinhauer, 1999; Vey *et al*., 1992). Currently a mutational analysis of the cleavage site of SeMNPV F is carried out to determine if cleavage of the protein may also be necessary for virus infectivity and activation of its membrane fusion potential.

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Chapter 3

FURIN IS INVOLVED IN BACULOVIRUS ENVELOPE FUSION PROTEIN ACTIVATION

SUMMARY

The *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) *Se8* gene was recently identified to encode the viral envelope fusion (F) protein. A 60 kDa C-terminal subunit (F_1) of the 76 kDa primary translation product of this gene was found to be the major envelope protein of SeMNPV budded virus (BV). (IJkel *et al.*, 2000). Using a specific inhibitor it is now shown that furin is involved in cleavage of the precursor envelope fusion (F_0) protein. BV produced in the presence of the inhibitor possesses the uncleaved F_0 protein, while a F protein with a mutation in the furin cleavage site was translocated to the plasma membrane, but lost its fusiogenic activity. These results indicate that cleavage of F_0 is required to activate the SeMNPV F protein and necessary for BV infectivity. Using specific antibodies against F_1 and against the putative N-terminus (F_2) of the primary translation product it is shown that the F protein is BV-specific and that BVs contain both the 60 kDa (F_1) and 21 kDa (F_2) cleavage products. In nonreducing SDS-PAGE both subunits migrate as a single 80 kDa protein, indicating that the subunits remain associated by a disulphide-linkage. In addition, the presence of the F protein predominately as monomer suggests that disulfide-links are not involved in oligomerization. Thus, the envelope fusion protein from group II NPVs of baculoviruses has properties similar to those from a number of vertebrate viruses.

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INTRODUCTION

The *Baculoviridae* are a family of large, enveloped double-stranded DNA viruses exclusively pathogenic to arthropods, predominantly insects in the order *Lepidoptera* (Adams and McClintock, 1991). Baculoviruses of the genus *Nucleopolyhedrovirus* (NPV) produce two distinct virion phenotypes: the occlusion-derived virus (ODV) and the budded virus (BV) (Volkman and Summers, 1977). ODVs are released from occlusion bodies due to the alkaline enviroment of the insect gut and able to infect the midgut epithelial cells by direct membrane fusion (Granados, 1978; Granados and Lawler, 1981; Horton and Burand, 1993). BVs infect insect cells via receptor mediated endocytosis and are responsible for the systemic spread of the virus in the insect (Hefferon *et al*., 1999; Volkman and Goldsmith, 1985). Upon acidification of the endosome, the viral and endosomal membranes fuse, thereby allowing entry of the nucleocapsid into the cytoplasm (Blissard and Wenz, 1992; Hefferon *et al*., 1999). For group I NPVs, e.g. *Autographa californica* (Ac)MNPV, *Bombyx mori* (Bm)NPV and *Orygyia pseudotsugata* (Op)MNPV, this fusion is mediated by the envelope fusion protein GP64 (Blissard and Wenz, 1992; Kingsley *et al*., 1999; Plonsky *et al*., 1999). This protein is also required for efficient budding of newly synthesized nucleocapsids at the plasma membrane (Monsma *et al*, 1996; Oomens and Blissard, 1999). Baculoviruses, belonging to group II NPVs, e.g. SeMNPV (IJkel *et al*., 1999), *Lymantria dispar* (Ld)MNPV (Kuzio *et al*., 1999) and *Helicoverpa amigera* (Ha)SNPV (Chen *et al*., 2001), conspicuously lack a GP64-like protein.

Recently, a novel type of envelope fusion (F) protein was identified in BVs of group II NPVs, notably in SeMNPV (IJkel *et al*., 2000) and LdMNPV (Pearson *et al*., 2000). In case of SeMNPV this F protein is encoded by ORF8 (Se8) (IJkel *et al*., 1999). A C-terminal 60 kDa subunit of the 76 kDa primary translation product was found to be the major envelope protein of SeMNPV BV. Several protein bands with a molecular size in the 15-17 kDa range were found (IJkel *et al*., 2000), and one of these could represent the N-terminal subunit of the F protein. The involvement of furin in the cleavage was hypothesized by the presence of a furin-like proprotein convertase site, RSKR, immediately upstream of the N-terminus of the 60 kDa cleavage product (IJkel *et al*., 2000).

Cleavage of viral fusion proteins seems to be a general mechanism to activate these proteins and to produce infectious viruses. The cleavage takes place in front of a hydrophobic sequence, the “fusion peptide”. Exposure to low pH or receptor binding triggers a conformational change whereby the fusion peptide is believed to translocate to the top of the molecule and to insert into the target membrane (Klenk and Garten, 1994). A number of fusion proteins of vertebrate viruses are cleaved by furin. These include human cytomegalovirus glycoprotein B (Vey *et al*., 1995), human parainfluenza virus type 3 F (Ortmann *et al*., 1994), Ebola virus glycoprotein GP (Volchkov *et al*., 1998) and avian influenza virus HA (Stieneke-Grober *et al*., 1992). The furin-mediated cleavage yields in all these cases two subunits that are disulfide-linked. Furin is localized in the *trans*-Golgi Network (Vey *et al*., 1994) and cleaves after the consensus cleavage site R-X-R/K-R↓ (Hosaka *et al*., 1991; Nakayama, 1997). The arginine immediately upstream of

the cleavage site is the most essential amino acid (Nakayama, 1997). For the Ebola virus envelope protein GP it has been shown that changing this arginine into a lysine resulted in an uncleaved precursor protein (Volchkov *et al*., 1998). Cleavage of some fusion proteins can be inhibited by peptidyl-chloroalkylketones containing the R-X-K/R-R motif, which specifically bind to the catalytic site of furin (Garten *et al*., 1989; Hallenberger *et al*., 1992; Richt *et al*., 1998; Stieneke-Gröber *et al*., 1992).

In this paper evidence is provided by using a furin inhibitor and mutational analysis of the furin-like proprotein convertase site that cleavage of the SeMNPV BV F protein is mediated by furin and that this cleavage is required to activate the SeMNPV F protein to render infectious BVs. Furthermore, it is investigated whether both subunits of the primary translation product are present in the SeMNPV BV and whether these remain associated to each other.

MATERIALS AND METHODS

Cells, insects and viruses

The *Spodoptera frugiperda* cell-line IPLB-SF-21 (Vaughn *et al*., 1977), and the *S. exigua* cell-line Se301 (Hara *et al*., 1995) were cultured at 27°C in plastic tissue culture flasks (Nunc) in Grace's insect medium, pH 5.9-6.1, (GibcoBRL) supplemented with 10% foetal bovine serum (FBS). A culture of *S. exigua* insects was maintained according to Smits and Vlak (Smits and Vlak, 1988). The SeMNPV isolate (Gelernter and Federici, 1986) was originally obtained from Dr. B. A. Federici (Department of Entomology, University of California, Californica, USA) and was called SeMNPV-US1 (Muñoz *et al*., 1998). The AcMNPV-E2 strain (Smith and Summers, 1978) was originally obtained from Dr. M. D. Summers (Texas A&M University, College Station, Texas, USA).

Plasmid constructions

The coding regions of amino acids 1(M)-149(R) (F_2) and amino acids 150(G)-579(G) ($F_1\Delta$580-665) of ORF8 in the SeMNPV genome were amplified by high fidelity "Expand" long template PCR (Boehringer Mannheim) from plasmid pSe*Bgl*II-H (IJkel *et al*., 1999). Primers used to amplify $F_1\Delta$580-665 were 5'-TTTGGATCCCGGCCTTTTTAA TTTTA TGG-3' (underlined nucleotides (nt) generated a *Bam*HI site) and 5'-AATAAGC**TTA**ACCGATGCTGGAAAACCACGAAGAC-3' (underlined and bold nt generated a *Hin*dIII site and a translation stop codon, respectively). Primers used for amplifying F2 were 5'-TTGGGATCCTATGCTGCGTTTTAAAGTGATTGTG-3' (underlined nucleotides generated a *Bam*HI site) and 5'-ATTAAGC**TTA**GCGTTTAGAGC GTCTTTTCGTCG-3' (underlined and bold nt generated a *Hin*dIII site and a translation stop codon, respectively). The PCR products were cloned into the *Bam*HI and *Hin*dIII cloning sites of the expression vector pET28a (Novagen) to generate pET28-Se$F_1\Delta$580-665 and pET28-SeF_2.

PCR-based site-directed mutation of the arginine immediately upstream of the convertase cleavage site into a lysine (R149K) was performed according to the method of Sharrocks and Shaw (1992). The 5' primer 5'-TTATGGATCCATGCTGCGTTTTAAA

GTGATTGTG-3' and 3' mutagenic primer 5'-CCCATAAAATTAAAAAGGCCCTTTTTAG AGCGTCTTTTCGTCG-3' (underlined nt generated the mutation) were used in conjunction with plasmid p166AcV5-Se8 (IJkel *et al*., 2000) as template, to introduce the R149K mutation using high fidelity "Expand" long template PCR (Boehringer Mannheim). The PCR product was agarose gel purified and the single strand containing the mutation at the 3' end served as 5' mutagenic primer in a second PCR together with the 3' primer 5'-GAGAGGCACGGGCCACGAAAGG-3'. The second PCR product was cloned into pGEM-T (Promega) to generate pGEM-Se8(R149K). The plasmids p166AcV5-Se8(R149K) and p166AcV5-Se8(R149K)GFP were obtained by exchanging the *Bam*HI/*Stu*I fragment of pGEM-Se8(R149K) with that of p166AcV5-Se8 and p166AcV5-Se8GFP (IJkel *et al*., 2000), respectively.

Production of polyclonal antibodies

The proteins $F_1\Delta$580-665 and F_2 were expressed in *Escherichia coli* Bl21 cells containing the vectors pET28-Se$F_1\Delta$580-676 or pET28-SeF_2 and were purified as described previously (IJkel *et al*., 2001). The proteins were concentrated using a Centriprep-10 kDa filter device (Amicon) and dialyzed against PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Protein concentrations were determined with the Bio-Rad protein asssay (Bio-Rad).

Two chickens were injected intramuscularly with either 200 μg purified $F_1\Delta$580-665 or F_2 protein using a water in oil adjuvant. The chickens were boosted after 4 weeks with 150 μg purified protein. Two weeks after the booster, eggs were collected every day for 2 weeks. The egg yolk was diluted three times (w/w) in PBS containing 5.25% PEG-6000, 0.05% NaN_3. The mixture was centrifuged for 15 min at 2,250 x *g*. The supernatant was filtered through glass wool and PEG-6000 was added until 12% (w/v) was reached. After centrifugation for 15 min at 2,250 x *g*, the pellet was dissolved in water and centrifuged as before to remove debris. PEG-6000 was added to the solution until 12% (w/v) was reached and centrifuged 20 min at 2,250 x *g*. The pellet containing α-F_1 or α-F_2 was dissolved in 2.5 ml 0.9% NaCl per egg and stored at –20°C.

Purification of SeMNPV BV and ODV

Hemolymph-derived BVs were purified form SeMNPV-infected *S. exigua* fourth-instar larvae as described previously (IJkel *et al*., 2000) with some modifications. Briefly, 3 days post infection (p.i.) hemolymph was collected and clarified at 2,000 x *g* for 10 min at 4°C. The supernatant was passed through a 0.45 μm filter. BVs in the filtrate were pelleted through a 25% (w/w) sucrose cushion made up in 0.1 x TE (10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA) by centrifugation at 100,000 x *g* for 90 min at 4°C and resuspended in 0.1 x TE. BVs from cell culture supernatants were purified in a similar fashion. Se301 cells were infected with 0.1 $TCID_{50}$ units/cell of SeMNPV. Seventy-two h p.i. cell culture supernatants were clarified at 2,000 x *g* for 10 min at 4°C and passed through a 0.45 μm filter. BVs in the filtrate were pelleted and resuspended as above. ODVs were purified from polyhedra derived from SeMNPV infected *S. exigua* fourth-instar larvae as described previously (IJkel *et al*., 2000). The purity and integrity of BVs and ODVs were checked by electron microscopy.

Western blot analysis

For electrophoresis under reducing condition purified SeMNPV ODVs and BVs were disrupted in Laemmli buffer (125 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.8). For electrophoresis under nonreducing condition virions were disrupted in 125 mM Tris-HCl, 8% SDS, 37.5 mM iodoacetamide, 10% glycerol, 0.001% bromophenol blue, pH 6.8. Samples were denatured for 10 min at 95^{o}C. Proteins were electrophoresed in SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore) by semi-dry electrophoresis transfer (Ausabel *et al*., 1994). Membranes were blocked overnight at 4^{o}C in PBS containing 2% milk powder, followed by incubation for 1 h at room temperature (RT) with antiserum at a dilution of 1:1,000 in PBS containing 0.2% milk power. After washing three times 15 min in PBS-T (PBS containing 0.1% Tween-20), the membranes were incubated for 1 h at RT with horse radish peroxidase conjugated with rabbit anti-chicken immunoglubulin (Sigma) diluted 1:50,000 in PBS containing 0.2% milk powder. After washing three times 15 min in PBS-T, the signal was detected by ECL technology as described by the manufacturer (Amersham).

Furin inhibition assay

Se301 cells (2.0 x 10^5) were infected with 10 $TCID_{50}$ units/cell of SeMNPV or AcMNPV. At two h p.i. cells were washed twice with medium and incubated with medium containing 50 μM of the furin cleavage inhibitor decanoylated arginyl-valyl-lysyl-arginyl chloromethylketone (dec-RVKR-cmk) (Bachem). The control infections were incubated without dec-RVKR-cmk. Cell culture supernatants were collected 48 h p.i and infectious BV was quantified by a 50% tissue culture infective dose ($TCID_{50}$) assay (O'Reilly *et al*., 1992) on Se301 cells. The SeMNPV-infected cells were washed three times with medium without FBS and assayed for the presence of active fusion proteins in the plasma membrane by a syncytium formation assay as described below.

Syncytium formation assay

Syncytium formation assays were performed by transfection of 5.0 x 10^5 Sf21 cells with 5 μg DNA of p166AcV5-Se8 or p166AcV5-Se8(R149K) using Cellfectin (GibcoBRL). The empty vector p166BRNX-AcV5 (IJkel *et al*., 2000) was used as a negative control for the syncytium formation assay. Forty-eight h after transfection, cells were washed three times with medium without FBS, and treated for 2 min in acidic medium without FBS, pH 5.0. The acidic medium was removed and replaced with medium with FBS, pH 6.1. Syncytium formation was recorded by light microscopy 4 h after treatment with the acidic medium. Syncytium formation was positive when at least 4 nuclei were present in each syncytial mass.

Fluorescence microscopy

To investigate the cellular location of the mutant SeMNPV F protein, 1.0 x 10^5 Sf21 cells were grown on glass cover slips and transfected with 5 μg DNA of p166AcV5-Se8GFP or p166AcV5-Se8(R149K)GFP. As control for GFP expression the plasmid

p166AcV5-GFP (IJkel *et al*., 2000) was used. At 48 h post transfection the localization of GFP in the cells was examined with a Zeiss LSM510 (confocal) laser scanning microscope for fluorescence using an excitation wave length of 488 nm and an emission band pass filter of 505 - 530 nm.

Computer-assisted analysis

The SeMNPV F protein was analyzed for several protein motifs using different bioinformatic programs. N-terminal signal sequences were found with the program PSORT II (National Institute for Basic Biology [http://www.nibb.ac.jp]). Possible coiled-coil regions in the protein were predicted using the program PairCoil (version 1.0; MIT Laboratory for computer Science [http://www.lcs.mit.edu]). Comparisons between the SeMNPV F protein with other viral fusion proteins were done with the program FASTA (version 3; EMBL-European Bioinformatics Institute [http://www.ebi.ac.uk]). Sequence alignments were performed with the program ClustalW (EMBL-European Bioinformatics Institute [http://www.ebi.ac.uk]).

RESULTS

Inhibition of furin-mediated cleavage

The ORF8 (*Se8*) of SeMNPV was recently identified to encode the viral fusion (F) protein. A 60 kDa C-terminal subunit (F_1) of the primary *Se8* translation product was present in the envelope of BVs, which were purified from hemolymph of infected *S. exigua* larvae (IJkel *et al*., 2000). The amino acid sequence RSKR immediately upstream of the N-terminus of the 60 kDa subunit F_1 is consistent with a consensus cleavage site of furin-like endoproteases (IJkel *et al*., 2000) and suggests that F_1 is a cleavage product of a precursor F_0 of the envelope fusion protein. To demonstrate that F_0 is subjected to proteolytic cleavage by furin and that cleavage is important for infectivity, the furin inhibitor dec-RVKR-cmk was used. This inhibitor penetrates into cells (Garten *et al.,* 1989; Hallenberger *et al*., 1992; Richt *et al*., 1998; Stieneke-Gröber *et al*., 1992), and thus may inhibit the production of infectious SeMNPV BV.

Supernatants of SeMNPV infected Se301 cells (48 h p.i.), grown in the presence or absence of 50 μM dec-RVKR-cmk, were assayed for the amount of infectious progeny BV, using a $TCID_{50}$ assay. In the presence of the furin inhibitor the virus titer was reduced dramatically to 2% of the control (absence of the inhibitor) (Fig. 3.1A, lanes 1-2). This drop could be either caused by distortion in the BV assembly pathway, giving no new progeny BVs, or by progeny BVs that are not infectious anymore due to the presence of uncleaved fusion protein (F_0) in their envelopes. In a similar experiment with AcMNPV the furin inhibitor had no effect on the virus titer (Fig. 3.1A, lanes 3-4), indicating that the furin inhibitor did not affect the formation of BV per se.

To exclude the possibility that the drop in SeMNPV virus titer was due to an impairment of the SeMNPV BV assembly pathway, progeny BVs were isolated from cell cultures incubated in the presence or absence of dec-RVKR-cmk. Western analysis with polyclonal antibodies against F_1 (α-F_1) revealed the presence of a 60 kDa protein (F_1) in

both BV preparations (Fig. 3.1B, lanes 1-2). BV obtained from cell cultures incubated in the presence of the furin inhibitor possessed an additional 80 kDa protein (F_0) (Fig. 3.1B, lane 2). These experiments showed that blocking the furin-mediated cleavage of F_0 did not impair the assembly of BVs.

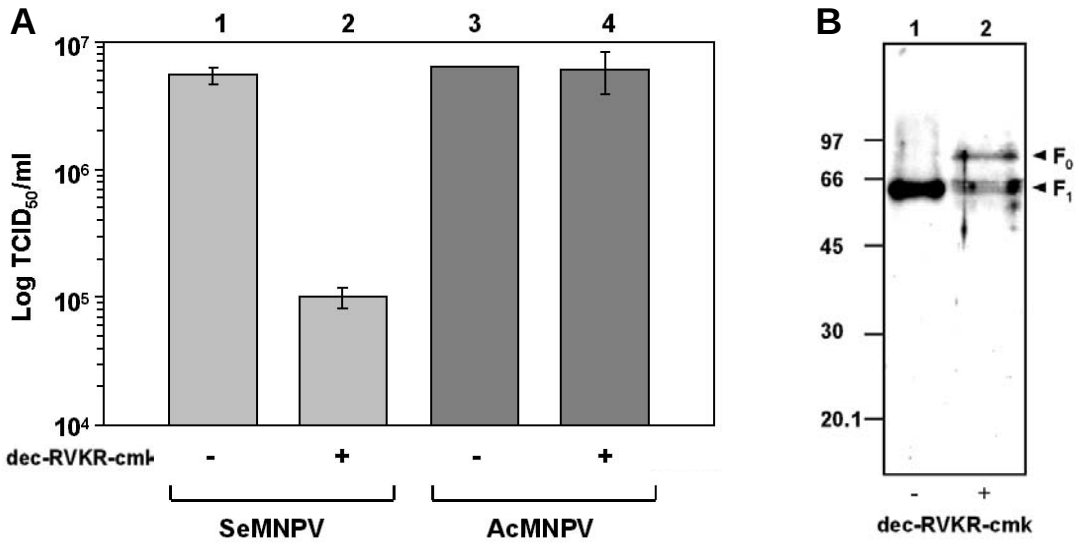

Figure 3.1 Effect of furin inhibitor dec-RVKR-cmk on SeMNPV and AcMNPV BV infectivity. A) Se301 cells were infected with SeMNPV (1, 2) or AcMNPV (3, 4) in the presence (2, 4) or absence (1, 3) of the furin inhibitor dec-RVKR-cmk. At 48 h p.i. the titer of progeny infectious BV in the cell culture supernatants was determined by $TCID_{50}$ assays. The data presented are means and standard deviations of three independent experiments. B) Western blot analysis of proteins obtained from SeMNPV BV, produced in the absence (-) (lane 1) or presence (+) (lane 2) of the furin inhibitor dec-RVKR-cmk. BVs were incubated for 10 min at 95°C in Laemmli buffer, and subjected to SDS-PAGE (12% polyacrylamide). Proteins were transferred onto an Immobilon-P membrane (Millipore), incubated with α-F_1, and detected with a chemiluminescent substrate. Size standards in kDa are indicated on the left. F_0 = precursor SeMNPV F protein; F = 60 kDa subunit of SeMNPV F protein.

SeMNPV F protein with mutated cleavage site is unable to trigger fusion

Previously, it was demonstrated that acidification of the medium of SeMNPV infected Se301 cells resulted in syncytium formation (IJkel *et al*., 2000). To determine whether inhibition of furin-mediated cleavage, would also inhibit activation of membrane fusion activity by the F protein, Se301 cells were infected with SeMNPV in the presence of dec-RVKR-cmk. After the pH drop to 5.0 cells were examined for their ability to form syncytia (48 h p.i.). Control cells infected in the absence of the furin inhibitor showed cell-to-cell fusion (Fig 3.2A), whereas in the presence of the furin inhibitor cell-to-cell fusion was not observed (Fig. 3.2B). This suggests that the uncleaved precursor F_0 is unable to trigger cell-to cell fusion.

To obtain direct evidence that the F_0 protein of SeMNPV has no fusiogenic activity, the consensus furin cleavage site RSKR of the F protein was mutated by changing the arginine (R) at amino acid residue 149 (upstream of the cleavage site) into a lysine (K). First of all it was examined whether the mutant F_{R149K} protein could be transported to the plasma membrane. Sf21 cells, instead of Se301 cells (Se301 cells are difficult to transfect and cell-to-cell fusion is difficult to monitor due to the multimorphic cell phenotypes found in this cell line), were transfected with plasmids, containing either

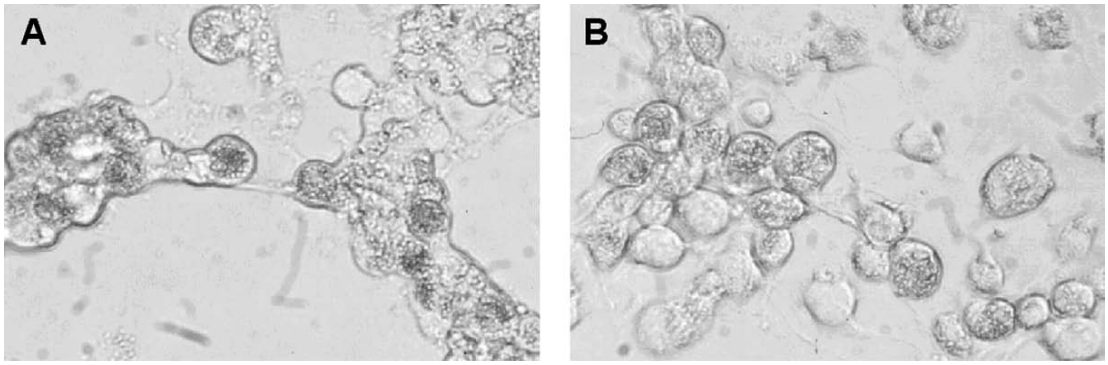

Figure 3.2 SeMNPV mediated fusion of Se301 cells. Cells were infected with SeMNPV in the absence (A) or presence (B) of the furin inhibitor dec-RVKR-cmk. Forty-eight hours p.i. cells were incubated for 2 min with medium, pH 5.0. Syncytium formation was scored 4 h after dropping the pH by phase-contrast microscopy.

wild-type F (p166AcV5-Se8GFP) or the mutant F_{R149K} (p166AcV5-Se8(R149K)GFP) ORF fused C-terminally to GFP under the control of an optimized OpMNPV *gp64* promoter to facilitate expression in insect cells (Blissard and Rohrmann, 1991). Both wild-type F and mutant F_{R149K} proteins were predominantly localized in the plasma membrane, 48 h after the transfection (Fig 3.3A-B). The GFP protein alone (p166AcV5-GFP) showed a homogeneous fluorescent signal in the cytoplasm and nucleus of Sf21 cells (Fig. 3.3C), suggesting that the mutation does not affect the transport of the F protein to the plasma membrane.

To examine whether the F_{R149K} protein is able to trigger cell-to-cell fusion upon lowering the pH of the medium, Sf21 cells were transfected with similar constructs without the C-terminal GFP fusion. Cells transfected with the wild-type F construct

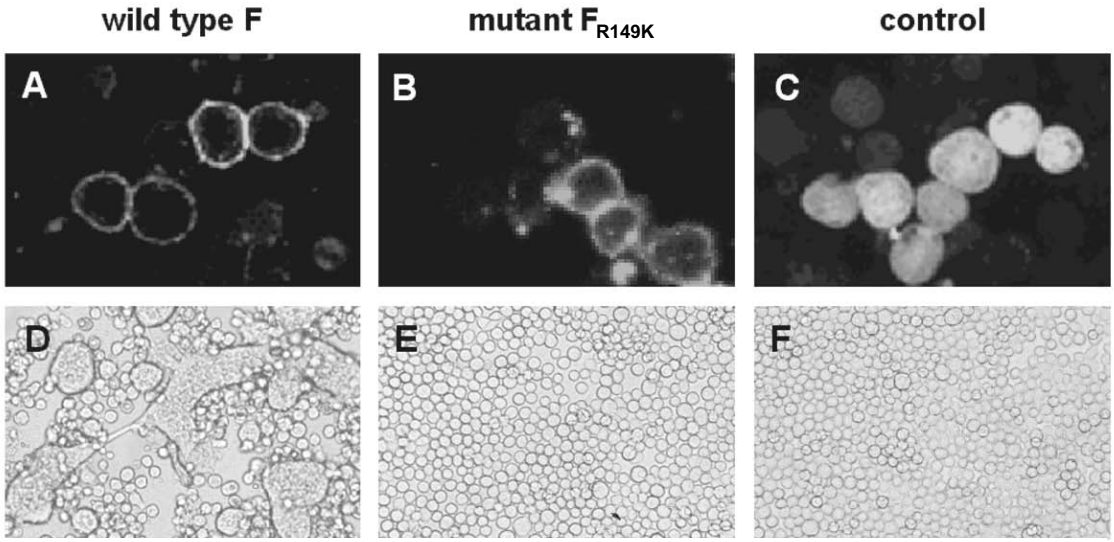

Figure 3.3 Localization studies with SeMNPV wild type F and mutant F_{R149K} GFP-fusion proteins (A-C) and their ability to mediate cell-to-cell fusion in Sf21 cells (D-F). For the localization studies, Sf21 cells were transfected with plasmid p166AcV5-Se8GFP (A), p166AcV5-Se8(R149K)GFP (B), or with control plasmid p166AcV5-GFP (C). Fluorescence was examined 48 h after transfection by confocal laser scanning microscopy. For pH-dependent membrane fusion, Sf21 cells were transfected with plasmid p166AcV5-Se8 (D), p166AcV5-Se8(R149K) (E), or control p166BRNX-AcV5 plasmid (F). Forty-eight h after transfection, cells were treated for 2 min with medium, pH 5.0. Four h after the pH drop syncytium formation was scored by phase-contrast microscopy.

(p166AcV5-Se8) clearly showed pH-dependent cell-to-cell fusion (Fig 3.3D). The F_{R149K} protein (p166AcV5-Se8(R149K)) did not trigger cell-to-cell fusion (Fig. 3.3E), similar to the cells transfected with the empty vector (p166BRNX-AcV5) (Fig. 3.3F). So, it is concluded that the SeMNPV F protein requires cleavage by furin to become active as a fusion protein.

SeMNPV BV contains both F protein subunits

Cleavage of the F_0 precursor will yield, besides the 60 kDa C-terminal cleavage product (F_1), a product of approximately 15 kDa (the N-terminal subunit minus the signal peptide) (F_2). Several proteins in the 15-17 kDa range have been reported from BVs purified from hemolymph of SeMNPV-infected *S. exigua* larvae (IJkel *et al*., 2000).

To determine whether SeMNPV BV contains both mature F subunits (F_1 and F_2) and whether cleavage of the SeMNPV F_0 protein occurs in tissue culture as well as in insects, BVs were purified from cell culture supernatants of SeMNPV-infected Se301 cells and from hemolymph obtained from SeMNPV-infected *S. exigua* larvae. Western analysis with polyclonal antibodies against F_1 (α-F_1) revealed the presence of a 60 kDa protein in BVs obtained from hemolymph as well as from cell culture (Fig. 3.4, lanes 1-2). Polyclonal antibodies specific against F_2 (α-F_2) detected a 21 kDa protein in both BV preparations (Fig. 3.4, lanes 4-5). Neither antibodies against F_1 nor against F_2 reacted with ODV derived proteins (Fig. 3.4, lanes 3 and 6), demonstrating that both F protein subunits are BV specific.

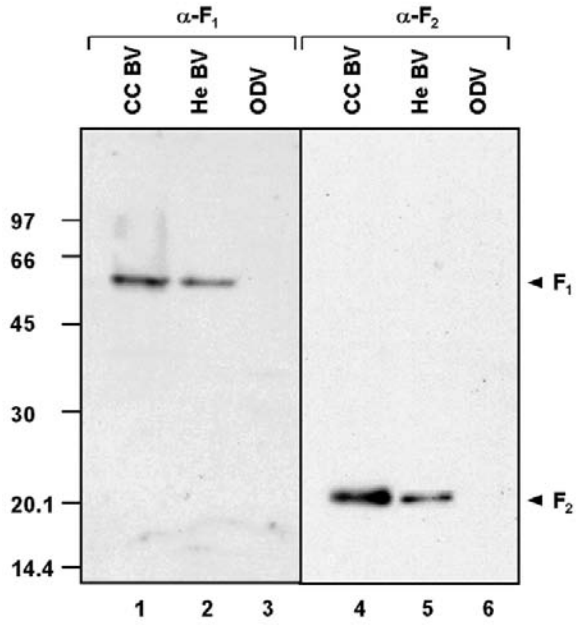

Figure 3.4 Western blot analysis of reduced proteins of SeMNPV virus preparations. Cell culture derived (CC) BVs, hemolymph derived (He) BVs and polyhedra derived ODVs were incubated for 10 min at 95°C in Laemmli buffer, and subjected to SDS-PAGE (12% polyacrylamide). Proteins were transferred onto an Immobilon-P membrane (Millipore), incubated with either α-F_1 (left panel) or α-F_2 (right panel) antibodies, and detected with a chemiluminescent substrate. Size standards in kDa are indicated on the left. F_1 = 60 kDa subunit of SeMNPV F protein; F_2 = 21 kDa subunit of SeMNPV F protein.

Processing and oligomerization of the SeMNPV F protein

The observation that both subunits, F_1 and F_2, were present in SeMNPV BV, raised the question whether these subunits interacted with each other. The possibility exists that these subunits remain noncovalently associated, like the fusion protein of the *Retroviridae* (Env) (Veronese *et al*., 1985), or covalently by disulfide-linkage as found for fusion proteins in members of the *Orthomyxoviridae* (HA), *Herpesviridae* (gB), and

Paramyxoviridae (F) (Lazarowitz *et al*, 1971; Meyer *et al*, 1990; Schied and Choppin, 1977).

To determine the type of interaction between F_1 and F_2, SeMNPV BV proteins were treated with iodoacetamide, which alkylates free sulfhydryl groups and thereby prevents artificial disulfide exchange during further treatment or electrophoresis (Gray, 1997). The proteins were denatured in the absence of reducing agents and electrophoresed in a 12% polyacrylamide gel. Western analysis with α-F_1 and α-F_2 detected in both cases a single protein with a molecular weight of 80 kDa ($F_{1,2}$) (Fig. 3.5A, lanes 1-2). The absence of free F_1 and F_2 indicates that these subunits remain covalently linked after cleavage.

To investigate whether F proteins from SeMNPV also form disulfide-linked oligomers, a nonreducing SDS-PAGE was performed, using a 6% polyacrylamide gel. The antibodies α-F_1 and α-F_2 detected a major monomeric form of 80 kDa (Fig. 3.5B lanes 1-2). The antibody α-F_2 detected also a possible dimeric form as a minor band of 155 kDa (Fig 3.5B, lane 2), higher multimeric forms were not detected.

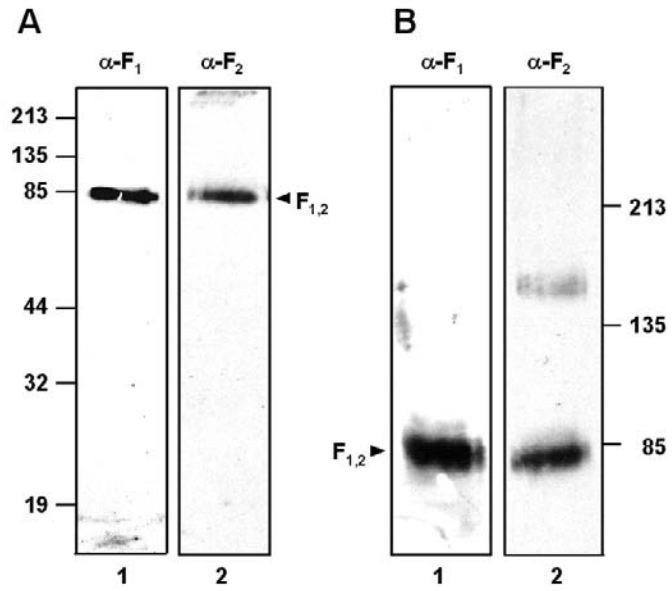

Figure 3.5 Western blot analysis of nonreduced proteins of SeMNPV BV. Cell culture derived BVs were incubated for 10 min at 95°C in the presence of 37.5 mM iodoacetamide and absence of 2-mercaptoethanol and electrophoresed in a 12% (A) or a 6% (B) SDS-polyacrylamide gel. Proteins were transferred onto an Immobilon-P membrane (Millipore), incubated with either α-F_1 or α-F_2, and detected with a chemiluminescent substrate. Size standards are indicated in kDa. $F_{1,2}$ = 60 and 21 kDa disulfide-linked subunits of SeMNPV F protein.

DISCUSSION

Post-translational cleavage of fusion proteins of enveloped viruses seems to represent a general mechanism used to regulate viral membrane fusion activity. All members of the *Orthomyxoviridae, Paramyxoviridae, Togoviridae, Coronaviridae, Retroviridae* and *Herpesviridae* express their fusion protein as a precursor, which has to be cleaved by host proteases to render infectious virus (Klenk and Garten, 1994). The cleavage usually takes place in front of a hydrophobic sequence, the “fusion peptide”. Exposure to low pH or binding to a receptor triggers a conformational change in which the fusion peptide is believed to translocate and insert itself into the target membrane (Klenk and Garten, 1994).

In this report it is demonstrated that SeMNPV, a representative of a group II NPV

within the *Baculoviridae*, also expresses its fusion protein as a precursor F_0. This precursor is post-translationally cleaved by insect cell furin into two disulfide-linked subunits, F_1 and F_2 to become active in tissue culture and in insects. The major envelope protein of HaSNPV BV, the product of *Ha133*, has also been identified and characterized (Chen *et al.,* 2001; M. Westenberg, and H. Z. Wang, unpublished data). This protein turned out to be also a C-terminal cleavage product of the SeMNPV F protein homolog. In contrast to the SeMNPV and HaSNPV F proteins, the LdMNPV BV F protein had been reported not to be cleaved (Pearson *et al*., 2000), despite the fact that it possesses a similar furin cleavage site at an analogous position, as in SeMNPV and HaSNPV. An 83 kDa fusion protein was detected in LdMNPV BV purified from cell culture supernatants. Tunicamycin treatment of the cells resulted in a LdMNPV fusion protein with a molecular weight of 72 kDa. The latter value is consistent with a predicted molecular weight of 75 kDa for the whole ORF minus the signal sequence (residues 1-16). Therefore it is most likely that Ld625Y cells are unable to properly cleave the fusion protein from LdMNPV BV. However the titers of progeny BVs from Ld625Y cells are even higher (Riegel and Slavicek, 1997) than that of SeMNPV in Se301 cells. So it is very possible that the fusion protein of LdMNPV can be activated during viral uptake by Ld625Y cells similar to the influenza virus A/WSN/33 (H1N1) in vertebrate cells. This virus possesses only the uncleaved hemagglutinin, which is cleaved during uptake presumably by an endosomal protease (Boycott *et al*., 1994).

The cleavage of the F_0 protein by furin was demonstrated by the use of a furin inhibitor at a concentration of 50 μM, which seemed to be the most effective concentration (data not shown). The titers of the progeny BVs produced in the presence of the inhibitor were about 2% of the control (Fig. 3.1A). The 2% residual infectious virus is in agreement with similar findings with other viral systems (Hallenberger *et al*., 1992; Ortmann *et al*., 1994; Richt *et al*., 1998), and is probably the result of the instability of the furin inhibitor in aqueous solutions. However, it cannot be excluded that a small proportion of the F_0 protein is cleaved by another unknown protease. Infected cells were also unable to form syncytia in the presence of the inhibitor (Fig. 3.2), which is consistent with earlier reports that cell-to-cell fusion after viral infection could be completely blocked by peptidyl-chloroalkylketones (Hallenberger *et al*., 1992; Stieneke-Grober *et al*., 1992). BV assembly was not impaired in the presence of the furin inhibitor (Fig. 3.1B). Nevertheless the amount of F_1 protein versus F_0 protein is not comparable with the drop in virus titer, but is in agreement with similar findings for influenza virus where the cleavage inhibition of HA_0 was not comparable to the drop in hemagglutination titer (Stieneke-Grober *et al*., 1992). For influenza viruses and group I NPVs it has been shown that at least three HA trimers (Blumenthal *et al*., 1996; Danieli *et al*., 1996) and approximately six GP64 trimers (Plonsky and Zimmerberg, 1996), respectively, cooperate in the formation of the fusion pore. So, the presence of even low amounts of F_0 in BVs can interfere with the mature fusion proteins in the fusion process.

The cleavage products, F_1 and F_2, are covalently associated, similar to the fusion proteins of members of the *Orthomomyxoviridae*, *Herpesviridae*, and P*aramyxoviridae* (Lazarowitz *et al*, 1971; Meyer *et al*, 1990; Schied and Choppin, 1977). The F_2 subunit of SeMNPV contains only a single cysteine residue (C94), which make this cysteine the

only candidate to form the disulfide-bridge with F_1. This cysteine residue is conserved among the F protein homologs of HaSNPV (Chen *et al*., 2001) and LdMNPV (Kuzio *et al*., 1999). In contrast, the F_1 subunit contains 15 cysteine residues. Ten of these are situated upstream of the predicted transmembrane region of which 9 are conserved among the group II NPVs (SeMNPV, LdMNPV, HaSNPV). The molecular weight of the F_1 subunit (60kDa) is in close agreement with the molecular weight of 59 kDa predicted from the DNA sequence. The F_2 subunit was detected in SDS-PAGE as a 21 kDa peptide (Fig 3.4). Upon maturation the F_2 subunit might go through some post-translational modification processes, including N-glycosylation and O-glycosylation (IJkel *et al*., 2000), which may explain the difference between the theoretical (15 kDa) and actual (21 kDa) size of this subunit.

Viral fusion proteins generally form higher-order oligomers (Hernandez *et al*., 1996). In most cases these oligomers are formed by noncovalent interactions. However, the envelope fusion protein GP64 of group I NPVs forms a trimer of disulfide-linked monomers (Oomens *et al*., 1995). The F protein of SeMNPV was predominantly present as a monomer (fig 3.5B). A minor band approximately twice the size of the monomer was also detected. Although this band could represent a dimeric form of the protein, most likely this is due to incomplete denaturation, since this band was only detected by α-F_2. Furthermore, treatment of GP64 of AcMNPV under the same conditions resulted in the simultaneous occurrence of trimers together with minor dimers and monomers (data not shown). Therefore, it is concluded that disulfide-links are most likely not involved in oligomerization of the F protein. The potential oligomeric structure of the SeMNPV F protein remains to be characterized.

The SeMNPV F protein is very similar in structure to the paramyxovirus fusion protein. Both consist of a small N-terminal subunit linked by a disulfide-bridge to the large C-terminal membrane-anchored subunit. The SeMNPV F protein has the highest homology with the paramyxovirus human respiratory syncytial virus fusion protein [P03420], with 12% amino acid identity and 36% similarity. Although the overall homology is not high (12%), predicted domains as α-helices and β-sheets, as well as the furin cleavage sites, the heptad repeats and the transmembrane regions are structurally conserved. Furthermore, the fusion protein of the paramyxoviruses contains a fusion peptide, which is located at the N-terminus of the large membrane anchored subunit. Presumably the SeMNPV F protein contains a putative fusion peptide at an analogous position. Amino acids 150-166 GLFNFMGHVDKYLFGIM contain nine hydrophobic residues (underlined) with hydrophobicity indices (H.I.) higher than 0.62 according to the normalized consensus scale of Eisenberg (Eisenberg, 1984). These hydrophobic residues can be modeled as a hydrophobic face in an α-helix. The average H.I. of the hydrophobic face is 1.1 and the total amino acid stretch has a H.I. of 0.5, which is consistent with other viral fusion proteins (White, 1992).

The paramyxovirus fusion proteins contain heptad repeats (HR1 and HR2) immediately adjacent to the fusion peptide and the transmembrane domain (Lamb, 1993). Previous studies with other viral fusion proteins suggested that these HRs form trimeric hairpin-like structures, with the HR2 regions packing in an antiparallel manner against the inner coiled-coil formed by the HR1 regions. The likely role of the hairpin

structure is to facilitate apposition of the viral and cellular membranes by bringing the fusion peptide, which inserts into the cellular membrane, close to the transmembrane segment, which is anchored in the viral membrane (Singh *et al*., 1999). The SeMNPV F protein also contains two putative HRs. The first HR is adjacent to the putative fusion peptide and is formed by amino acid residues 174-202 (HR1: **L**HM**L**ANT**T**NS**L**NSQ**V**KQ**L**NDE**L**IV**L**ADY**V**). The second putative HR is adjacent to the transmembrane domain and is formed by amino acid residues 521-549 (HR2: **V**DD**M**KIN**N**DL**E**KTN**L**HE**L**TSR**L**YD**L**RRR**I**). Further studies are in progress to confirm whether those motifs indeed are required for the fusion function.

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Chapter 4

F PROTEINS OF GROUP II NPVS ARE FUNCTIONALLY ANALOGOUS TO ACMNPV GP64

SUMMARY

GP64, the major envelope glycoprotein of budded virions of the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), is involved in viral attachment, mediates membrane fusion during virus entry, and is required for efficient virion budding. Thus, GP64 is essential for viral propagation in cell culture and in animals. Recent genome sequences from a number of baculoviruses show that only a subset of closely related baculoviruses have *gp64* genes, while other baculoviruses have a recently discovered unrelated envelope protein named F. F proteins from *Lymantria dispar* (Ld)MNPV (LdF) and *Spodoptera exigua* (Se)MNPV (SeF) mediate membrane fusion and are therefore thought to serve roles similar to that of GP64. To determine whether F proteins are functionally analogous to GP64 proteins, the *gp64* gene from an AcMNPV bacmid was deleted and F protein genes from three different baculoviruses were inserted. In addition, also the envelope protein genes from vesicular stomatitis virus (VSV) and Thogoto virus were inserted. Transfection of the *gp64*-null bacmid DNA into Sf9 cells does not generate infectious particles, but this defect was rescued by introducing either the F protein gene from LdMNPV or SeMNPV or the G protein gene from VSV. These results demonstrate that baculovirus F proteins are functionally analogous to GP64. Because baculovirus F proteins appear to be more widespread within the family and are much more divergent than GP64 proteins, *gp64* may represent the acquisition of an envelope protein gene by an ancestral baculovirus. The AcMNPV pseudotyping system provides an efficient and powerful method for examining the functions and compatibilities of analogous or orthologous viral envelope proteins, and it could have important biotechnological applications.

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INTRODUCTION

The baculoviruses are a large family of enveloped DNA viruses that are pathogenic to invertebrates, infecting primarily insects in the order *Lepidoptera*, but also certain members of the *Diptera* and *Hymenoptera* (Blissard *et al*., 2000; Friesen and Miller, 2001). Baculoviruses have supercoiled, double-stranded DNA genomes of approximately 80 to 180 kbp. Baculoviruses are classified into two genera, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs), and the NPVs can be further subdivided into groups I and II based on phylogenetic studies of many genes (Bulach *et al*., 1999; Hayakawa *et al*., 2000; Herniou *et al*., 2001).

GP64 is the major envelope protein of the budded-virus (BV) form of group I NPVs such as *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Orgyia pseudotsugata* (Op)MNPV. GP64 is involved in viral attachment and is required for low-pH-mediated membrane fusion during virus entry (Blissard and Wenz, 1992; Hefferon *et al*., 1999). GP64 is also required for efficient budding of the BV (Oomens and Blissard, 1999). Thus, GP64 is required for propagation of infection in cell culture and in *Trichoplusia ni* larvae (Monsma *et al*., 1996). Although GP64 proteins have been identified in other group I NPVs, recent data from the complete genomic sequences of a number of baculoviruses suggest that *gp64* genes are not present in group II NPVs or GVs. An envelope protein gene that is unrelated to *gp64* was recently identified in *Lymantria dispar* (Ld)MNPV (Pearson *et al*., 2000) and *Spodoptera exigua* (Se)MNPV (IJkel *et al*., 2000), and homologs of this gene have been identified in other group II NPVs and GVs. This protein has been named the F (fusion) protein (Westenberg *et al*., 2002). F proteins from LdMNPV (ORF 130, or LdF) and SeMNPV (ORF 8, or SeF) are present in the virion membrane and mediate pH-triggered membrane fusion (IJkel *et al*., 2000; Pearson *et al*., 2000). Like envelope proteins from some other virus groups, the baculovirus F proteins are cleaved internally by cellular proprotein convertases such as furin, and cleavage is necessary for fusion activity (Westenberg *et al*., 2002). These observations suggest that F proteins may represent a functional analog of GP64 in some (perhaps all) baculoviruses that do not have a *gp64* gene. To experimentally address this possibility, the *gp64* locus was deleted of a bacterial artificial chromosome containing an AcMNPV genome (bacmid) (Luckow *et al*., 1993) and inserted F protein genes from two group II NPVs and a GV. It is shown that the F protein genes from LdMNPV and SeMNPV can rescue the *gp64* knockout in AcMNPV. In addition, it was shown show that the predicted furin cleavage site present in the SeMNPV F protein is required for processing of the SeMNPV F protein and rescue of the *gp64*-null virus. Also the functions of orthomyxovirus and rhabdovirus envelope proteins were examined in the *gp64*-null pseudotyping system.

MATERIALS AND METHODS

gp64-null AcMNPV bacmid

The *gp64* gene of an AcMNPV bacmid (bMON14272; Invitrogen) was deleted by using a modification of the method reported by Bideshi and Federici (Bideshi and

Federici, 2000). A chloramphenicol resistance gene (*cat*) cassette was amplified by PCR with primers P5'Spe(ChlR) (5'-ACTAGTGCTGGATCGGGCCCTAAATACCTG-3') and P3'(ChlR)Bgl-II (5'-AGATCTTTACGCCCCGCCCTGCCACTCATC-3') and with plasmid pRE112 as a template (Edwards *et al*., 1983). The PCR product was cloned into the pCR-II-Blunt vector (Invitrogen) to generate plasmid pChl^{R}-CRIIblunt. The insert containing the *cat* cassette was excised from pChl^{R}-CRIIblunt by *Spe*I and *Bgl*II digestion and was used to replace the *Spe*I-*Bgl*II fragment (the *gp64* gene) in pAcEcoHΔSma, a plasmid containing the AcMNPV *gp64* locus and flanking sequences (Monsma *et al*., 1996). The resulting plasmid, pAcEcoHΔSma,gp64(Chl^{R}), was digested with *Eco*RI and *Hin*dIII; 60 ng of the gel-purified *Eco*RI-*Hin*dIII fragment containing the *cat* cassette was cotransformed with 1 µg of bMON14272 into electrocompetent BJ5183 cells (Stratagene); and colonies resistant to both kanamycin and chloramphenicol were selected.

Donor plasmids containing envelope protein genes

Genes (*LdF, SeF*, *PxF, VSV-G* and *gp75*) encoding heterologous viral envelope proteins and AcMNPV *gp64* (as a positive control) were amplified by PCR using Vent DNA polymerase (New England Biolabs) and cloned downstream of the AcMNPV *gp64* promoter on a modified pBacGus5 plasmid (Novagen), pmBG5. pmBG5 was modified by digesting pBacGus5 with *Nco*I and *Sma*I to remove sequences encoding His-tags and protease cleavage sites. The 5' overhang was filled in with Klenow fragment and religated, so that the AcMNPV *gp64* early-plus-late promoter was immediately upstream of a multiple cloning site and a ß-glucuronidase gene (GUS) reporter driven by a *p6.9* late viral promoter. As an example, the AcMNPV *gp64* open reading frame (ORF) was PCR amplified with primers P5'Bam2327Acgp64 (5'-GGATCCAAGATGGTAAGCGCTAT TGTTTT-3') and P3'AcEcoHdstopE (5'-GAATTCTTAATATTGTCTATTACGGTTTCTA-3') by using pAcEcoHΔSma as a template, digested with *Bam*HI and *Eco*RI, and cloned into similarly digested pmBG5. The *gp64* promoter and ORF were subsequently PCR amplified and subcloned into pΔFBgus(R), a donor plasmid of pFastBac1 (Invitrogen) from which the *polyhedrin* promoter had been removed and replaced with the GUS cassette from pBacGUS5. Genes for heterologous envelope proteins were similarly amplified, cloned into pmBG5, and subcloned into pΔFBgus(R). In some cases, genes (*LdF, PxF,* and *gp75*) were placed under the control of the stronger *polyhedrin* promoter to increase expression. For the *PxF*, *VSV-G, gp75* and *LdF* constructs under the control of the *gp64* promoter, the *p6.9* promoter-GUS cassette was inserted in the orientation opposite that shown in Fig. 4.1 (with the GUS transcription oriented toward Tn7R). All clones containing PCR-derived sequences were confirmed by sequencing. To confirm expression of PxF, sequences encoding a c-myc epitope tag were fused to the 3' end of the *PxF* ORF. Primers P5'Px996 (5'-GACCCTCGATTTCATGACGCTGTG-3') and P3'Px26cmycEcoRI (5'-GTGAATTCCTACAGATCCTCTTCTGAGATGAGTTTTTGTTCC AGCGGTTTAAGTATCG-3') were used to amplify the 3' end of *PxF* and to add a c-myc epitope tag by PCR using pΔFBgusPx26 as a template. The PCR product was cloned into the pCR-4-Blunt vector (Invitrogen), released by *Sac*I and *Eco*RI, and used to

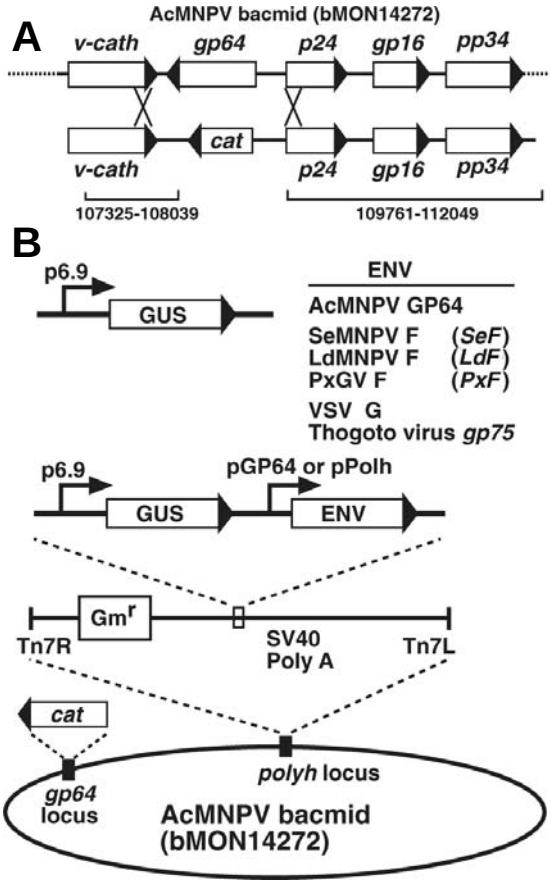

Figure 4.1 (A) Strategy for generation of a *gp64*-null AcMNPV bacmid by homologous recombination in *E. coli*. The *gp64* locus of the AcMNPV bacmid (bMON14272) is shown above the *Eco*RI-*Hin*dIII fragment that was used as a transfer vector to replace the *gp64* locus with a chloramphenicol resistance gene (*cat*). Sequences included for homologous recombination (107325 to 108039 and 109761 to 112049) are indicated (Ayres *et al*., 1994). (B) Strategy for insertion of envelope protein gene constructs into the *polyhedrin* locus of the *gp64*-null AcMNPV bacmid. Inserts include a *p6.9* promoter-GUS reporter and no envelope protein gene (top) and a *p6.9* promoter-GUS reporter plus envelope protein genes (ENV) under the control of either the *gp64* promoter (pGP64) or the *polyhedrin* promoter (pPolh) (center). Envelope protein gene cassettes were inserted into the *att b* site (indicated by right and left insertion sites, Tn7R and Tn7L) in the *polyhedrin* locus by Tn*7*-based transposition. Envelope proteins and genes (italicized) are listed on the right.

replace the 3' end of the *PxF* gene in pΔFBgusPx26, generating plasmid pΔFBgusPx26c-myc.

Transpositions of inserts from donor plasmids into the *gp64*-null bacmid were confirmed by gentamicin resistance, blue-white screening according to the BAC-to-BAC manual (Invitrogen), and PCR using a primer corresponding to sequences in the GUS reporter gene in the donor plasmid (P-gus-jm [5'-GTGAAGAGTATCAGTGTGCATG-3']) in combination with a primer corresponding to bacmid sequences adjacent to the transposition site (either the M13/pUC forward primer [5'-CCCAGTCACGACGTTGTAAA ACG-3'] or the M13/pUC reverse primer [5'-AGCGGATAACAATTTCACACAGG-3']).

Transfection-infection assay

DNAs were prepared from 1.5 ml cultures of 2 to 3 independent colonies carrying the bacmid with the inserted heterologous envelope protein gene according to the BAC-to-BAC manual (Invitrogen) and were analyzed in parallel. For transfections, 12.5% of each DNA preparation was used to transfect 9.0 x 10^5 Sf9 or Sf9^{Op1D} (Plonsky *et al*., 1999) cells in a 6-well plate by calcium phosphate precipitation. At 72 h (protocol 1) or 10 days (protocol 2) posttransfection, the supernatant (passage 1) was centrifuged for 10 min at 2,200 x *g* to remove debris, and this clarified supernatant (50 or 500 µl) was used to infect 9.0 x 10^5 Sf9 cells for 48 h (protocol 1) or 72 h (protocol 2). Both transfected and infected cells were stained for GUS activity (according to the BAC-to-BAC manual, Invitrogen) to monitor transfection efficiency and to detect infection by virions generated in transfected cells. Supernatants from infected Sf9 cells (passage 2) were stored at 4°C. In all cases where rescue was not observed by using protocol 1, no rescue was

observed by using protocol 2. As a control to confirm that no lethal mutation had been acquired during propagation of the AcMNPV bacmid in *Escherichia coli*, all bacmid DNAs were also used to transfect Sf9^{Op1D} cells, which express a GP64 protein (Plonsky *et al*., 1999) and the supernatants from those transfections were used to infect Sf9 cells.

Transfections into CHO cells were performed with Lipofectin (Invitrogen), and 3 µg of DNA was used to transfect 70% confluent cells in 60-mm plates according to the manufacturer's instructions.

Virus amplification and preparation

Viruses carrying envelope protein genes that rescued the *gp64*-null phenotype were amplified by infecting one T150 flask containing 1.0 x 10^7 Sf9 cells with 500 µl of passage 2 supernatant, and cells were split every 3 to 5 days for 10 to 13 days. Viruses carrying envelope proteins that did not rescue the *gp64* deletion were amplified in a similar manner but with Sf9^{Op1D} cells and Sf9^{Op1D} cell-derived supernatant. Amplified pseudotyped viruses were titered on Sf9^{Op1D} cells by a $TCID_{50}$ (50% tissue culture infective dose) assay (O'Reilly *et al*., 1992) and scored for infection by examining cells for GUS expression. Budded virions were purified by centrifugation through a 25% sucrose pad, and Western blot analysis was performed as described previously (Mangor *et al*., 2001).

RESULTS

Disruption of the *gp64* gene in an AcMNPV bacmid

The critical roles that GP64 plays in AcMNPV budding and propagation of infection were demonstrated by early physical and functional studies (Blissard and Wenz, 1992; Hefferon *et al*., 1999; Hohmann and Faulkner, 1983; Leikina *et al*., 1992; Volkman *et al*., 1984) and by studies of *gp64*-null viruses that were generated by homologous recombination in insect cells (Monsma *et al*., 1996; Oomens and Blissard, 1999). To determine whether the recently identified baculovirus F proteins serve as functional analogs of GP64 in baculoviruses with no *gp64* gene, an AcMNPV bacmid (Luckow *et al*., 1993) was modified by first deleting the *gp64* gene and then inserting heterologous envelope protein genes. The *gp64* gene of the AcMNPV bacmid bMON14272 was deleted by homologous recombination in *recA*$^+$ *E. coli* BJ5183 cells by using a linear *Eco*RI-*Hin*dIII fragment of DNA containing a *cat* cassette flanked by 2,288 bp of sequences upstream, and 714 bp of sequences downstream, of the *gp64* locus (Fig. 4.1A). Three colonies resistant to kanamycin and chloramphenicol were selected and confirmed by PCR to contain the desired recombinant *gp64*-null bacmid (data not shown). All three *gp64*-null bacmids failed to propagate an infection after transfection into Sf9 cells, while in parallel experiments, the original bacmid was capable of initiating a spreading infection (data not shown). *gp64*-null bacmid DNA was then transformed into DH10β cells containing a helper plasmid (pMON7124) that encodes a Tn*7* transposase (Luckow *et al*., 1993). The resulting *E. coli* strain (gp64$^-$ bacmid-DH10B+pMON7124) was used to insert reporter and envelope protein genes into the *gp64*-null bacmid for subsequent studies. To examine the abilities of several heterologous envelope proteins

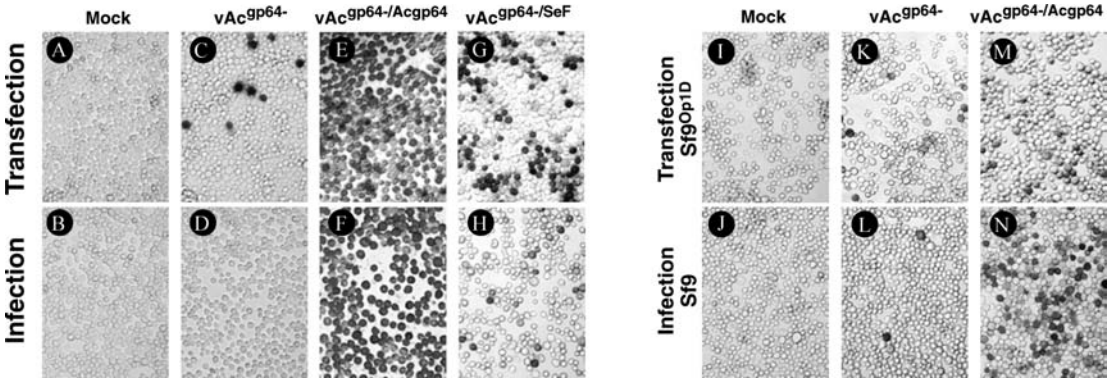

Figure 4.2 Transfection-infection assay for viral propagation. Sf9 cells were transfected with the indicated bacmids, incubated for 72 h, and stained for GUS activity (A, C, E, and G). Supernatants from the transfected cells (top panels) were used to infect Sf9 cells (B, D, F, and H), which were incubated for 48 h and then stained for GUS activity. Staining of cells in the lower panels (infected cells) indicates that infectious virions were generated in the transfected cells. Bacmids that fail to propagate an infection in Sf9 cells can propagate an infection in cells expressing OpMNPV GP64 ($Sf9^{Op1D}$). $Sf9^{Op1D}$ cells were transfected with the indicated bacmids (I, K, and M) and incubated for 72 h; then supernatants were transferred to Sf9 cells and stained for GUS activity after 48 h (J, L, and N).

to rescue the *gp64*-null AcMNPV, each heterologous envelope protein gene was inserted into the *polyhedrin* locus of the above *gp64*-null bacmid by using a donor plasmid and Tn*7*-based transposition (Luckow *et al*., 1993). Each donor plasmid contained a GUS reporter gene plus the heterologous envelope protein gene under the control of the AcMNPV *gp64* promoter. In some cases, the envelope protein gene was also placed under the control of the much stronger *polyhedrin* promoter (Fig. 4.1B). As a positive control, the AcMNPV *gp64* gene was inserted into a donor plasmid. A donor plasmid containing no envelope protein gene served as a negative control. The resulting bacmids were named vAc^{gp64-} (no *gp64* gene), $vAc^{gp64-/Acgp64}$ (*gp64* reinserted), and $vAc^{gp64-/env}$ ("env" stands for the heterologous *envelope* protein gene that was inserted) (Fig. 4.1B).

Following the insertion of each heterologous envelope protein gene, bacmid DNAs from at least two independent colonies were used to separately transfect Sf9 cells, and cells were scored for the spread of infection and generation of infectious virions by a transfection-infection assay (see Materials and Methods). After transfection of Sf9 cells with each bacmid construct, culture supernatants were harvested from transfected cells at 72 h (or, alternatively, at 10 days) posttransfection and used to infect a second group of Sf9 cells, which were incubated for 48 h (or, alternatively, for 72 h). If infectious virions are produced from the transfected cells, infection should be detected in the second group of cells. Both transfected and infected cells were stained for GUS reporter activity to detect entry and infection by the virus. Our results show that although the *gp64*-null (vAc^{gp64-}) bacmid entered cells by transfection and the late promoter was activated, infectious virions were not produced and infection did not spread (Fig. 4.2C and D). In parallel experiments using the *gp64*-null bacmid that was repaired by reinsertion of *gp64* ($vAc^{gp64-/Acgp64}$), infection spread in both transfected and infected cells (Fig. 4.2E and F). Furthermore, transfection of the *gp64*-null bacmid into $Sf9^{Op1D}$ cells (which express GP64) (Plonsky *et al*., 1999) also resulted in generation of infectious virions (Fig. 4.2K and L), indicating that the defect can be rescued by supplying the GP64 protein in *trans*. Thus,

the inability of the *gp64*-null bacmid to propagate an infection is due to the lack of GP64 and not to possible second-site mutations.

Envelope proteins from group II NPVs rescue an AcMNPV *gp64*-null bacmid

To determine whether F proteins from heterologous baculoviruses could rescue the *gp64*-null bacmid, three F protein genes under the control of the AcMNPV *gp64* promoter were made and inserted into the *gp64*-null bacmid (Fig. 4.1B and Table 4.1). Transfection of Sf9 cells with *gp64*-null bacmids carrying the F protein gene from SeMNPV or LdMNPV resulted in rescue of the *gp64*-null AcMNPV and production of infectious virions (Fig. 4.2G and H; Table 4.1, vAc$^{gp64\text{-}/SeF}$ and vAc$^{gp64\text{-}/LdF}$). In contrast, the *gp64*-null bacmid carrying the F protein gene from the *Plutella xylostella* (Px)GV (Table 4.1, vAc$^{gp64\text{-}/PxF}$) (Hashimoto *et al*., 2000) did not produce infectious virions. To ensure that the PlxyGV F protein can be expressed in Sf9 cells, a bacmid containing sequences encoding the PlxyGV F protein with a c-myc epitope tag (vAc$^{gp64\text{-}/PxFc\text{-}myc}$) was generated. After transfection of the vAc$^{gp64\text{-}/PxFc\text{-}myc}$ bacmid into Sf9 cells, the c-myc-tagged PlxyGV F protein was detected by Western blot analysis of infected cell lysates (data not shown), indicating that the PlxyGV F protein could be expressed. However, neither the wild type nor the c-myc-tagged PlxyGV F protein rescued *gp64*-null AcMNPV, suggesting that PlxyGV F was unable to substitute for GP64 function. To determine whether a stronger promoter driving expression of the PlxyGV F protein could result in rescue of the *gp64* deletion, a *gp64*-null bacmid containing the *PxF* gene under the control of the *polyhedrin* promoter (vAc$^{gp64\text{-}/pPolh\text{-}PxF}$) was tested. Although a parallel LdMNPV *f* gene construct (under *polyhedrin* promoter control) complemented GP64 function, the PlxyGV *f* gene construct did not (Table 4.1). When both bacmids encoding PxF were transfected into Sf9^{Op1D} cells, infectious virions were produced (Table 4.1), indicating that the lack of virion production in Sf9 cells was due to lack of a functional envelope protein and not to a second-site mutation. Thus, our results show that the F protein genes from LdMNPV and SeMNPV rescued a *gp64*-null bacmid, but the F protein gene from PlxyGV did not.

Table 4.1 Pseudotyped gp64-null bacmids and viruses

gp64 -null bacmid	Envelope protein	Promoter	Rescue	Titer (IU/ml)[a] in:	
				Sf9 cells	Sf9^{Op1D} cells
Controls					
vAc$^{gp64\text{-}}$	None		-	NA	8.3×10^6
vAc$^{gp64\text{-}/Acgp64}$	AcMNPV GP64	*gp64*	++	2.9×10^8	ND
Baculovirus F proteins					
vAc$^{gp64\text{-}/SeF}$	SeMNPV F	*gp64*	+	2.5×10^7	ND
vAc$^{gp64\text{-}/SeFR149K}$	SeMNPV F_{R149K}	*gp64*	-	NA	2.2×10^7
vAc$^{gp64\text{-}/LdF}$	LdMNPV F	*gp64*	+	5.0×10^6	8.3×10^6
vAc$^{gp64\text{-}/pPolh\text{-}LdF}$	LdMNPV F	*polyh*	+	8.3×10^4	6.9×10^4
vAc$^{gp64\text{-}/PxF}$	PxGV F	*gp64*	-	NA	1.9×10^6
vAc$^{gp64\text{-}/pPolh\text{-}PxF}$	PxGV F	*polyh*	-	NA	1.4×10^7
Other viral Env proteins					
vAc$^{gp64\text{-}/VSV\text{-}G}$	VSV G	*gp64*	+	5.9×10^7	ND
vAc$^{gp64\text{-}/Thogp75}$	Thogoto GP75	*gp64*	-	NA	2.8×10^6
vAc$^{gp64\text{-}/pPolh\text{-}Thogp75}$	Thogoto GP75	*polyh*	-	NA	ND

[a] NA, not applicable; ND, not determined.

Thogoto virus GP75 is incorporated into AcMNPV virions but does not rescue a *gp64*-null bacmid

Two envelope proteins from viruses unrelated to baculoviruses were also examined in these studies: Vesicular stomatitis virus (VSV) G protein and Thogoto virus GP75. VSV-G shows no sequence similarity to either GP64 or F proteins but was included as a positive control, since a previous study showed that it was able to rescue a *gp64* deletion (Mangor *et al*., 2001). In agreement with the findings of that study, the VSV G gene rescued the *gp64* deletion in AcMNPV (Table 4.1). Although the Thogoto virus is a single-stranded RNA virus (orthomyxovirus), the Thogoto virus GP75 envelope protein shares 28% amino acid sequence identity with the AcMNPV GP64 protein and therefore appears to be derived from a common ancestor (Morse *et al*., 1992). Therefore it was asked whether GP75 could rescue a *gp64*-null AcMNPV bacmid. The Thogoto virus *gp75* gene, under the control of either the *gp64* promoter or the *polyhedrin* promoter, failed to rescue the *gp64* deletion in AcMNPV (Table 4.1). Expression of Thogoto virus GP75 in transfected Sf9 cells and the presence of GP75 in pseudotyped virions were confirmed by Western blot analysis (Fig. 4.3A and B). Moreover, when $Sf9^{Op1D}$ cells were transfected with either the $vAc^{gp64\text{-}/Thogp75}$ or the $vAc^{gp64\text{-}/pPolh\text{-}Thogp75}$ bacmid, the virus could be propagated (Table 4.1), confirming that the bacmid was otherwise replication competent. Because Thogoto virus GP75 was expressed in infected Sf9 cells and can be incorporated into AcMNPV budded virions, these results suggest that Thogoto virus GP75 is not able to functionally substitute for GP64 in the context of an

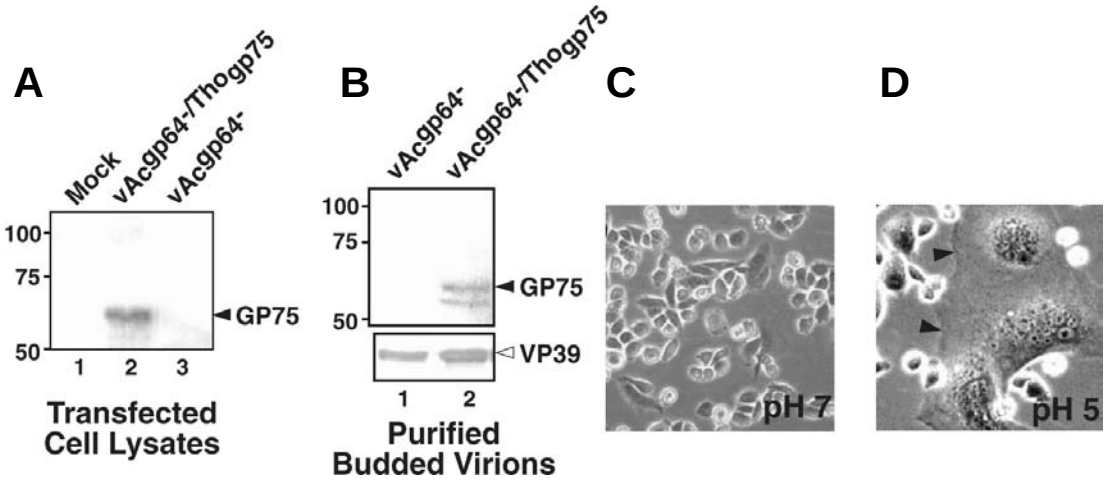

Figure 4.3 Thogoto virus GP75 protein expression, detection in AcMNPV budded virions, and membrane fusion activity. (A) Western blot analysis of GP75 expression in Sf9 cells that were either mock transfected (lane 1) or transfected with the $vAc^{gp64\text{-}/Thogp75}$ (lane 2) or $vAc^{gp64\text{-}}$ (lane 3) bacmid and examined at 72 h posttransfection. GP75 was detected by using an affinity-purified antibody generated against a GP75 peptide (N-KERAHEKSKDLPFGNK-C). (B) Western blot detection of GP75 protein in purified budded virions ($vAc^{gp64\text{-}}$ or $vAc^{gp64\text{-}/Thogp75}$) generated in $Sf9^{Op1D}$ cells. An anti-capsid protein (VP39) antibody was used as an internal control (lower panel). (C and D) Syncytium formation assay. CHO cells were transfected with a plasmid (pRC-THO) expressing GP75 under the control of a cytomegalovirus early promoter. At 24 h posttransfection, cells were incubated in phosphate-buffered saline at pH 7.0 (C) or pH 5.0 (D) for 15 min; then they were examined for syncytium formation after 90 min. Large syncytial masses containing many nuclei are indicated by arrowheads. Cells transfected with the empty vector (pRC-CMV) did not show syncytium formation (data not shown).

AcMNPV infection. To determine whether GP75 was functional in Sf9 cells, a membrane fusion (syncytium formation) assay was performed in Sf9 cells, using the donor plasmid construct that expresses GP75 under the control of the AcMNPV *gp64* promoter. Transiently expressed GP75 did not mediate detectable low-pH-triggered membrane fusion (data not shown). Since Thogoto virus is a tick-borne virus that replicates in a variety of mammals (Davies *et al*., 1986), also the GP75 was tested for syncytium formation in mammalian cells. When GP75 was expressed in CHO cells and similarly assayed, pH-triggered membrane fusion was readily detected (Fig. 4.3C and D). Thus, GP75 mediated membrane fusion in mammalian cells but did not appear to be functional in insect Sf9 cells. This may explain the inability of GP75 to rescue the *gp64*-null bacmid.

Infectious virion production by pseudotyped viruses

To more thoroughly characterize the ability of certain envelope proteins to substitute for GP64 in pseudotyped *gp64*-null viruses, one-step growth curves were performed. Infectious virus production from the repair virus (containing wild-type *gp64* reinserted into the *polyhedrin* locus) was similar to that from wild-type AcMNPV (Fig. 4.4), indicating that the use of the bacmid itself and the introduction of envelope genes into the *polyhedrin* locus did not have a significant negative effect on infectious virion production. Virus production by the VSV-G-pseudotyped virus was approximately 1 log unit lower than that by the wild-type AcMNPV (Fig. 4.4). Virus production by viruses pseudotyped with the F protein from either SeMNPV or LdMNPV was approximately 2 log units lower than that by control viruses (Fig. 4.4). The presence of VSV-G and SeMNPV F proteins in purified budded virions was also confirmed (Fig. 4.5, lanes 1 and 2). Although LdF antibodies were not available, the GP64-null phenotype was confirmed (Fig. 4.5, lane 3), and the genotypes of all viruses were confirmed by PCR analysis (data not shown).

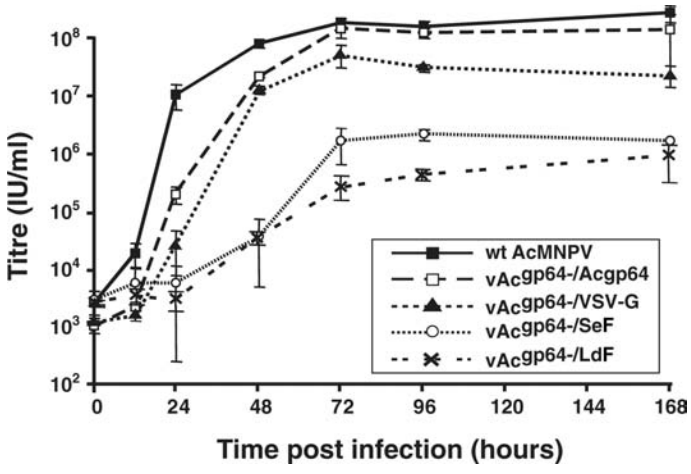

Figure 4.4 One-step growth curves are shown for wild type AcMNPV, the repair virus vAc$^{gp64-/Acgp64}$, and AcMNPV *gp64*-null viruses pseudotyped with VSV-G (vAc$^{gp64-/VSV-G}$), SeF (vAc$^{gp64-/SeF}$), or LdF (vAc$^{gp64-/LdF}$). Sf9 cells were infected at a multiplicity of infection of 5, and supernatants were harvested at the indicated times postinfection and titered on Sf9^{Op1D} cells. Each data point represents the average from three separate infections (error bars, standard deviations), except for the data point for the SeF-pseudotyped virus at 168 h postinfection, which represents a single infection.

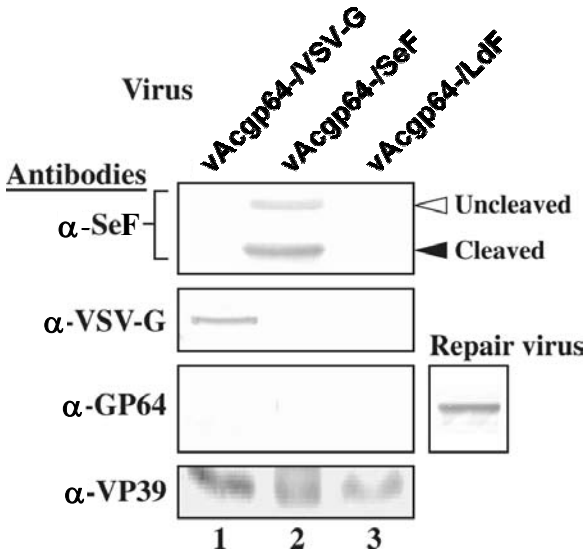

Figure 4.5 Western blot analysis of pseudotyped virions. A series of Western blots of purified virions from pseudotyped viruses vAc$^{gp64-/VSV-G}$ (lane 1), vAc$^{gp64-/SeF}$ (lane 2), and vAc$^{gp64-/LdF}$ (lane 3) were probed with antibodies α-SeF (against SeMNPV F_1 protein, the 60-kDa SeF cleavage product) (Westenberg *et al*., 2002), α-VSV-G (Mangor *et al*., 2001), and α-GP64 (monoclonal antibody AcV5) Hohmann and Faulkner, 1983). An anti-nucleocapsid antibody (α-VP39) was used as an internal control for each preparation of purified budded virions (Mangor *et al*., 2001). GP64 was not detected from pseudotyped viruses but was detected in a positive control shown on the right (Sf9 cell lysates infected with the repair virus, vAc$^{gp64-/Acgp64}$). Positions of uncleaved and cleaved SeMNPV F are indicated.

Proteolytic cleavage of the SeMNPV F protein is essential for rescue of the *gp64*-null bacmid

Recently, a proprotein convertase (PPC or furin) cleavage site was identified in a number of baculovirus F proteins (IJkel *et al*., 200). The consensus furin cleavage site [R-X-(R/K)-R] is conserved at a similar position in F proteins from group II NPVs and GVs. Recent biochemical and mutagenic studies of the SeMNPV F protein strongly suggested that the SeMNPV F protein is cleaved by a PPC or furin protease (Westenberg *et al*., 2002). To determine whether cleavage at the furin cleavage site is essential for rescue of the *gp64*-null virus by the SeMNPV F protein, a *gp64*-null bacmid expressing a SeMNPV F protein with a point mutation in the furin cleavage site (SeF_{R149K}) was generated (Fig. 4.6A). This point mutation, an arginine-to-lysine substitution in a critical residue in the consensus furin cleavage site, was previously shown to prevent F protein cleavage (Westenberg *et al*., 2002). Using two protocols for testing *gp64* complementation, it was found that the F protein containing the R149K mutation did not complement the *gp64*-null bacmid (see Table 4.1). However, the SeF_{R149K}-pseudotyped virus can be propagated in $Sf9^{Op1D}$ cells, and titers of approximately 2 x 10^7 IU/ml can be achieved in these cells (Table 4.1, vAc$^{gp64-/SeFR149K}$). Western blot analysis of SeF_{R149K}-pseudotyped virions shows that SeF_{R149K} can be detected in these preparations. Western blot analysis of SeF_{R149K}-pseudotyped virions shows that SeF_{R149K} can be detected in these preparations (Fig.4.6B lane 3), indicating that SeF_{R149K} can be incorporated into the virion. In these preparations, SeF_{R149K} was detected as a protein of approximately 75 kDa. In contrast, a 60-kDa processed form of SeF was the major protein form detected in virions pseudotyped with the wild-type SeMNPV F protein (Fig. 4.6B, lane 1) and in wild-type SeMNPV virions (IJkel *et al*., 2000). These results demonstrate that the putative furin cleavage site is necessary both for processing of this F protein and for complementation of the *gp64*-null virus in Sf9 cells.

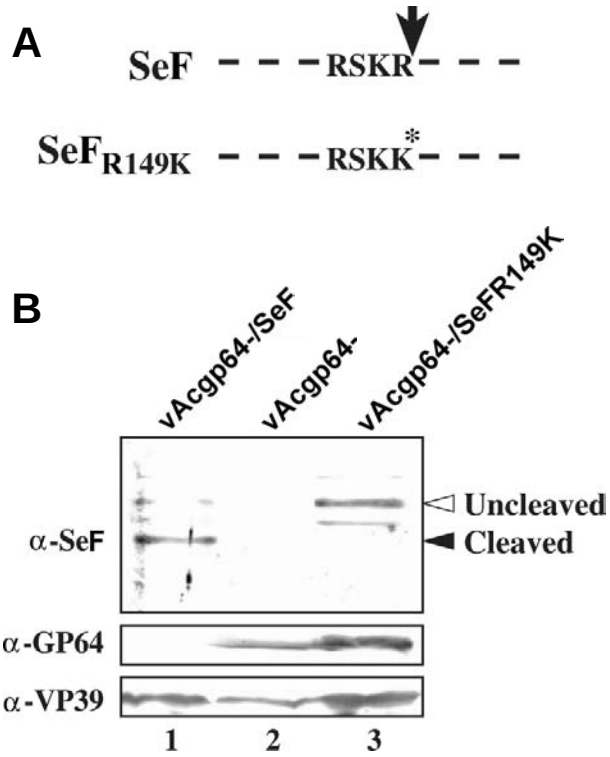

Figure 4.6 (A) Diagram of the furin cleavage site in the SeMNPV F protein (SeF) and the SeF_{R149K} mutant. RSKR represents the furin cleavage site in the wild-type SeMNPV F protein (SeF), and RSKK represents the furin cleavage site containing a single amino acid substitution (SeF_{R149K}). (B) Western blot analysis of *gp64*-null budded virions pseudotyped with SeF or SeF_{R149K}. Virion preparations (lanes 1 to 3) were probed with antibodies directed against the SeMNPV F protein (α-SeF), GP64 (α-GP64), and VP39 (α-VP39). Virions of $vAc^{gp64-/SeF}$ were generated in infected Sf9 cells (lane 1), whereas virions of vAc^{gp64-} (lane 2) and $vAc^{gp64-/SeFR149K}$ (lane 3) were generated in infected $Sf9^{Op1D}$ cells. The latter virions therefore contain OpMNPV GP64 (lanes 2 and 3). The VP39 capsid protein was included as an internal control. Positions of cleaved and uncleaved forms of Se8 are indicated.

DISCUSSION

The GP64 envelope protein is necessary for the critical first steps (attachment and entry) and the essential final steps (assembly and budding) in the infection cycle of AcMNPV. In this study, functional similarities between GP64 and the recently discovered baculovirus F proteins was examined. It was shown that an AcMNPV *gp64*-null virus can be rescued by F proteins from LdMNPV and SeMNPV (group II NPVs). The *gp64*-null viruses pseudotyped with F proteins from LdMNPV and SeMNPV generated substantially lower virus titers than wild-type AcMNPV. This suggests that the degree of compatibility between these F proteins and other AcMNPV or host proteins may not be optimal. Such incompatibilities might result in (i) inefficient virion budding, (ii) inefficient virus binding to host cell receptors, or (iii) inefficient membrane fusion and virus release from endosomes during virus entry. In spite of the differences in efficiency, these F proteins were able to substitute at least partially for all of the essential functions of GP64 and resulted in the production of infectious viruses. Thus, F proteins are functionally analogous to GP64. In contrast to the F protein genes from SeMNPV and LdMNPV, the PlxyGV gene did not rescue the *gp64*-null AcMNPV. Although it is possible that the PlxyGV F protein was inefficiently expressed, believe this is unlikely because both the LdMNPV and SeMNPV F proteins were expressed in quantities sufficient to rescue the *gp64*-null bacmid, and expression of an epitope-tagged PlxyGV F protein was detected (data not shown). Therefore, it is plausible that the inability of the PlxyGV F protein gene to rescue the *gp64*-null AcMNPV may be related to the greater evolutionary distance between AcMNPV and PlxyGV, resulting in less-compatible interactions between the F protein and other AcMNPV or host cell proteins. Consistent with this hypothesis,

LdMNPV and SeMNPV F proteins are similar in length (676 and 665 amino acids, respectively) and more highly conserved (38% identity), whereas the PlxyGV F protein is only 544 amino acids long, and the PlxyGV and LdMNPV F proteins are only 24% identical. Thus, our results from pseudotyping experiments with heterologous F proteins suggest that functional complementation reflects evolutionary distance.

It is of special interest that group I NPVs such as AcMNPV and OpMNPV (which carry *gp64* genes) also have genes encoding F protein homologs (Ac23 and Op21, respectively). One hypothesis to explain the existence of two different but functionally similar envelope proteins (GP64 and F proteins) within the family *Baculoviridae*, plus the presence of both GP64 and F protein homologs in certain group I NPVs, is the possibility that *gp64* is a recent acquisition by an ancestor of the group I NPVs (Rohrmann and Karplus, 2001). In this scenario, many or most of the functions of the ancestral AcMNPV F protein homolog may have been replaced by the GP64 protein, thus releasing all or portions of the AcMNPV F protein homolog from selection pressure. Consistent with this hypothesis, AcMNPV and OpMNPV F protein homologs appear to be less well conserved than F proteins from group II NPVs and GVs. Even though group I and II NPVs are more closely related phylogenetically (Herniou *et al*., 2001), the AcMNPV F protein (Ac23) and the LdMNPV F protein are only 13% identical. In contrast, the LdMNPV and PlxyGV F proteins are approximately 24% identical (Rohrmann and Karplus, 2001). In addition, the essential furin cleavage site that is present in LdMNPV and SeMNPV F proteins is absent in the predicted Ac23 and Op21 proteins. Furthermore, while F proteins from group II NPVs can functionally replace AcMNPV GP64, the F protein homolog of AcMNPV (Ac23) cannot compensate for a *gp64* deletion in AcMNPV (Monsma *et al*., 1996; Oomens and Blissard, 1999). While F proteins from group II NPVs and GVs likely serve an essential role in infectivity, the function of the more divergent F protein homologs in group I NPVs remains unknown. However, the observation that AcMNPV and OpMNPV F gene homologs are intact ORFs suggests that they may provide a beneficial function. Consistent with this hypothesis, Pearson *et al*. (2001) recently reported that the OpMNPV F homolog (Op21) is expressed and colocalized with GP64 in the budded virion membrane. Baculovirus F protein homologs were also recently identified in a subgroup of apparent retroviruses. This subgroup, referred to as insect errantiviruses (Malik *et al*., 2000), appears to have evolved from invertebrate long terminal repeat-containing retrotransposons that acquired an envelope protein gene, to become infectious retroviruses (Song *et al*., 1994; Tanda *et al*., 1994). The envelope protein genes of the Ty3/Gypsy group of retrotransposons from *Drosophila melanogaster* and the TED retrotransposon from *T. ni* (Ozers and Friesen, 1996) are homologs of the baculovirus F protein genes, and it has recently been suggested (Malik *et al*., 2000) that these invertebrate retrotransposons acquired their envelope protein gene from a baculovirus. In addition, a possible cellular homolog of the baculovirus F protein gene has been identified in *Drosophila* (Rohrmann and Karplus, 2001). Exchange of genetic material is known to occur between baculoviruses and their hosts, and also between coinfecting baculoviruses (Fraser *et al*., 1983; Maeda *et al*., 1993; Miller and Miller, 1982). A recent study by Herniou *et al*. (Herniou *et al*., 2001) suggested that baculovirus genomes were highly fluid, with gene rearrangements and multiple gene

content changes occurring during their evolution. Of 17 genes that are thought to be unique to group I NPVs, 4 encode structural proteins (Herniou *et al*., 2001). Thus, *gp64* and possibly other structural protein genes in group I NPVs may represent gene acquisitions from the host or from other viruses. The acquisition of new envelope proteins represents a mechanism by which some viruses are known to enhance their fitness. For example, orthomyxoviruses use reassortment to increase their diversity, and the acquisition of new envelope protein genes permits them to avoid immune detection. Although it is not clear if or why GP64 would provide a selective advantage, might speculate that acquisition of GP64 could have increased the efficiency of virus-receptor interactions, virus entry, or virus budding. Alternatively, GP64 may have facilitated the recognition of a different host cell receptor(s) and increased the range of cells or tissues infected. Thus, acquisition of this gene may have resulted in either more productive infections, or an increase or change in cell types infected.

In addition to F proteins, also asked whether GP75 from Thogoto virus could functionally complement GP64. The fact that Thogoto virus GP75 shows 28% amino acid sequence identity to AcMNPV GP64 (Morse *at al*., 1992) is striking and suggests that GP64 and GP75 proteins have a common evolutionary origin, even though orthomyxoviruses and baculoviruses do not appear to share a common evolutionary origin. Since Thogoto virus is vectored to mammals by arthropods (ticks), an intriguing possible scenario is that an ancestor of the Thogoto virus acquired a GP64 homolog either from the arthropod host genome or from a host coinfected with a baculovirus (Morse *at al*., 1992). However, other possible scenarios cannot be eliminated. Since it is likely that the GP64 and GP75 proteins have a common evolutionary origin, it is asked in the present study whether GP75 could substitute for GP64 in AcMNPV. It is found that GP75 was expressed and appropriately transported in infected Sf9 cells, and GP75 was also found in pseudotyped virions. However, GP75 did not rescue the *gp64*-null AcMNPV. In subsequent experiments membrane fusion activity in Sf9 cells expressing GP75 was not detected, while, in contrast, GP75 mediated membrane fusion in mammalian CHO cells. These observations suggest that while GP75 and GP64 share functional similarity (i.e., they are both membrane fusion proteins), GP75 may be somehow incompatible with the Sf9 host cells or with the AcMNPV entry or budding apparatus. This incompatibility may reflect GP64 and GP75 adaptations to the different host and viral systems.

In summary, a versatile genetic system is developed and used to examine the function and compatibility of heterologous envelope protein genes in the context of an AcMNPV baculovirus infection. Using this system, it was demonstrated that F proteins from group II NPVs can rescue infectivity and budded virus production in the absence of *gp64*. It was also found that Thogoto virus GP75 did not substitute for GP64 in AcMNPV, although the two proteins share 28% amino acid identity. In contrast, the unrelated but highly promiscuous VSV G protein effectively rescued a *gp64*-null AcMNPV.

The bacmid-based pseudotyping system developed for these studies will be useful for future studies aimed at understanding the nature of envelope protein interactions necessary for efficient entry and budding, and for pseudotyping AcMNPV for potential medical and biotechnological applications. Examples of such applications might include

pseudotyping AcMNPV with modified envelope proteins in order to modulate the host range of AcMNPV as a biocontrol agent, or to target specific cells or tissues for efficient or restricted delivery of genes for insect transgenesis or for gene therapy.

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Chapter 5

ON THE RESCUE OF BACULOVIRUS MUTANTS LACKING THE F GENE BY GP64

SUMMARY

The genus *Nucleopolyhedrovirus* (NPVs) of the *Baculoviridae* can be phylogeneticaly divided into two groups. This division can also be made on the basis of their budded virus (BV) envelope fusion protein. Group I NPVs, e.g. *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), are characterized by a GP64-like major envelope fusion protein. This BV protein is involved in attachment of viruses to cells, fusion of viral envelope with host cell membrane and is required for efficient budding of progeny nucleocapsids. Group II NPVs e.g. *Spodoptera exigua* (Se)MNPV and *Lymantria dispar* (Ld)MNPV have an envelope fusion protein, unrelated to GP64 and named F. In contrast to GP64, F proteins are found in all baculoviruses, but they are not functional in group I NPVs. AcMNPV, lacking GP64, can be pseudotyped by the SeMNPV and LdMNPV F proteins, suggesting that the F proteins are functionally analogous to GP64. In the current study evidence is provided that this functional analogy is not reciprocal. Transfection of an *f*-null SeMNPV bacmid in Se301 did not result in the production of infectious BVs. This defect was corrected by introduction of the SeMNPV *f* gene, but not of the AcMNPV *gp64* gene. This suggests that F proteins from group II NPVs may contain additional functions in BV formation, which are lacking in the GP64-type of fusion protein.

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INTRODUCTION

The *Baculoviridae* are a family of large, enveloped double-stranded DNA viruses that are pathogenic to arthropods, predominantly insects in the order *Lepidoptera*, but also to certain members of the *Diptera*, *Hymenoptera* and *Decapod*a (Adams and McClintock, 1991). The family encompasses two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). The NPVs can be further divided into group I and II NPVs, based on phylogenetic data (Bulach *et al*., 1999; Hayakawa *et al*., 2000; Herniou *et al.*, 2001; Herniou *et al.*, 2003). Baculoviruses produce two distinct virion phenotypes: occlusion-derived virus (ODV) and budded virus (BV) (Volkman and Summers, 1977). ODVs are present in occlusion bodies and are able to infect midgut epithelial cells by direct membrane fusion (Granados, 1978; Granados and Lawler, 1981; Horton and Burand, 1993). BVs infect insect cells via receptor-mediated endocytosis and are responsible for the systemic spread of the virus in the insect (Hefferon *et al*., 1999; Volkman and Goldsmith, 1985). The BVs of group I NPVs, e.g. *Autographa californica* (Ac)MNPV and *Orgyia pseudotsugata* (Op)MNPV, contain a major envelope protein, GP64. This protein is involved in attachment of BVs to the cell, is required for low-pH-triggered membrane fusion during virus entry, and is necessary later in the process of infection for efficient budding of progeny nucleocapsids (NCs) into the hemolymph or cell culture supernatant (Blissard and Wenz, 1992; Hefferon *et al*., 1999; Oomens and Blissard, 1999). Recent data from complete genomic sequences of a growing number of baculoviruses suggest that all group I NPVs possess a *gp64*–like gene, while group II NPVs and GVs lack this gene. For two group II NPVs, e.g. *Spodoptera exigua* (Se)MNPV and *Lymantria dispar* (Ld)MNPV, it has been shown that low-pH-mediated membrane fusion is mediated by a novel type of envelope fusion protein, called F (IJkel *et al*., 2000; Pearson *et al*., 2000). Like several mammalian viral envelope fusion proteins, the baculovirus F protein has to be posttranslationally cleaved by a proprotein convertase (furin) to become fusiogenic (Lung *et al*., 2002; Westenberg *et al*., 2002). Homologs of the *f* gene have been identified in other group II NPVs, in GVs and in members of the insect retrovirus family *Errantiviridae*, but do also exist in group I NPVs (Herniou *et al*., 2003; Malik *et al*., 2000; Rohrmann and Karplus, 2001; Terzian *et al*., 2001). In the genome of group I NPVs an *f* homolog is present (*Ac23*-homologs). Its translation product is found on the envelope of BVs, but it is not required for viral replication or pathogenesis (IJkel *et al*., 1999; Lung *et al*., 2003; Pearson *et al*., 2001).

Recently it was shown that the F proteins of SeMNPV and LdMNPV were capable of functionally substituting for GP64 in AcMNPV (Lung *et al*., 2003). An AcMNPV bacmid lacking the *gp64* gene was unable to produce BVs after transfection into insect cells, while this defect could be rescued by insertion of the SeMNPV and LdMNPV *f* genes (Lung *et al*., 2002; Mangor *et al*., 2001). GP64 homologs are also found on Thogoto and Dhori viruses, which are tick transmitted orthomyxoviruses replicating both in ticks and mammals (Morse *et al.,* 1992; Freedman-Faulstich and Fuller, 1990). Therefore it has been suggested that the group I NPVs has acquired the *gp64* gene either from the host or from another virus, thereby acquiring a selective advantage and obviating the need

for a functional F protein. We experimentally addressed this hypothesis by pseudotyping a group II NPV (SeMNPV) with GP64. The *f* gene of SeMNPV was deleted using site-specific mutagenesis of an infectious SeMNPV bacmid (Pijlman *et al*., 2002). Transfection of this *f*-null bacmid into insect cells revealed that the virus is no longer able to propagate an infection. Reinsertion of the *f* gene rescued infectivity, but strikingly insertion of the *gp64* gene failed to rescue infectivity.

MATERIALS AND METHODS

Cells, insects and virus

The *Spodoptera exigua* cell line Se301 (Hara *et al*., 1995) and the *Spodoptera frugiperda* cell line IPLB-SF-21 (Vaughn *et al*., 1977) were cultured at 27^{o}C in plastic tissue culture flasks (Nunc) in Grace's insect medium, pH 5.9-6.1 (Invitrogen) supplemented with 10% fetal bovine serum (FBS). The SeMNPV isolate (Gelernter and Federici, 1986) was originally obtained from Dr. B.A. Federici (Department of Entomology, University of Californica) and was called SeMNPV-US1 (Muñoz *et al*., 1998).

Deletion of SeMNPV *f* gene by ET recombination in *E. coli*

For deletion mutagenesis of the *f* gene of the SeMNPV-US1 bacmid SeBAC10 (Pijlman *et al*., 2002), 75 to 77-bp primers were designed with on the 5' end 50 nt corresponding to the 50 bp flanking the deletion target region on the SeMNPV genome. Forward primer was 5'-TTTGGTCGTCGTCGTCGTCGTTGAAATGATACCCTTTGTCGTTGAACTGGCCTAGGTTTAAGGGCACCAATAACTG-3', with viral flanking sequences (5' UTR) from nt 12248 to 12297 according to the SeMNPV complete genome sequence (IJkel *et al*, 1999). Reverse primer was 5'-ATACATTATATATTGTTTTATTTTACTCTACTACTATTACAATCAATCGGCCTAAGGTTCCTGTGCGACGGTTAC-3', with viral flanking sequences (3' UTR) from nt 14545-14496. The 3' ends of the primers anneal to the chloramphenicol resistance gene (*cat)* of pBeloBac11 (Shizuya *et al.*, 1992; Wang *et al*., 1997a) and a *Bsu*36I site was designed in between the viral and *cat* sequence (underlined sequence).

PCR on pBeloBac11 was performed using high-fidelity Expand long-template PCR (Roche). The expected 1050 bp PCR fragment was gel-purified and digested with *Dpn*I to eliminate residual template DNA. After a second round of gel purification about 500 ng of PCR product was used for transformation of electrocompetent *E. coli* DH10β cells containing both SeBAC10 and the plasmid pBAD-$\alpha\beta\gamma$ promoting homologous recombination as described before (Pijlman *et al*., 2002). The altered sequence of the recombinant bacmid, designated SeBACΔF, was confirmed by PCR using primers of the regions flanking the *f* gene.

Donor plasmid construction

The SeMNPV *polyhedrin* gene with its own promoter was cloned as a *Sma*I/*Hin*dIII fragment (Pijlman *et al*., 2002) in the *Sma*I and *Kpn*I sites of pFastBac Dual

(Invitrogen) generating pFBSePolh Dual. The SeMNPV *f* gene and the AcMNPV *gp64* gene both under the control of the *gp64* promoter were cloned as *Xba*I/*Eco*RI and *Xba*I/*Sst*I fragments from pΔFBgusSe8 and pΔFBgusGP64 (Lung *et al*., 2002) into the *Sma*I and *Sst*I sites of pFBSePolh Dual. In this procedure the AcMNPV p10 and polyhedrin promoters were removed, and plasmids pFBSepolh-SeF(*pGP64*) and pFBSepolh-GP64(*pGP64*) were obtained, respectively.

The 250 bp upstream of the SeMNPV *f* open reading frame (ORF) were amplified with primers 5'-AAACCCGGGTTTGGTCGTCGTCGTCGTCGTTG-3' and 5'-TAAGGATCCTATTTTGCTTGCGACTCGGTTCTC-3' (underlined sequences generate a *Sma*I and a *Bam*HI restriction sites respectively) from pSe*Bgl*II-H (IJkel *et al*., 1999) as template using high-fidelity Expand long-template PCR (Roche). The PCR fragment was cloned into the *Sma*I and *Bam*HI site of pFBSePolh Dual, thereby removing the AcMNPV p10 and polyhedrin promoters, and generating pFBSepolh-(*pSeF*). The SeMNPV *f* gene and the AcMNPV *gp64* gene were cloned as *Bam*HI /*Not*I and *Eco*RI/*Eco*RI fragments from pΔFBgusSe8 and pΔFBgusGP64 (Lung *et al*., 2002) into the *Bam*HI and *Not*I sites of pFBSePolh-(*pSeF*), generating pFBSePolh-SeF(*pSeF*) and pFBSePolh-GP64(*pSeF*), respectively. For the generation of control bacmids a vector was generated containing only the SeMNPV *polyhedrin* gene behind its own promoter. This was done by removing the SeMNPV *f* promoter as *Sma*I/*Stu*I fragment from pFBSepolh-(*pSeF*), generating pFBSepolh.

Transfection of SeMNPV bacmids

The inserts of the donor plasmids were transposed into the *att*Tn7 transposition sites of the SeMNPV bacmids SeBacΔF or SeBAC10 according to the Bac-to-Bac manual (Invitrogen). Transposition was confirmed by PCR using the M13/pUC forward primer (5'-CCCAGTCACGACGTTGTAAAACG-3') in combination with the M13/pUC reverse primer (5'-AGCGGATAACAATTTCACACAGG-3') and M13/pUC forward primer in combination with a primer corresponding to the sequence of the Gm^R gene, BAC-control (5'-AGCCACCTACTCCCAACATC-3').

Se301 cells (5.0×10^5) were seeded into 35 mm tissue culture plates (Nunc). The cells were transfected with approx. 1 μg of bacmid DNA using 10 μl of Cellfectin (Invitrogen). After 5 days the cells were transferred to a T75 flask (Nunc) and subsequently every 5 days one-third of the cells were transferred to a new T75 flask until 90% of the cells contained polyhedra. The insect cell supernatant was clarified by centrifugation at 4,000 x *g* for 10 min and subsequently passed through a 0.45 μm filter. The presence of infectious BVs in the supernatant was investigated by infecting 1.0×10^6 Se301 cells in a T25 flask with 500 μl of the supernatant. Finally, the BVs genotype was verified by PCR.

Syncytium formation assay

The expression of GP64 from the donor plasmids pFBSePolh-GP64(*pGP64*) and pFBSePolh-GP64(*pSeF*) was examined by a syncytium formation assay (Sf21-Sf21 fusion). Sf21 cells (1.0×10^6) were seeded into 35 mm tissue culture plates (Nunc) and transfected with 10 μg of donor plasmid DNA using Cellfectin (GibcoBRL). The donor

plasmid pΔFBgusGP64 (Lung *et al.*, 2002) and plasmid p166AcV5-GFP (IJkel *et al*., 2000) were used as positive and negative controls for the syncytium formation, respectively. The plasmid p166AcV5-GFP was also used to determine the transfection efficiency. Forty-eight h after transfection, cells were washed three times with 2 ml Grace's medium (pH 6.1) without FBS, and afterwards cells were treated for 2 min in 1 ml acidic Grace's medium at pH 5.0. The acidic medium was removed and replaced by 2 ml Grace's (pH 6.1) with 10% FBS. Syncytium formation was scored and observed by light microscopy 4 h after treatment with the acidic medium.

RESULTS

Disruption of the *f* gene in a SeMNPV bacmid

The envelope fusion protein GP64 of group I NPVs plays a critical role in budding and propagation of infection (Hefferon *et al*., 1999; Monsma *et al*., 1996; Oomens and Blissard, 1999). Group II NPVs do not have a *gp64* gene, but have an *f* gene encoding the envelope fusion protein (IJkel *et al.,* 2000; Pearson *et al*., 2000). Recently, it has been shown that a *gp64*-null AcMNPV bacmid, unable to propagate an infection upon transfection into Sf9 cells, could be rescued by the *f* gene of certain group II NPVs (Lung *et al*., 2002). To determine if the reverse was also possible, a recently constructed SeMNPV bacmid (Pijlman *et al*., 2002) was modified by deleting the *f* gene and inserting the AcMNPV *gp64* gene. The *f* ORF and the promoter of the SeMNPV bacmid SeBac10 were deleted by ET recombination (Muyrers *et al*., 1999) using a PCR amplified DNA fragment containing a *cat* cassette flanked by 50 bp of sequence 200 bp upstream of the start codon and 50 bp downstream of the stop codon. (Fig. 5.1). Bacterial colonies resistant for chloramphenicol (insert) and kanamycin (bacmid) were selected. The colonies were analyzed for successful recombination by PCR using primers flanking the recombination sites and by sequencing the amplified DNA fragments (data not shown).

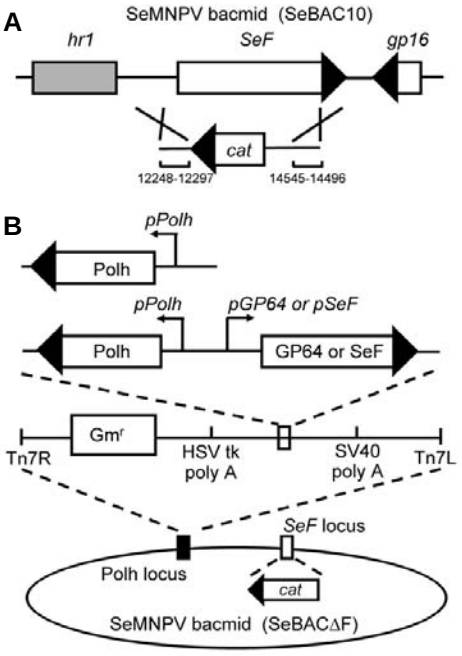

Figure 5.1 (A) Strategy for the generation of an *f*-null SeMNPV bacmid by ET recombination in *E. coli*. The *SeF* locus in the SeMNPV bacmid SeBac10 is shown above. A PCR amplified DNA fragment containing the chloramphenicol resistance gene (*cat*) flanked by 50 bp SeMNPV sequences on both sites was used to substitute the *f* gene for the *cat* gene. Sequences included for the homologous recombination (122480 to 12297 and 1454 to1449612) are indicated (IJkel *et al*., 1999) (B) Strategy for insertion of gene cassette constructs into the *polyhedrin* locus of the *f*-null SeMNPV bacmid. Inserts include the SeMNPV *polyhedrin* gene without the *f* gene (top), and the SeMNPV *polyhedrin* gene plus the AcMNPV *gp64* (GP64) or SeMNPV *f* (SeF) genes either under the control of the AcMNPV *gp64* promoter (*pGP64*) or the SeMNPV *f* promoter (*pSeF*) (center). The cassettes were inserted into the *att*Tn7 sites (indicated by the right and left insertion sites, Tn7R and Tn7L) in the *polyhedrin* locus by Tn*7*-based transposition (Pijlman *et al*., 2002).

The generated SeBacΔF DNA was transformed into DH10β together with the plasmid pMON7124 that encodes a Tn7 transposase, which can facilitate transposition of gene cassettes into the bacmid (Luckow *et al*., 1993).

To investigate if the *f* gene is necessary for efficient budding and propagation of infection, the SeMNPV polyhedrin gene was inserted into the SeBac10 and SeBacΔF bacmids at its original locus by Tn7-based transposition to mark successful infection (Luckow *et al*., 1993). Se301 cells were transfected with the generated bacmids SeBac10/Sepolh and SeBacΔF/Sepolh. As a control, wt SeMNPV DNA was also transfected in parallel. polyhedra were found 1 week post transfection (p.t.) in cells initially transfected with SeMNPV wild type DNA, while two weeks p.t. approx. 90 % of the cells contained polyhedra (Fig. 5.2A). A significant delay was observed for cells transfected with SeBac10/Sepolh, where the 90 % was reached after approximately 3 weeks p.t. (Fig. 5.2B). The presence of infectious BVs in the supernatants of the polyhedra containing cells was demonstrated by infecting healthy Se301 cells with the supernatants (data not shown). Cells initially transfected with SeBacΔF/Sepolh did contain polyhedra at 3 weeks p.t. (Fig. 5.2C), but subculturing of the cells, revealed that the virus did not spread. Therefore it can be concluded that the SeMNPV *f* gene is essential for virus propagation.

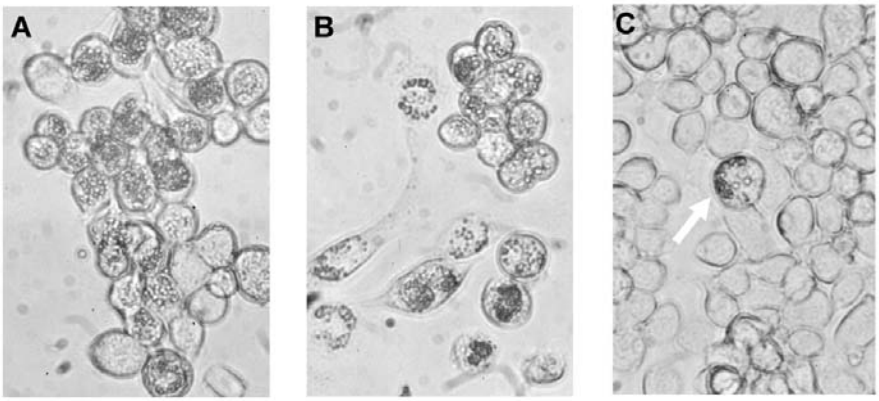

Figure 5.2 Infection of SeMNPV in Se301 cells after transfection of 1 μg SeMNPV wild type (A) or bacmid DNA (B and C). Cells were subcultured every 5 days for a maximum period of 4 weeks or until 90 % of the cells contained polyhedra. Infected cells containing polyhedra observed 2 weeks p.t. for wild type SeMNPV DNA (A), 3 weeks p.t. for SeBac10/Sepolh DNA (B) and polyhedra in the initially transfected cells (indicated by the arrow) 3 weeks p.t. for SeBacΔF/Sepolh (C).

The *f*-null SeMNPV bacmid is not rescued byGP64

The inability to propagate an infection upon transfection with the *f*-null SeMNPV bacmid shows that the *f* gene is essential for BV production. To find out if the propagation defect could be rescued, the *f* gene was reinserted via a repair bacmid, SeBacΔF/Sepolh-SeF(*pSeF*), generated by transposing the *f* gene under the control of its own promoter with the *polyhedrin* gene into SeBacΔF. Transfection of the bacmid resulted in the formation of polyhedra in the majority of cells three weeks p.t. (Fig. 5.3A) and the production of infectious BVs was demonstrated upon passaging the supernatant (data not shown). This shows that the *f*-null SeMNPV bacmid could be rescued and that a viable virus was generated. The experiment further shows that the homologous repeat

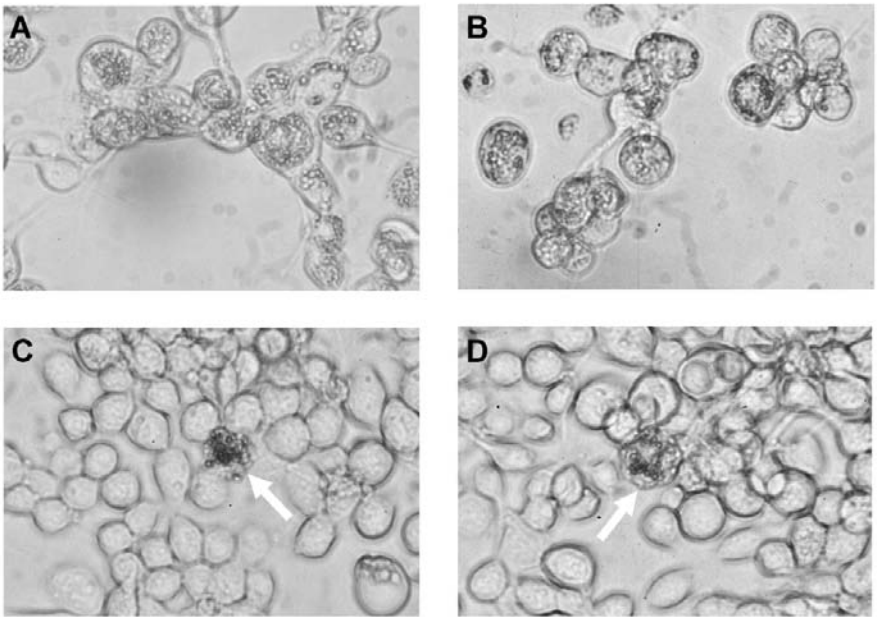

Figure 5.3 Infection of Se301 cells after transfection of 1 μg SeMNPV bacmid DNA. Cells were subcultured every 5 days for a maximum period of 4 weeks or until 90 % of the cells contained polyhedra. Infected cells containing polyhedra observed 3 weeks p.t. for SeBacΔF/Sepolh-SeF(*pSeF*) (A) and SeBacΔF/Sepolh-SeF(*pGP64*) (B), and polyhedra in the initially transfected cells (indicated by arrows) 3 weeks p.t. for SeBacΔF/Sepolh-GP64(*pSeF*) (C) and SeBacΔF/Sepolh-GP64(*pGP64*) (D).

region *hr1*, essential for viral replication (Broer *et al*., 1998) and *gp16*, expressing a nuclear membrane protein with unknown function (Gross *et al*., 1993), both flanking the *f* gene in SeMNPV, were not likely affected by the deletion of *f*.

To investigate if the AcMNPV GP64 is also able to rescue *f*-null SeMNPV viruses, the *gp64* gene under the control of the SeMNPV *f* promoter together with the polyhedrin gene as marker, were transposed into SeBacΔF generating SeBacΔF/Sepolh-GP64(*pSeF*). Polyhedra were observed 3 weeks p.t. in Se301 cells, which were initially transfected with the bacmid (Fig. 5.3B), but the infection did not spread to other cells. To exclude the possibility that this was not due to low GP64 expression being governed by the SeMNPV *f* promoter, or due to less optimal replication of the bacmid, two other bacmids were constructed. Bacmids SeBacΔF/Sepolh-SeF(*pGP64*) and SeBacΔF/Sepolh-GP64(*pGP64*) were generated, in which either the F protein or the GP64 protein would be expressed under the control of the AcMNPV *gp64* promoter. Viral infection was noticed when cells were transfected with *f*-null bacmid expressing the F protein (Fig. 5.3C), indicating that the *gp64* promoter is also able to drive gene expression in a SeMNPV virus. Despite this observation, rescue of *f*-null SeMNPV bacmid by *gp64* under the control of its own promoter was not detected as polyhedra were only observed in the initially transfected cells (Fig. 5.3D).

Expression of GP64 in insect cells

A syncytium formation assay was performed to investigate whether the incapability of GP64 to rescue the *f*-null SeMNPV bacmid was not due to the inability to express the GP64 protein from the expression cassette, which was transposed into the

bacmids. Sf21 cells were transfected with donor plasmids pFBSePolh-GP64(*pSeF*) and pFBSePolh-GP64(*pGP64*). The donor plasmid pΔFBgusGP64 (Lung *et al*., 2002) was used as a positive control for GP64, while the plasmid p166AcV5-GFP (IJkel *et al*., 2000), containing *gfp* downstream of the OpMNPV *gp64* promoter, was used as a negative control as well as to determine the transfection efficiency. Fluorescence microscopy observation of cells transfected with the control plasmid p166AcV5-GFP showed that approximately 5% of the cells was transfected (Fig 5.4B). Cells transfected with the plasmid pΔFBgusGP64 (Fig. 5.4A) as well as with the two donor plasmids pFBSePolh-GP64(*pSeF*) (Fig. 5.4B) and pFBSePolh-GP64(*pGP64*) (Fig. 5.4C) showed pH-dependent cell-to-cell fusion, while these syncytia were not observed with cells transfected with p166AcV5-GFP (Fig. 5.4D). So, GP64 could in principle be expressed from the *f*-null SeMNPV bacmids containing AcMNPV *gp64* under the control of either the SeMNPV *f* or AcMNPV *gp64* promoter. Thus, these studies suggest that GP64 may not be able to rescue an *f*-null SeMNPV mutant.

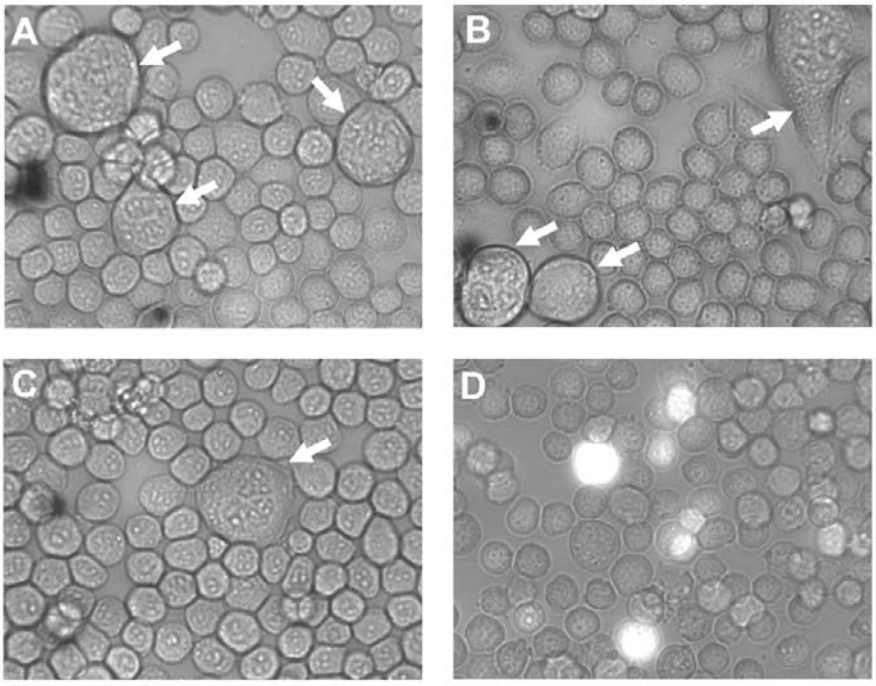

Figure 5.4 GP64-triggered cell-to-cell fusion of Sf21 cells transfected with donor plasmids. Cells (1 x 10^6) were transfected with 10 μg of plasmid pΔFBgusGP64 (A), pFBSePolh-GP64(*pSeF*) (B), pFBSePolh-GP64(*pGP64*) (C) and p166AcV-GFP (D). At 48 h p.t. cells were treated with Grace's medium pH 5.0. Cells were observed for syncytium formation 4 h after de pH drop by phase contrast microscopy. Cells transfected with p166AcV-GFP (D) are shown as an overlay of a fluorescence and phase-contrast image. The arrows indicate the formed syncytia.

DISCUSSION

Pseudotyping of viruses with heterologous envelope fusion proteins is a commonly used method to alter the host range of the virus (Landau *et al*., 1991). In this respect the Vesicular stomatitis virus G (VSV-G) protein has been successfully incorporated in AcMNPV BVs (Barsoum *et al*., 1997) and could even substitute GP64

(Mangor *et al*., 2001), although viral propagation in insect cells was greatly reduced. GP64 is involved in viral attachment to host cells, is required during virus entry and is necessary for efficient budding of progeny nucleocapsids (Blissard and Wenz, 1992, Hefferon *et al*., 1999; Oomens and Blissard, 1999). GP64 is present in all group I NPVs. Group II NPVs lack the GP64 protein and use F proteins as envelope fusion protein (IJkel *et al*., 2000; Pearson *et al*., 2000). It has previously been shown that AcMNPV could be pseudotyped with certain group II NPV F proteins, which substituted for the functions of GP64 (Lung *et al*., 2003). This indicated that the group II NPV F proteins are functionally analogous to GP64 in an AcMNPV virion. In this study, however, it is found that the AcMNPV GP64 protein does not functionally substitute for the F protein in SeMNPV. The lack of viral propagation could be rescued by reinsertion of a SeMNPV *f* gene in an *f*-null SeMNPV bacmid, confirming that the *f* gene is essential for BV production and systemic spread of the virus of a group II NPV. This in contrast to the truncated F homolog (Ac23) in the group I NPV AcMNPV, where this protein can be deleted without effecting the viral replication or pathogenesis in cell culture or infected animals (Lung *et al*., 2003).

The inability of the *f*-null bacmid to propagate an infection could not be rescued by the introduction of AcMNPV *gp64* neither downstream of the authentic SeMNPV *f* promoter nor downstream of the AcMNPV *gp64* promoter. However, the ability to express GP64 from those bacmids was demonstrated by a syncytium formation assay using the donor plasmids as expression cassettes (Fig. 5.4). To exclude the possibility that the propagation was affected by a deletion in the bacmid that could have been generated during transposition and transformation in *E. coli,* four different bacmid clones were transfected into insect cells. However, none of the bacmids was able to propagate an infection, while the *f*-repair SeMNPV bacmid was able to do so each time.

Despite the observation that GP64 is not able to pseudotype SeMNPV in this studies, it has been demonstrated that GP64 meets the requirements of an envelope glycoprotein, which is independently able to insert in a membrane and form envelopes, i.e. pseudotyped lentiviruses (Kumar *et al.,* 2003). The Human immunodeficiency virus type 1 (HIV-1) does not require its envelope protein (Env) for virion budding, however, the generated virions are not infectious. Budding of retroviruses seems to occur at ordered lipid microdomains, called lipid rafts (Briggs *et al*., 2003). It is well possible that when expressed in mammalian cells GP64 may end up in these lipid rafts and thereby in the envelope of lentiviruses. Recent experiments, however, indicate that GP64 does not seem to be associated with lipid rafts in insect cells (Zhang *et al*., 2003).

In contrast to HIV-1, the major envelope protein E2 of alphaviruses is absolutely required for efficient budding (Owen and Kuhn, 1997). One hypothesis to explain the synergistic roles of various proteins in the budding process is the push-pull model (Mebatsion *et al*., 1996). The push represents the role of the matrix and perhaps other proteins on the inner surface of the plasma membrane and the pull represents the role of the membrane proteins within and on the exterior of the membrane. The concerted or synergistic effects of the two components may accomplish budding. In this respect, it is well possible that SeMNPV and perhaps all group II NPV nucleocapsids require a special interaction with the SeMNPV F protein to provoke budding, while this interaction

is not needed for the incorporation of the SeMNPV F protein in AcMNPV BVs. A possible candidate for this interaction may be the cytoplasmic tail domain (CTD) of the group II NPV F-like proteins. This CTD ranges in length from about 54 to 78 amino acids. In GP64-like proteins this domain is much smaller, 3 to 8 amino acids and probably inert. The long CTDs of the F-like proteins could possess one or more specific protein motifs required for the interaction with the viral nucleocapsids, while these motifs are absent in the rather short CTDs of GP64-like proteins.

The involvement of CTDs of viral envelope proteins in the budding process has indeed been supported for a number of other viruses, including Sendai virus (Ali and Nayak, 2000), Influenza virus A (Bilsel *et al*., 1993), VSV (Robison and Whitt, 2000), Mason-Pfizer monkey virus (Song *et al*., 2003) and Semliki Forest virus (Zhao *et al*., 1994). It is plausible that there is an interaction between the CTD of F and the viral nucleocapsid protein, which is absent in group I NPVs. The putative protein Se107 (ORF 107 in SeMNPV) with a nuclear localization prediction and only present in group II NPVs (Herniou *et al*., 2003) can be a good candidate for such a protein. Group II NPVs share another unique gene, *Se30*. The putative translation product of this gene seems to have a signal peptide and transmembrane domain and therefore it is well possible that this protein is translocated to the cell membrane and acts in conjunction with F in virion assembly and virus budding. Further studies are in progress to confirm whether the CTD of F is important for the budding process.

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Chapter 6

FUNCTIONAL ANALYSIS OF THE PUTATIVE FUSION DOMAIN OF THE BACULOVIRUS F PROTEIN

SUMMARY

Group II nucleopolyhedroviruses (NPVs), e.g. *Spodoptera exigua* (Se)MNPV, lack a GP64-like protein that is present in group I NPVs, but have an unrelated envelope fusion protein named F. In contrast to GP64, the F protein has to be activated by a posttranslational cleavage mechanism to become fusiogenic. In several vertebrate viral fusion proteins the cleavage activation generates a new N-terminus which forms the so-called “fusion peptide”. This fusion peptide inserts in the cellular membrane, thereby facilitating apposition of the viral and cellular membrane upon sequential conformational changes of the fusion protein. A similar peptide has been identified in NPV F proteins at the N-terminus of the large membrane anchored subunit F_1. The role of individual amino acids in this putative fusion peptide on viral infectivity and propagation was studied by mutagenesis. Mutant F proteins with single amino acid changes as well as an F protein with a deleted putative fusion peptide were introduced in *gp64*-null AcMNPV BVs. None of the mutations analyzed had an major effect on the processing and incorporation of F proteins in the envelope of BVs. Only two mutants, one with a substitution for a hydrophobic residue (F152R) and one with a deleted putative fusion peptide, were completely unable to rescue the *gp64*-null mutant. Several nonconservative substitutions of other hydrophobic residues and the conserved lysine residue had only an effect on the infectivity. In contrast to what was expected from vertebrate virus fusion peptides, alanine substitutions for glycines did not show any effect.

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INTRODUCTION

The *Baculoviridae* are a family of large, enveloped, double stranded DNA viruses that are exclusively pathogenic for arthropods, predominantly insects of the order *Lepidoptera* (Adams and McClintock, 1991). Baculoviruses are classified into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). The NPVs can be phylogenetically subdivided into group I and II NPVs (Bulach *et al*., 1999; Hayakawa *et al*., 2000; Herniou *et al*., 2001; Herniou *et al*., 2003). The BV phenotype of group I NPVs contains a GP64-like major envelope glycoprotein. This protein is involved in viral attachment to host cells (Hefferon *et al*., 1999), triggers low-pH-dependent membrane fusion during BV entry by endocytosis (Blissard and Wenz, 1992; Kingsley *et al*., 1999; Plonsky *et al*., 1999; Volkman and Goldsmith, 1985) and is required for efficient budding from the cell surface (Monsma *et al*., 1996; Oomens and Blissard, 1999).

In contrast, BVs of group II NPVs and GVs lack a homolog of GP64. The low-pH-dependent membrane fusion during BV entry by endocytosis is triggered in this case by a so-called F protein (IJkel *et al*., 2000; Pearson *et al*., 2000). F homologs are also found as envelope protein of the insect errantiviruses, while cellular homologs are found in the fruitfly *Drosophila melanogaster* and in the African malaria mosquito *Anopheles gambiae* (Malik *et al*., 2000; Rohrmann and Karplus, 2001). Unlike GP64, the F proteins are structurally similar to fusion proteins from several vertebrate viruses such as orthomyxo- and paramyxoviruses. Recently, it has been shown that the GP64 protein in BVs of *Autographa californica* (Ac)MNPV can be replaced by the F protein of group II NPVs (Lung *et al*., 2002), implying that F is functionally analogous to GP64.

Like several mammalian viral envelope fusion proteins, the baculovirus F protein has to be posttranslationally cleaved by a proprotein convertase (furin) to become fusiogenic (Westenberg *et al*., 2002). Also for some errantiviruses it has been shown that the envelope protein is posttranslationally cleaved (Ozers and Friesen, 1996; Song *et al*., 1994; Tanda *et al*., 1994). Cleavage seems to be a general mechanism for viruses to activate their fusion proteins (Klenk and Garten, 1994). In a number of virus families this cleavage occurs in front of a strongly hydrophobic sequence, the so-called “fusion peptide” (White, 1990; White, 1992). These fusion peptides are believed to translocate upon cleavage to the top of the protein and to insert into the target membrane after exposure to low pH or receptor binding. This translocation facilitates the apposition of viral and cellular membranes upon further conformation changes of the fusion protein (Klenk and Garten, 1994). Comparison of available F protein sequences reveals a conserved strongly hydrophobic domain with the consensus sequence ΦGXΦBΦΦGXK ΦΦΦGXΦDXXDXXXΦ where Φ represents hydrophobic amino acids, B stands for an aspartic acid or an asparagine residue, and X represents any amino acid (Fig. 6.1). This domain is preceded by a furin-like cleavage site in the F proteins from group II NPVs, GVs and errantiviruses, while this domain is more or less absent in the remnant F protein from group I NPVs and in the cellular homologs (Rohrmann and Karplus, 2001). This strongly hydrophobic sequence at the N-terminus of the membrane-anchored SeMNPV F_1 fragment has all the characteristics of a fusion peptide (White, 1992). It is well-conserved within the virus family (Fig. 6.1), when modeled in α-helix, it displays one

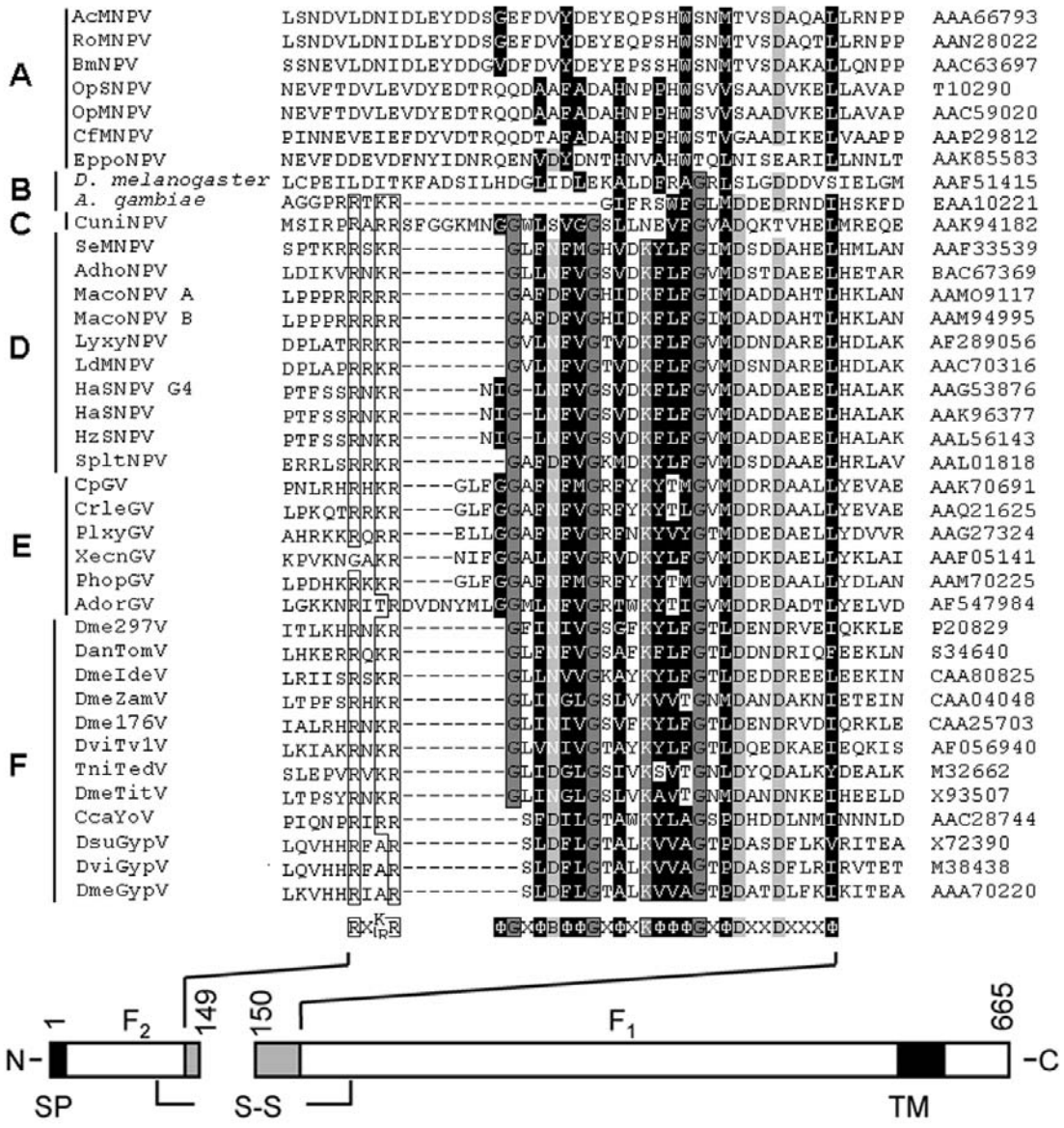

Figure 6.1 Amino acid sequence alignment of the SeMPNV F protein domains near the protease cleavage site with the corresponding domain of the F proteins of Group I NPVs (A), Dipteran (B), Lepidopteran NPVs (C), Group II NPVs (D), GVs (E), Errantiviruses (F). Virus and dipteran abbreviations, and Genbank accession numbers of the F proteins are shown on the left and right, respectively. The conserved amino acids are boxed. The consensus sequence is shown below the alignment, in which B represents D or N, Φ indicates a hydrophobic amino acid (H.I. ≥ 0.12) and X represent any amino acid. A schematic presentation of the SeMNPV F protein, with the consensus sequence shown in gray boxes, is shown at the bottom. N (N-terminus), C (C-terminus), SP (signal peptide), TM (transmembrane domain), S-S (disulfide bridge).

face with a hydrophobic index (H.I.) of about 0.9, according to the normalized consensus scale of Eisenberg (1984), and a backface with hydrogen bonding potential, and it contains glycines on one side of the helix (Fig. 6.2A).

The role in the fusion process of the three conserved charged amino acids in the putative fusion peptide, immediately downstream of the proprotein convertase cleavage site of the *Lymantria dispar* (Ld)MNPV F protein has already been studied by site-directed mutagenesis (Pearson *et al.,* 2002). In this paper site-directed mutagenesis was

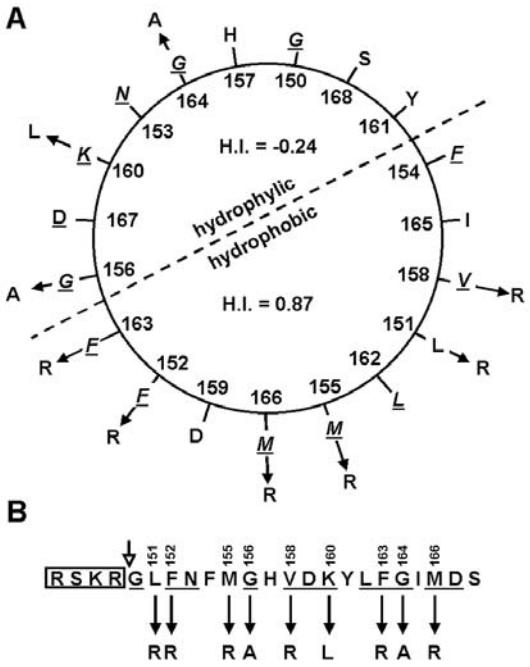

Figure 6.2 (A) Helical wheel presentation of the first 21 N-terminal amino acids of the SeMNPV F_1 fragment. The helix is typically amphiphatic, with relatively polar amino acids (H.I. ≤ 0.48) above the dotted line, with an average H.I. of –0.24 and except for one aspartatic acid residue (D) non-polar amino acids (H.I. > 0.62) below the dotted line with an average H.I. of 0.87. (B) Linear presentation of the first 21 N-terminal amino acids of the SeMNPV F_1 fragment preceded the consensus furin recognition sequence (box). The open arrow indicates the cleavage site. Underlined amino acids are conserved as shown in Fig. 6.1. Solid arrows indicate the point mutations as well as the substitutions described in this study.

used to investigate the role in viral propagation and infectivity of the conserved glycines and lysine as well as the hydrophobic amino acids in the putative fusion peptide of the SeMNPV F protein. Several conservative and nonconservative substitutions were introduced in the fusion domain and their impact on viral propagation and infectivity was examined by using a recently developed AcMNPV pseudotyping system in which the envelope fusion protein GP64 can be replaced by the heterologous envelope fusion protein F of SeMNPV (Lung *et al.,* 2002). The viral propagation of mutant infectious viruses as well as the amount of BV production gives further credence for the role of the conserved domain of the baculovirus F protein as “fusion peptide”.

MATERIALS AND METHODS

Cells

Spodoptera friguperda cell lines IPLB-Sf21 (Vaughn *et al.*, 1977) and $Sf9^{Op1D}$ (Plonsky *et al.*, 1999) (provided by Dr. G. W. Blissard, Ithaca NY, USA) were cultured at 27°C in plastic culture flasks (Nunc) in Grace's insect medium, pH 5.9 to 6.1 (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS).

Donor plasmids containing envelope protein genes

A silent mutation was introduced into the coding sequence of the SeMNPV F ORF to generate a *Nde*I cloning site. Therefore nucleotide 525 in the *f* ORF was changed from C to T by PCR-based site-directed mutation, according to the method of Sharrocks and Shaw (1992). The 5' mutagenic primer 5'-CGACGCTCACGAACTGCA**T**ATGCTCGCCAACACCACAA-3' (underlined and boldface sequences represent a *Nde*I site and the mutation, respectively) and the 3' primer 5'-GAGAGGCACGGGCCACGAAAGG-3' (primer downstream of *PmeI* site) were used in conjunction with plasmid

pΔFBgusSe8 (Lung *et al.*, 2002) as template using *Pfu*-polymerase (Promega). The PCR product (346 bp) was agarose gel-purified, and the single strand containing the mutation at the 3' end served as a 3' mutagenic primer in a second PCR reaction with 5' primer 5'-TTATGGATCCATGCTGCGTTTTAAAGTGAT-3' (underlined represent a *Bam*HI site) using high-fidelity "Expand" long-template polymerase (Roche). The second PCR product (862 bp) was cloned into pGEM-T (Promega) to generate the intermediate plasmid pGEM-SeF^{NdeI}. Plasmid pΔFBgusSeF^{NdeI} was obtained by swapping the *Bam*HI/*Pme*I fragment of pGEM-SeF^{NdeI} with the same fragment of pΔFBgusSe8.

Mutations and deletions in the coding sequence of SeF, comprehending amino acids (a.a.) 151-170 were performed as follows. For every mutant a 5' phosphorylated primer pair (Table 6.1) was used with pGEM-SeF^{NdeI} as template and *Pfu*-polymerase (Promega) to amplify the entire vector. Finally the 5' ends of the PCR products were ligated to its own 3' ends generating a new restriction endonuclease site at the junction (Table 6.1). Clones containing the additional restriction site were sequenced were sequenced to confirm the mutation. The obtained mutations in pGEM-SeF^{NdeI} were introduced into pΔFBgusSeF^{NdeI} by swapping the *Bam*HI/*Nde*I fragments.

Table 6.1 Primer pairs generating the desired mutation and a restriction endonuclease (RE) site.

Mutant	5' primer[a,b]	3' primer[a,b]	RE site
$SeF_{\Delta 151\text{-}170}$	5'-GC**C**CACGAACTGCATATGCTCGCC-3'	5'-GCCGCGTTTAGAGCGTCTTTTCG-3'	*Nar* I
SeF_{L151R}	5'-CC**GC**TTTAATTTTATGGGACACGTCG-3'	5'-CCGCGTTTAGAGCGTCTTTTC-3'	*Not* I
SeF_{F152R}	5'-CCTT**CG**TAATTTTATGGGACACGTCG-3'	5'-CC**T**CGTTTAGAGCGTCTTTTCGTC-3'	*Stu* I
SeF_{M155R}	5'-**GC**GGACACGTCGACAAATATCTG-3'	5'-GGAAATTAAAAAGGCCGCGTTTAG-3'	*Sac* II
SeF_{G156A}	5'-CGT**G**GACAAATATCTGTTTGGCATTATG-3'	5'-TGT**G**CCATAAAATTAAAAAGGCCGCGTTT-3'	*Eco*72I
SeF_{V158R}	5'-CGACAAATATCTGTTTGGCATTATG-3'	5'-**CGA**TGTCCCATAAAATTAAAAAGGCCGCGTTT-3'	*Nru* I
SeF_{K160L}	5'-**TCT**ATATCTGTTTGGCATTATGGACAG-3'	5'-TC**T**ACGTGTCCCATAAAATTAAAAAGG-3'	*Bgl* II
SeF_{F163R}	5'-CTTGCGTGGCATTATGGACAGCGAC-3'	5'-TACTTGTCGACGTGTCCCATAAAA-3'	*Sca* I
SeF_{G164A}	5'-**CG**ATTATGGACAGCGACGACGCTCAC-3'	5'-C**G**AACAGATATTTGTCGACGTG-3'	*Nru* I
SeF_{M166R}	5'-**CGC**GACAGCGACGACGCTCACGA-3'	5'-AATGCCAAACAGATATTTGTCGAC-3'	*Nru* I

[a]Underlined sequences of primer pairs are generating restriction sites after blunt-end selfligation of the PCR products.

[b]Mutations are indicated in boldface.

Transfection-infection assay

The *gus* reporter gene controlled by the *p6.9* promoter and the mutant SeMNPV *f* genes under control of the *gp64* promoter from pΔFBgusSeF^{NdeI} were transposed into the *att*Tn7 transposon integrase site of a *gp64*-null AcMNPV Bacmid (provided by Dr. G. W. Blissard) (Lung *et al*, 2002) according to the Bac-to-Bac manual (Invitrogen). Transpositions of inserts from donor plasmids were confirmed by PCR using a primer corresponding to the gentamycin resistance gene of the donor plasmid (P-gen-RV [5'- A GCCACCTACTCCCAACATC-3']) in combination with a primer corresponding to the bacmid sequence adjacent to the transposition site (M13/pUC forward primer [5'-CCCA GTCACGACGTTGTAAAACG-3']). Bacmid DNA, positive in the PCR reaction, was electroporated into DH10β cells to eliminate the helper plasmid and of some residual untransposed bacmid DNA.

Approximately 1 μg DNA of each recombinant bacmid was transfected into 1.5 x 10^6 Sf21 or Sf9^{Op1D} cells using 10 μl Cellfectin (Invitrogen). At 5 days posttransfection (p.t.) transfected cells were stained for GUS activity according to the Bac-to-Bac protocol

(Invitrogen) to monitor transfection efficiency. The supernatant was clarified for 10 min at 2,200 x *g* and subsequently filter-sterilized (0.45 μm pore size). One-fourth (500 μl) of the supernatant was used to infect 2.0 x 10^6 Sf9 or $Sf9^{Op1D}$ cells, respectively. At 72 hours postinfection (p.i.) cells were split into two portions. Cells of one portion were stained for GUS activity; the other portion used at 10 days p.i. to monitor viral propagation. As positive control for transfection and infection served the *gp64*-positive bacmid, while the *gp64*-null bacmid was used as negative control for transfection and infection (Lung *et al*., 2002). The SeF_{R149K} bacmid was used as negative control for F protein cleavage (Lung *et al*., 2002).

BV amplification and preparation

Viruses carrying *f* genes that rescued the *gp64*-null phenotype were amplified by infecting 1.0 x 10^7 Sf21 cells with 500 μl of cell supernatant from 10 days p.i. Viruses carrying *f* genes that did not rescue the *gp64* deletion in Sf21 cells were amplified in a similar manner using $Sf9^{Op1D}$ cells. Cells were split every 3 to 5 days until all cells were infected. Amplified pseudotyped viruses were titrated on $Sf9^{Op1D}$ cells by a 50% tissue culture infective dose ($TCID_{50}$) assay (O'Reilly *et al*., 1992) and scored for infection by examining cells for GUS expression.

The genotypes of the pseudotyped viruses were confirmed by PCR on purified BV DNA, using primers P-SeF-mutant-FW [5'-GGCGTTGACGGTCGAGGCTAAAT-3' and P-SeF-mutant-RV [5'-GTGCATCGCTTTTTCGGTGAGAGG-3'] to amplify a DNA fragment containing the incorporated restriction site (see Table 6.1). The amplified DNA fragment was subsequently subjected to restriction enzyme analysis.

One step growth curves

To follow infectious BV production from viruses carrying mutant *f* genes that rescued the *gp64*-null phenotype, viral growth curves were generated by collecting infected cell supernatants. Sf21 cells (1.5 x 10^5 cells per well; 24-wells plate) were infected at a m.o.i. of 5.0 $TCID_{50}$ units/cell or 0.5 $TCID_{50}$ units/cell for 1 h at 27°C. After infection the inoculum was removed and fresh 0.5 ml medium was added to the cells. At 0, 24, 48, 72, 69 and 144 h p.i the infected cell supernatants were collected. For each time point p.i., and each virus sample, duplicate samples were generated. The quantity of infectious BVs in the samples was determined by $TCID_{50}$ assays on $Sf9^{Op1D}$ cells. A third sample was generated for those duplicated samples differing in titers with a factor 2.5 or more.

Western blot analysis

BVs were amplified by infecting 1.0 x 10^7 Sf21 or $Sf9^{Op1D}$ at a m.o.i of 0.5 $TCID_{50}$ units/cell. Cells were split every 3 to 5 days until all cells were infected. BVs were purified from the supernatants as described previously (Westenberg *et al.,* 2002). Equal amounts of BVs, determined by the Bradford method (Bradford, 1976), were disrupted in Laemmli buffer (125 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.8) and denatured for 10 min at 95°C. Proteins were electrophoresed in SDS-polyacrylamide gels and

transferred onto Immobilon-P membranes (Millipore) by semi-dry electrophoresis transfer (Ausabel *et al*., 1994). Membranes were blocked overnight at 4°C in PBS containing 2% milk powder, followed by incubation for 1 h at room temperature (RT) with either Pab α-F1 and α-F2 (Westenberg *et al*., 2002), Mab AcV5 (Hohmann and Faulkner, 1983) or Pab α-VP39 (provided by Dr. A. L. Passarelli, Manhattan KS, USA) (Thiem and Miller, 1990) all in a 1:1000 dilution in PBS containing 0.2% milk power. After washing three times for 15 min in PBS-T (PBS containing 0.1% Tween-20), the membranes were incubated for 1 h at RT with horseradish peroxidase conjugated appropriate secondary antibody (Sigma, DAKO) in PBS containing 0.2% milk powder. After washing three times for 15 min in PBS-T, the signal was detected by ECL technology as described by the manufacturer (Amersham).

Computer-assisted analysis

Protein comparisons with entries in the updated GenBank/EMBL, were performed with FASTA and BLAST programs (Altschul *et al*., 1997; Pearson, 1990). Sequence alignments were performed with the program ClustalW (EMBL-European Bioinformatics Institute [http://www.ebi.ac.uk]) and edited with the Genedoc Software (Nicholas *et al.,* 2002). The α-helix predictions were performed with the Protean software of DNASTAR using the Garnier-Robson method (Garnier *et al*., 1978).

RESULTS

Pseudotyping *gp64*-null AcMNPV with *f* mutants

To examine the role of the N-terminus of the SeMNPV F_1 fragment in viral propagation and infectivity, mutational analysis on this domain was performed. The hydrophilic arginine was substituted for hydrophobic amino acids (Fig. 6.2A and B, and Table 6.1). Five of these hydrophobic amino acids (F^{152}, M^{155}, V^{158}, F^{163} and M^{166}) are conserved and one (L^{151}) is nonconserved among F homologs of group II NPVs, GVs en errantiviruses (Fig. 6.1). Alanine was substituted for glycines (G^{156} and G^{164}) and a hydrophobic leucine was substituted for the conserved lysine (K^{160}). Each mutation was marked by the presence of a new restriction site. Furthermore an F mutant with a deletion in the conserved region (a.a. 151-170) of the fusion peptide was also constructed. It was then determined whether the F protein mutants could rescue BV propagation of a *gp64*-null AcMNPV bacmid, relative to the native F protein (Lung *et al*., 2002) when transfected into Sf21 cells. Mutant *f* genes under control of the AcMNPV *gp64* promoter and a *gus* reporter gene downstream of the baculovirus *p6.9* promoter were inserted simultaneously by Tn7 based transposition (Luckow *et al*., 1993) into the *gp64*-null AcMNPV bacmid. Sf21 cells were transfected with the constructed bacmids together with various control bacmids: *gp64*-null AcMNPV bacmids containing (1) no envelope fusion protein gene (ΔF) (negative control), (2) AcMNPV *gp64* (positive control), (3) SeMNPV *f*, (4) SeMNPV f^{NdeI} (*f* gene with silent mutation creating a *Nde*I cloning site) and (5) SeMNPV f_{K149R} (*f* gene mutant negative in F_0 cleavage). Five days p.t. cells were stained for GUS activity (Fig. 6.3A^1-O^1). The presence of infectious BVs in

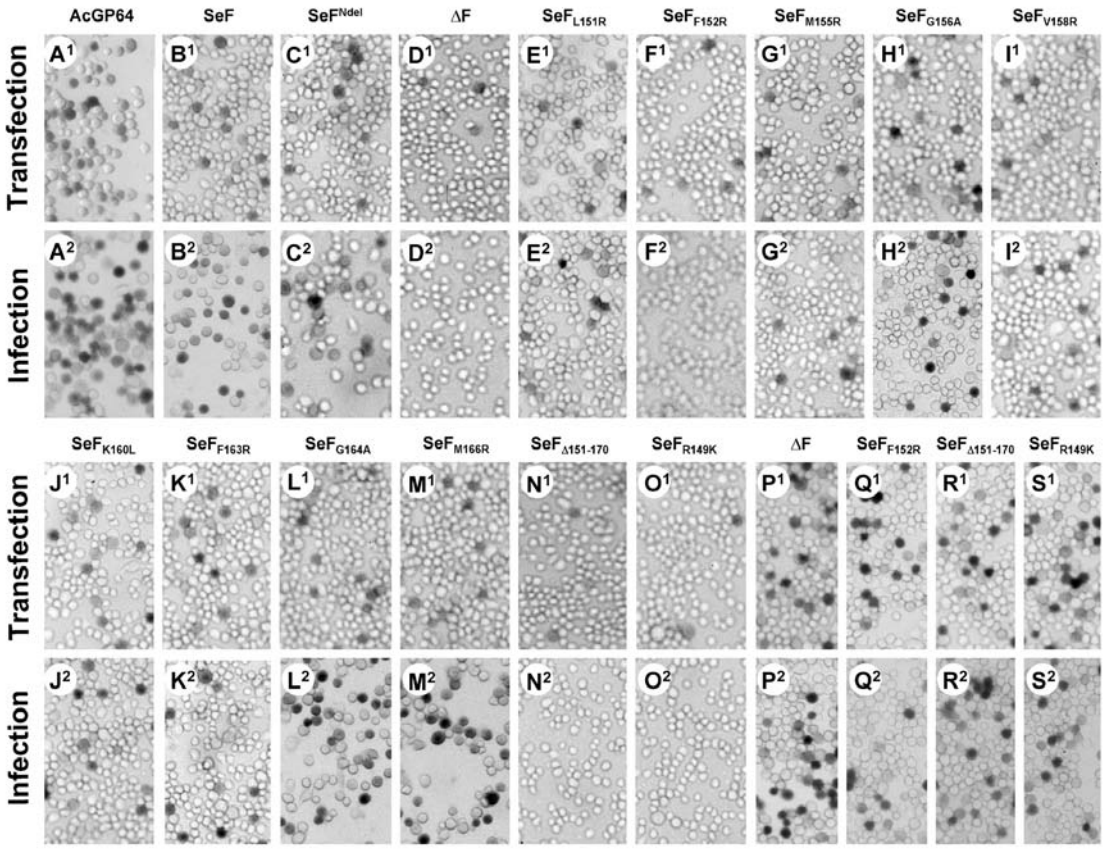

Figure 6.3 Transfection-infection assays for viral propagation. Sf21 cells were transfected with indicated mutant *gp64*-null bacmids pseudotyped with *f* genes, incubated for 5 d, and stained for GUS activity (A^1- O^1). Supernatants from the transfected cells were used to infect Sf21 cells (A^2- O^2), which were incubated for 72 h and subsequently stained for GUS activity. Stained cells (infected cells) indicate that infectious virions were generated in the transfected cells. Bacmids that fail to propagate an infection in Sf21 cells (D^2, F^2, N^2, and O^2) were propagated in cells expressing constitutively OpMNPV GP64 ($Sf9^{Op1D}$). $Sf9^{Op1D}$ cells were transfected with indicated *gp64*-null bacmids (P^1-S^1) and incubated for 5 days; then supernatants were transferred to $Sf9^{Op1D}$ and stained for GUS activity after 72 h (P^2-S^2). Indicated *gp64*-null AcMNPV bacmids are pseudotyped with: AcGP64 (AcMNPV *gp64*), SeF (SeMNPV *f*), SeF^{NdeI} (SeMNPV *f* with silent mutation generating a *Nde*I restriction site), ΔF (no envelope fusion gene), SeF_{L151R}, SeF_{F152R}, SeF_{M155R}, SeF_{G156A}, SeF_{V158R}, SeF_{K160L}, SeF_{F163R}, $SeF_{G164A,}$ SeF_{M166R} (SeMNPV *f* with mutations causing amino acid substitution in the putative fusion peptide as indicated in subscript), $SeF_{\Delta 151\text{-}170}$ (SeMNPV *f* with a deletion encoding amino acid 151-170), SeF_{R149K} (SeMNPV *f* with mutations causing an amino acid substitution in the furin cleavage site).

the supernatant was determined by passaging the supernatants to new Sf21 cells. Three days p.i. infected cells were demonstrated by their GUS activity (Fig. 6.3A^2-O^2). The bacmids SeF_{L151R}, SeF_{M155R}, SeF_{G156A}, SeF_{F152R}, SeF_{V158R}, SeF_{K160L}, SeF_{F163R}, SeF_{G164A} and SeF_{M166R} were all able to produce infectious viruses (Fig. 6.3E-M), like SeF^{NdeI} (Fig. 6.3C) and the positive controls, AcGP64 and SeF (Fig. 6.3A and B). The bacmids SeF_{F152R} and $SeF_{\Delta 151\text{-}170}$ (Fig. 6.3F and N), and both negative controls (ΔF and $SeF_{K149R,}$ Fig. 6.3D and O) were not able to produce infectious viruses, as expected. However, when those bacmids were transfected into $Sf9^{Op1D}$ cells, which constitutively express the *Orgyia pseudogata* (Op)MNPV GP64 protein to pseudotype AcMNPV (Plonsky *et al.*, 1999), infectious BVs could be demonstrated (Fig. 6.3P-S), indicating that the defect in BV propagation was attributable to the expression of an inactive fusion protein.

BVs carrying an envelope fusion protein gene that rescued the *gp64*-null phenotype were amplified by infecting Sf21 cells; those BVs that were not rescued were amplified by using $Sf9^{Op1D}$ cells as described in Materials and Methods. The amplified viruses were all subjected to PCR analysis to verify the introduced mutations in their *f* gene (Fig. 6.4). For viruses containing the mutant *f* genes PCR fragments of similar sizes were obtained, whereas the $SeF_{\Delta 151\text{-}170}$ mutant did show a much smaller fragment (Fig. 6.4A). Along with the desired mutations an additional restriction site was introduced in the *f* genes to mark the mutation (see Table 6.1), allowing the analysis of the resulting PCR fragments by restriction enzyme analysis (Fig. 6.4B). From the patterns it could be concluded that the *gp64*-null AcMNPV viruses contained the correct mutations.

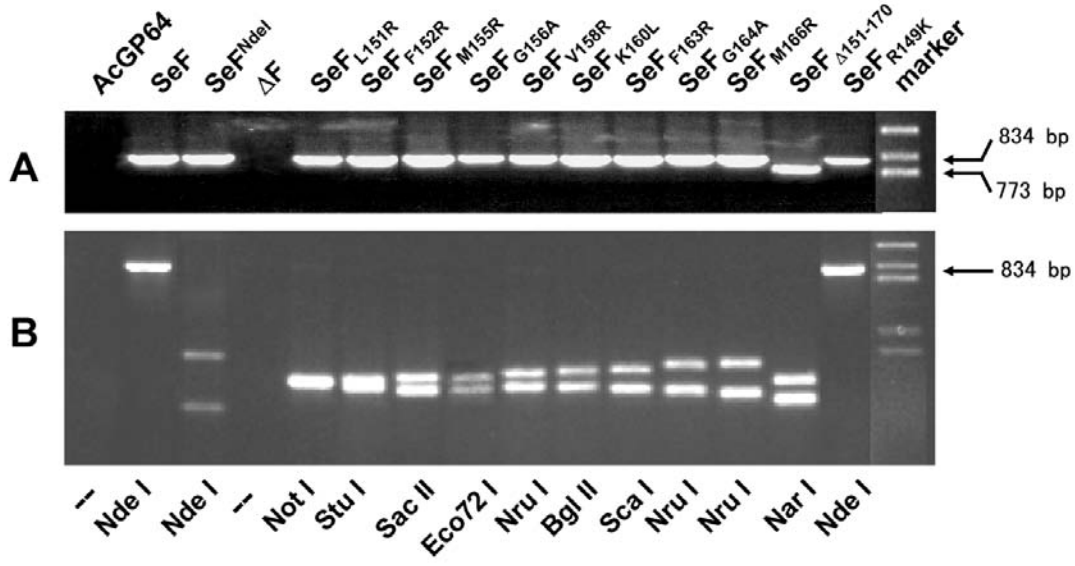

Figure 6.4 PCR and restriction enzyme analyses of purified BV DNA to verify the genotype of indicated pseudotyped vAc^{gp64-} viruses as described in Fig. 6.3. (A) PCR with *f* gene specific primer pairs used to examine mutant viral DNAs amplifying an 834 pb fragment, when the virus contains the *f* gene, except for $SeF_{\Delta 151\text{-}170}$ where a 773 bp fragment was amplified. B) Restriction enzyme analysis of PCR amplified DNA fragments. Mutant F genes have besides the incorporated *Nde*I site an additional restriction site, which is used to distinguish the viruses (Table 6.1). The SeF_{R149K} mutant has neither a *Nde*I site nor an additional restriction site.

Western analysis of *f* gene pseudotyped *gp64*-null AcMNPV viruses

The effect of the various mutations on the processing and incorporation of the F protein in BVs was determined by Western analysis (Fig. 6.5). An antibody against the major nucleocapsid protein (α-VP39) served as internal control to determine the amount of BVs used in the analysis. Results indicated that all mutant F proteins were present in BVs. The amount of BVs for each recombinant virus was estimated to be similar, except for SeF_{V158R} (lane 9) and SeF_{K160L} (lane 10) where less BVs were used (Fig. 6.5D). The AcMNPV GP64 protein could be detected with the repair virus (Fig. 6.5C, lane 1) as well as for the viruses propagated in $Sf9^{Op1D}$ cells (Fig. 6.5C, lanes 4, 6, 14 and 15). Western analysis using antibodies against the SeMNPV F_1 and F_2 indicated that the incorporation of the F protein in the BVs was reduced for SeF_{F152R}, SeF_{M155R}, SeF_{M166R} and SeF_{R149K} (Fig.

6.5A and B, lanes 6, 7, 13 and 15, respectively) compared to that of native SeF protein (Fig. 6.5A and B, lane 1). Furthermore, all mutant F proteins, except SeF_{R149K} (Fig. 6.5A, lane 15) lacking a furin cleavage site, were properly cleaved into F_1 (Fig. 6.5A) and F_2 (Fig. 6.5B) fragments.

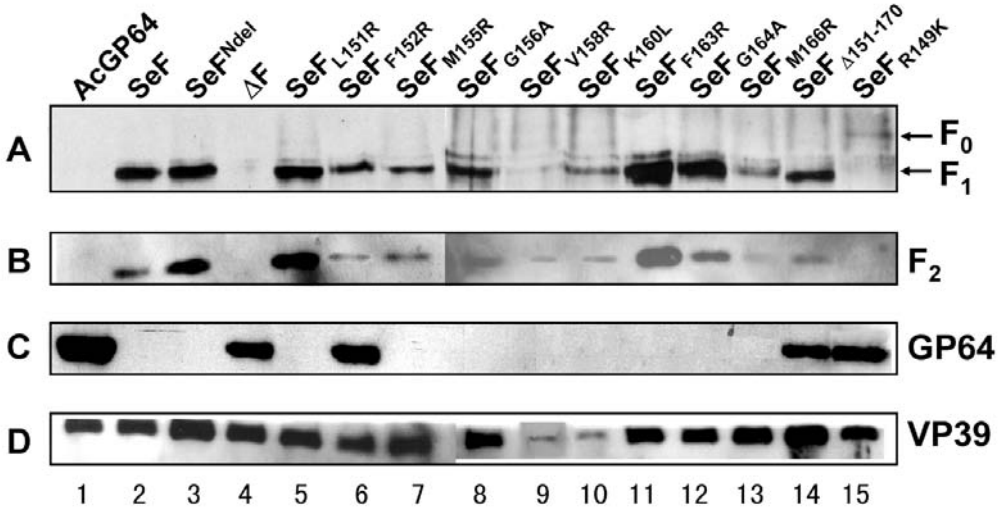

Figure 6.5 Western blot analysis of *gp64*-null BVs pseudotyped with indicated (mutant) *f* genes as described in Fig. 6.3. Infectious BVs were generated in Sf21 cells (lanes 1-3, 5, 6 and 8-13) and BVs, which are not able to propagate in Sf21 cells, were propagated in $Sf9^{Op1D}$ cells (lanes 4, 7, 14 and 15). Blots were probed with antibodies (A) α-F1 and (B) α-F2 (Westenberg *et al*., 2001), (C) α-GP64 (monoclonal antibody AcV5 (Hohmann and Faulkner, 1983)). (D) An anti-nucleocapsid antibody (α-VP39) was used as an internal control for each preparation of purified budded virions (Thiem and Miller, 1990)

Viral infectivity of *f* gene pseudotyped *gp64*-null AcMNPV viruses

To characterize the effect of the mutations on viral infectivity of the mutant *gp64*-null viruses, one-step growth curves were performed. Sf21 cells were infected at a m.o.i. of 5.0 $TCID_{50}$ units/cell. At different time points p.i. the amount of infectious BVs was determined (Fig. 6.6A). The viral titers for the native SeF and the SeF^{NdeI} mutant were not significantly different indicating that the silent mutation to generate the *Nde*I site had no effect on BV infectivity and propagation. The one-step growth curve for SeF^{NdeI} is therefore left out from the graph (Fig. 6.6). Except for SeF_{V158R}, the viral titers of the other mutants did not significantly differ from that of SeF. The viral titer of SeF_{V158R} remained significant lower at all time points compared to that of the authentic SeF. This indicates that the mutations had hardly any effect on the amount of BV production over time. A different behavior was seen when Sf21 cells were infected at a m.o.i. of 0.5 $TCID_{50}$ units/cell (Fig. 6.6B), so that not all cells were infected in the first round. In this experiment the effect of the mutations on viral infectivity could be determined, since the amount of BV production over time was not altered, except for SeFV158R. Also this time the growth curve of SeF^{NdeI} was almost identical to that of SeF (data not shown). In the situation of 0.5 $TCID_{50}$ units/cell only the growth curves of SeF_{L151R}, SeF_{G164A} and SeF_{M166R} were similar to that of SeF. The curve of SeF_{G156A} shows a drop in titer between 48 and 72 h p.i., but at 144 h p.i. the titer was raised to levels similar to that of SeF. The viral titers of SeF_{M155R}, SeF_{V158R}, SeF_{K160L} and SeF_{F163R} showed a dramatic decrease compared to

that of SeF. Thus, mutations in the fusion peptide affect viral infectivity (Fig. 6.6B), presumably the dynamics of viral spread, rather than viral production (Fig. 6.6A).

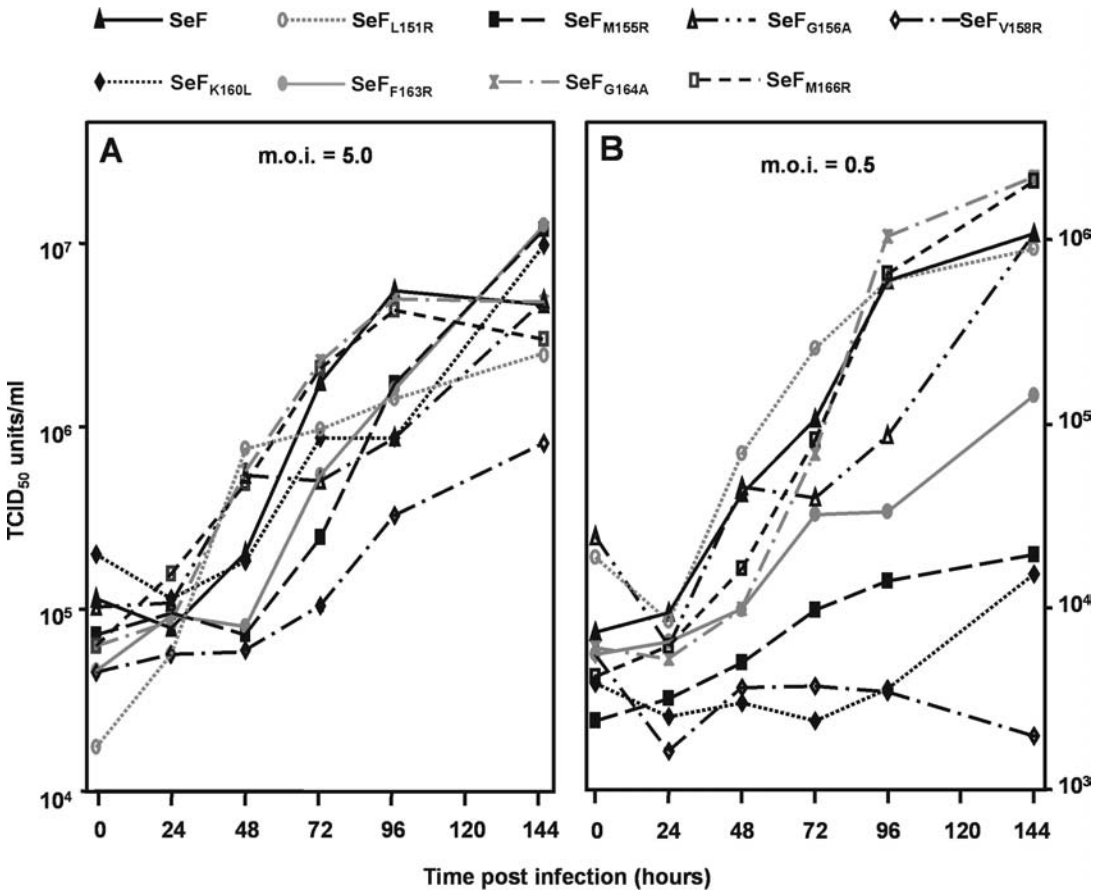

Figure 6.6 One-step growth curves are shown for *gp64*-null AcMNPV viruses pseudotyped with wild type SeF and SeF mutants as described in Fig. 6.3. Sf21 cells were infected with a multiplicity of (A) 5.0 or (B) 0.5 $TCID_{50}$ units/cell and supernatants were harvested at indicated times postinfection and titrated on $Sf9^{Op1D}$ cells. Each data point represents the average of two or three independent infections. Standard deviations bars are not presented for better interpretation of the graphs.

DISCUSSION

Posttranslational cleavage is a general mechanism to activate the fusion proteins of enveloped viruses (Klenk and Garten, 1994). Recently it has been shown that this is also the case for the baculovirus F protein, where two subunits are generated, F_1 and F_2 (Lung *et al*., 2002; Westenberg *et al*., 2002). Cleavage of envelope fusion proteins usually occurs in front of a conserved hydrophobic region, the “fusion peptide”. The N-terminus of the SeMNPV membrane-anchored domain (F_1) contains striking similarities with the fusion peptides of other viruses: (i) the domain is well conserved among its “functional” homologs, (ii) it is relatively hydrophobic, (iii) can be modeled as an amphipathic helix, and (iv) it contains conserved glycines at one side of the helix (Fig. 6.1 and 6.2). The helical nature of the corresponding region of LdMNPV F has recently been determined by circular dichroism (Pearson and Rohrmann, 2003).

However, there are also some striking differences with vertebrate viral fusion peptides. Fusion peptides of the latter are rich in alanines, while the first alanine (residue 22) in the SeMNPV F_1 N-terminus is found outside the conserved region. Another difference is that N-terminal fusion peptides of most vertebrate viral fusion proteins are generally apolar, whereas the SeMNPV F_1 N-terminus contains six polar amino acids (N^{153}, H^{157}, D^{159}, K^{160}, D^{167}, and S^{168}). Other baculovirus F proteins have even up to nine polar amino acids in this region (Fig.6.1). However, the fusion peptide of Influenza HA also contains two to three polar residues (Martin *et al*., 1999). It is very well possible, that the polar amino acids force the N-terminus of F_1 to insert in the membrane in a more perpendicular angle, with the polar amino acids to the hydrophilic side of the phospholipids, compared to other fusion peptides.

The importance of the SeMNPV F_1 N-terminus for virus infection and propagation was investigated by a series of amino acid substitutions. AcMNPV virions, lacking gp64, were pseudotyped with mutant f genes and assayed (see Table 6.2 for summarized results). A similar experimental system has been used to analyze the effect of mutations in the Ebola virus GP fusion peptide through pseudotyping the Vesicular stomatis virus (VSV) lacking its own fusion protein (Ito *et al*., 1999). The F protein with a deletion of the N-terminus ($SeF_{\Delta 151\text{-}170}$) was not able to produce infectious virus (Fig. 6.3), in spite of its ability to be incorporated in BVs and the occurrence of the posttranslational cleavage (Fig. 6.5). This suggests that this domain is not involved in virion assembly, but plays an important role in baculovirus entry in cells.

Table 6.2 Summary of obtained results.

gp64-null bacmid	Rescue	PCR	Western		viral propagation	
			F incorporation	F cleavage	m.o.i. = 5.0	m.o.i. = 0.5
Controls						
AcGP64	+++	*V*	NA	NA	+++	+++
SeF	++	*V*	++	+	++	++
SeF_{R149K}	-	*V*	+	-	NA	NA
ΔF	-	*V*	NA	NA	NA	NA
Mutants						
SeF^{NdeI}	++	*V*	++	+	++	++
SeF_{L151R}	++	*V*	++	+	++	++
SeF_{F152R}	-	*V*	+	+	NA	NA
SeF_{M155R}	+	*V*	+	+	++	+
SeF_{G156A}	++	*V*	++	+	++	++
SeF_{V158R}	+	*V*	++	+	+	+
SeF_{K160L}	+	*V*	++	+	++	+
SeF_{F163R}	+	*V*	++	+	++	+
SeF_{G164A}	++	*V*	++	+	++	++
SeF_{M166R}	++	*V*	++	+	++	++
$SeF_{\Delta 151\text{-}170}$	-	*V*	++	+	NA	NA

V =in agreement with the expectancy; NA= not applicable; +++ = good; ++ = fair; + = bad; - = no

Glycines as well as their conserved positions in the fusion peptides seem to be important for the function of viral fusion proteins (Cross *et al*., 2001b). It is supposed that glycine residues might provide the proper balance of amphipathicity necessary for merging viral and cell membrane or might be important for an oblique insertion of the fusiogenic peptide into the target membrane (Horth *et al*., 1991). In this study the specific role of two conserved glycine residues in the putative fusion peptide of SeMNPV F was addressed by converting these into alanines. These substitutions might increase

the stability of the possible a-helical conformation of the F_1 N-terminus. The G to A mutations in the Sendai virus fusion peptide led to increased fusion activity of the fusion protein (Horvath and Lamb, 1992), while for the Semliki Forest virus E1 protein it caused fusion at a lower pH (Levy-Mintz and Kielan, 1991). With the SeMNPV F protein the G to A mutations showed no notable effect. However, these results are in line with results obtained for the Ebola virus GP fusion peptide (Ito *et al*., 1999), where one of the mutations of the glycines also had no effect on the viral titer and the incorporation of the fusion protein in virions.

Arginine was substituted for conserved as well as nonconserved hydrophobic residues in the fusion peptide of the SeMNPV F protein. Introduction of a polar residue in the hydrophobic face of the amphiphatic helix is expected to result in either a shorter (SeF_{L151R}, SeF_{M166R}), narrower (SeF_{F152R}, SeF_{M155R}, SeF_{F163R}) or in a disrupted (SeF_{V158R}) hydrophobic face. The alterations can possibly disturb the helical conformation or the insertion of the helix in the host membrane and hence may have an effect on infectivity. Leucine was substituted for the conserved polar lysine (SeF_{K160L}), which decreases the hydrogen bonding potency of the back-face of the helix. Despite all the substitutions there was no notable effect on incorporation of F proteins in BVs and on the processing of the mutant F proteins (Fig. 6.5). Only, SeF_{F152R} was not able to produce infectious virus (Fig. 6.3) suggesting a critical role of this amino acid in fusion. Similar results with an F to R conversion have been obtained for the Ebola GP protein (Ito *et al*., 1999).

The virions pseudotyped with the SeF_{M155R}, $SeF_{F163R,}$ SeF_{V158R} (hydrophilic substitutions) and SeF_{K160L} (hydrophobic substitution) genes were all impaired in their virus propagation dynamics (Fig. 6.6B). For SeF_{M155R} this could be caused by a reduced incorporation of F protein in BVs (Fig. 6.5 lane 7). In contrast, when cells were infected with a higher dose, only the viral titer of virus pseudotyped with SeF_{V158R} was significant lower compared to that of native SeF (Fig. 6.6A). Such a V to R conversion has been shown to reduce fusion activity of the Murine Leukemia virus fusion protein (Jones and Risser, 1993). However, western analysis suggested that the amount of BVs produced is extremely low (Fig. 6.5 lane 9). It is very well possible that this is due to a defect in transport of the protein to the cell membrane, caused by incorrect folding of the protein.

The mutations L151R and M166R did not result in a significant drop in viral titers, although the incorporation of SeF_{M166R} in BVs was somewhat affected. The L^{151} and M^{166} residues are the first and the last hydrophobic amino acids of the putative fusion peptide and this suggests that the F protein can properly function with a smaller hydrophobic face. However, this does not imply that the borders of the putative fusion peptide of SeMNPV F are well-defined, because the two conserved aspartic acid residues D^{167} and D^{170} also appeared to be important for the fusiogenic activity of the LdMNPV F protein (Pearson *et al*., 2002a).

The N-terminal domain of the SeMNPV F_1 subunit is this most likely involved in the entry and infectivity of BVs and further credence is given for the role of this domain as “fusion peptide”. Future biochemical studies involving the 3-D structure of the fusion peptide should indicate how the active fusion peptide is folded and how the behavior of the site-specific mutant fusion peptides can be explained. Experiments to this end are in progress.

Chapter 7

GENERAL DISCUSSION

Introduction

Baculoviruses are studied for their (i) application as biological insecticide to control different insect pests (Moscardi, 1999), (ii) as gene expression vectors to produce large quantities of foreign proteins in insects and insect cells (Luckow, 1991), and (iii) as delivery system for gene therapy in vertebrates (Gosh *et al*., 2002; Pieroni and La Monica, 2001). In contrast to the well-studied, prototypic baculovirus AcMNPV, the host-range of the majority of baculoviruses is restricted to a limited number of related invertebrate hosts or even a single host (Adams and McClintock, 1991; Federici, 1997; Federici and Maddox, 1996; Summers 1977). This latter situation is also the case for SeMNPV (Onstad, 2003), the topic of this thesis. Specificity, i.e. host range, is an important feature of a virus in particular to regulate its spread and, in terms of biosafety, for its use as biological insecticide. In view of this, it is of much interest to study SeMNPV and its interaction with its host, *Spodoptera.*

Genetic approaches are very useful to unravel mechanisms of infection, but are necessarily based on detailed genomic information. Until 1999 only the genomes of the group I NPVs AcMNPV and OpMNPV were sequenced (Ahrens *et al.,* 1997; Ayres *et al*., 1994). At that time it was generally believed that all baculoviruses would encode homologs of the viral major envelope glycoprotein GP64. This protein was first found in AcMNPV, and is involved in (i) virion attachment to insect cells (Hefferon *et al*., 1999), (ii) fusion between viral and cellular membranes (Blissard and Wens, 1992; Kingsley *et al*., 1999; Plonsky *et al*., 1999; Volkman and Goldsmith, 1985), and (iii) budding of progeny virions (Monsma *et al.,* 1996; Oomens and Blissard, 1999). In 1999 the complete genome sequences of XecnGV, LdMNPV and SeMNPV became available (Hayakawa *et al*., 1999; IJkel *et al.,* 1999; Kuzio *et al*., 1999), revealing that all three baculoviruses lacked a *gp64* gene. With even more baculoviral genome sequences available (Table 1.1) the majority appears not to have a *gp64* gene, but do produce budded viruses (BVs).

This enigma was clarified by the discovery and the mode of action of a novel type of baculovirus envelope fusion protein, called F, as reported in this thesis. This protein has functional analogy to GP64 and to other class I viral fusion proteins, as found with coronaviruses, filoviruses, orthomyxoviruses, paramyxoviruses and retroviruses (Bosch *et al*., 2003; Dutch *et al*., 2000). The F protein is a typical class I viral fusion protein with respect to (i) its synthesis as a single chain precursor, (ii) its activation upon proteolytic cleavage into two fragments, (iii) the existence of a putative N-terminal fusion peptide and (iv) two heptad repeats in the membrane-anchored cleavage fragment and (v) its possible trimeric oligomerization with a triple-stranded coiled coil (Colman and Lawrence, 2003). The newly identified baculovirus F protein's ancestral relationship with

other viruses and its role in baculovirus entry is discussed and compared with that of well characterized class I viral fusion proteins.

F protein evolution

Baculovirus F proteins are encoded by *f* genes, which have been found in all group II NPVs sequenced to date. For SeMNPV (IJkel *et al*., 2000), LdMNPV (Pearson *et al*, 2000), SpltMNPV (Zhang *et al*., 2001) and HaSNPV (G. Long, personal communication) it has been demonstrated that their respective F proteins are able to induce plasma membrane fusion when expressed in insect cells. It is very likely that all NPVs that lack GP64 have F as alternative fusion protein to mediate viral entry into host cells. Granuloviruses also have a putative *f* gene. Computer predicted analysis of the PlxyGV F protein identified a putative furin cleavage site as well as a putative fusion peptide within the protein. However the F protein of PlxyGV appeared to be fusion incompetent (IJkel and Westenberg, unpublished data) being unable to rescue the infectivity of a *gp64*-null AcMNPV virus (Chapter 4). It is possible that granuloviruses require an additional protein to mediate viral entry, which will act in conjunction with F to trigger the fusion process. The latter has been seen for most paramyxoviruses. The paramyxovirus fusion protein (F) usually requires co-expression of their receptor binding protein (H, HN or G) to initiate membrane fusion (Morrison, 2003).

Baculoviruses that use GP64 as their fusion protein do also contain an *f* gene. These F homologs are not able to induce syncytium formation (Chapter 2; Pearson *et al*., 2000). Furthermore, they do not contain a furin cleavage site and a putative fusion peptide (Chapter 6). Nevertheless, the F homolog of OpMNPV is a BV envelope protein and has a similar location as GP64 on this envelope (Pearson *et al.,* 2001). It is possible that the F protein is the original baculovirus fusion protein and that an ancestral NPV acquired a *gp64* gene thereby obtaining a selective advantage obviating the need for F as fusion protein, but still having an advantage for the preservation of the remnant *f* gene. The *f* gene can be deleted from the genome of AcMNPV without effecting viral propagation *in vitro*, but insects infected with this *f*-null AcMNPV virus died at later times postinfection than wildtype AcMNPV (Lung *et al.,* 2003).

Another indication pointing to a “recent” acquisition of GP64 by group I NPVs is that the baculovirus GP64 homologs are between 70-92 % identical in amnio acid sequence, whereas the F homologs of group I and II NPVs are more diverged (50-96 % and 38-100 % amino acid sequence identity, respectively). Furthermore, GP64 shows moderate homology (24-27 % identical) to the envelope glycoproteins of Thogoto virus and Dhori virus. These are negative-strand ssRNA arboviruses, belonging to the family *Orthomyxoviridae*. These viruses can replicate in ticks as well as in mammalian cells (Freedman-Faulstich and Fuller, 1990; Morse *et al.,* 1992). Baculoviruses have also been found in dipterans (Becnel *et al*., 2003), in particular mosquitos. The latter are arbovirus vectors and gene transfer may have occurred between these arboviruses and baculoviruses in a dually infected dipteran host. However, it is equally possible that the arboviruses have acquired the *gp64* gene from a baculovirus. Arboviruses do not have a DNA stage during replication and therefore transfer of GP64 to the baculovirus by recombination seems to be a rather remote impossibility. The use of baculovirus *gp64*

mRNA as template to produce a viral RNA segment seems to be a more plausible explanation.

Homologs of the baculovirus *f* gene have also been found in the genomes of the fruit fly *Drosophila melanogaster* (Adams *et al.,* 2000) and the African malaria mosquito *Anopheles gambiae* (Holt *et al*., 2002). However, the F protein homolog of *D. melanogaster* seemed not to be associated with the plasma membrane, but was found in cytosolic compartments of the cells (Lung and Blissard, 2003). This location might be governed by a peroxisomal target signal consensus sequence R/K-L/I-$(X)_5$-H/Q-L (McNew and Goodman *et al*., 1996) within the protein amino acid sequence RLNNGVIQL in this host-derived F homolog.

The envelope protein (Env) from insect retroviruses belonging to the family *Errantiviridae* also shows relatedness to the baculovirus F protein (Malik *et al*., 2000; Rohrmann and Karplus, 2001). Retroviruses are believed to originate from long terminal

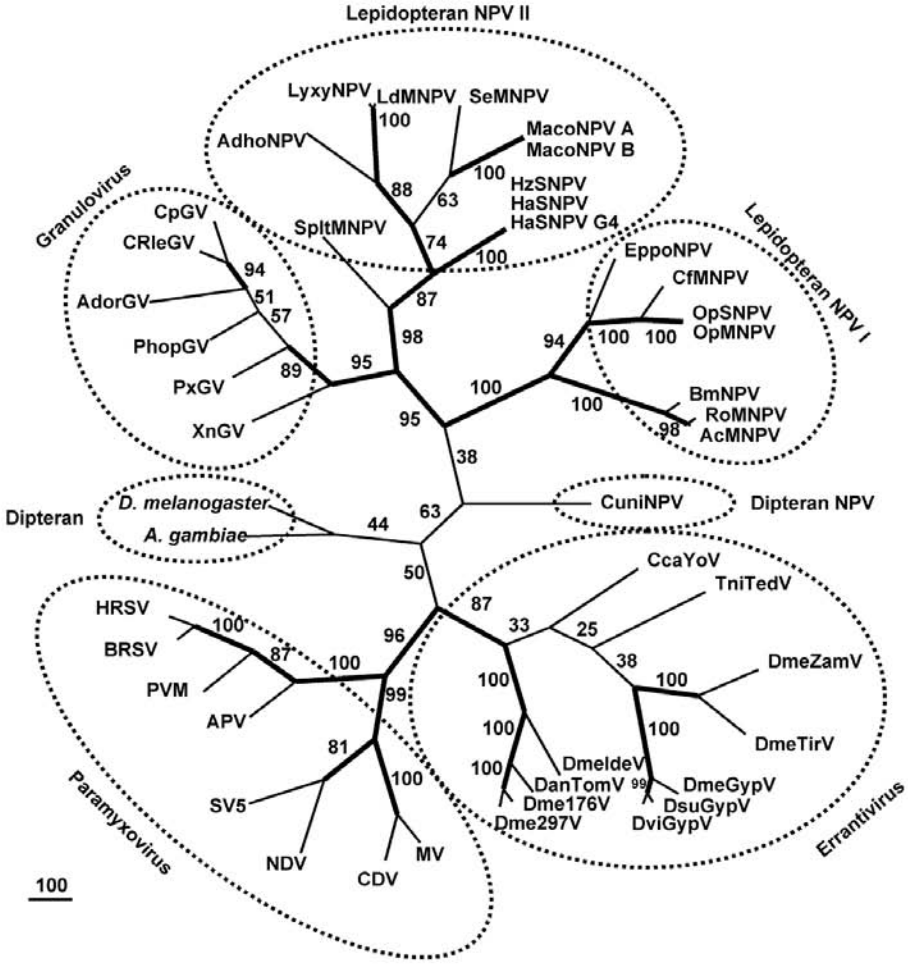

Figure 7.1 Bootstrap analysis (200 replicates) of unrooted phylogenetic trees of F proteins constructed with PAUP heuristic search algorithm. Numbers at the branches indicate frequency of clusters and thick lines indicate frequencies over 70%. The bar at the bottom equals a branch length of 100. Accession numbers of F protein homologs of baculoviruses, errantivirus and dipteran species are shown in Fig. 6.1. F protein homologs of paramyxoviruses used with their accession numbers in brackets: HRSV: Human respiratory syncytial virus (AAB38520), BRSV: Bovine respiratory syncytial virus (NP_0408055), PVM: Pneumovirus of mice (P35949), APV: Avian pneumovirus (AAG30839), SV5: Simian virus 5 (P04849), NDV: Newcastle disease virus (AAO46783), CDV: Canine distemper virus (AAG30919), MV: Measles virus (AAL29688).

repeat (RTL) retrotransposons (Pélisson *et al.,* 1997); the only difference to retrotransposons is that retroviruses contain an envelope protein gene (*env*) and can escape from the nucleus to re-insert host DNA in a neighbouring cell or even other individual. Retrotransposons have been found in the genome of both baculoviruses (Arends and Jehle, 2002; Friesen and Nissen, 1990, Miller and Miller, 1982) and their hosts (Friesen *et al*., 1986). Hence, it is very well possible that the RTL retrotransposons have acquired their *env* gene from a baculovirus, thereby becoming a retrovirus, and dipteran species might have picked up the F homolog as a consequence of a retrovirus infection.

The baculovirus F protein exhibits also some striking homology with the envelope fusion proteins of paramyxoviruses (Fig 7.1). As discussed in Chapter 3, the SeMNPV F protein is 12% identical and 36% similar to the Human respiratory syncytial virus (HRSV) F protein, and the predicted secondary structures are more or less conserved. A recent study (Misseri *et al.,* 2003) predicted that the three-dimensional structures of the F protein homologs of group II NPVs, GVs and in particular that of the errantiviruses, all generated by computer modeling, show significant similarities to the X-ray determined structure of the Newcastle disease virus (NDV) F protein (Chen *et al*., 2001). The evolutionary relatedness of these viruses is very speculative, but it may be possible that fusion proteins originate from a common ancestral (viral) protein and that the baculoviral and paramyxoviral F proteins evolved under a more or less equal structural pressure.

Baculovirus taxonomy

The family of *Baculoviridae* is divided in two genera, *Granulovirus* (GV) and *Nucleopolyhedrovirus* (NPVs) (van Regenmortel *et al.,* 2000). The NPVs are divided in two groups: group I and group II NPVs. This division is based upon phylogenetic studies using amino acid sequences of the *polyhedrin/granulin* (*Zanotto et al*., 1993), *egt* (Chen *et al*., 1997), *lef-2* (Chen *et al*., 1999) and *DNA polymerase* (Bulach *et al*., 1999) genes. To date 22 baculovirus genomes are completely sequenced and baculovirus taxonomy can now be based on 30 conserved genes (Herniou *et al.,* 2003). These analyses revealed that the NPVs can be divided in three groups: lepidopteran group I and group II NPVs and a dipteran NPV group. Only a single baculovirus (CuniNPV) (Alfonso *et al.*, 2001) has been assigned to the dipteran NPV group, but with time more baculoviruses infecting dipteran species will be analyzed. Recent information of an NPV of the sawfly *Neodiprion lecontei* (NeleNPV) suggests even a fourth NPV group (Lauzon *et al*., 2003).

From single gene-based phylogenetic analysis no definitive conclusions on the taxonomic position of a virus can be made. However, phylogeny based on the baculovirus envelope fusion proteins resulted in a similar grouping of the baculoviruses. These phylogenetic analysis studies further suggests that the F protein is a good marker to classify new baculoviruses to one of the four baculovirus groups known to date.

The gene order of conserved genes in baculovirus genomes can also be used as phylogenetic marker (Herniou *et al.,* 2001; Hu *et al*., 1998). It is conceivable that a certain gene clustering might be in some way important for virus replication, e.g. a transcriptional constraint. The baculovirus *f* gene is not situated within a conserved gene cluster (Fig. 7.2). However, evolutionary/phylogenetic relationships among baculoviruses can also be derived from the gene order surrounding the *f* gene. Based on this gene

arrangement the group II NPVs could be further subdivided and now six baculovirus groups can be made consisting of (i) the group I NPVs, (ii) the SpltMNPV, HaSNPV and HzSNPV (group II NPV A), (iii) SeMNPV and MacoNPV (group II NPV B), (iv) LdMNPV and AdhoNPV (group II NPV C), (v) the dipteran NPV CuniNPV, and (vi) the GVs. Apparent is the more or less similar gene arrangement of SpltMNPV, HaSNPV and HzSNPV and the group I NPVs. The phylogenetic distance of their F proteins to those of the group I NPVs is much smaller than to that of the other group II NPV F proteins (Fig. 7.1). More baculovirus genomes sequence are required to further substantiate this grouping.

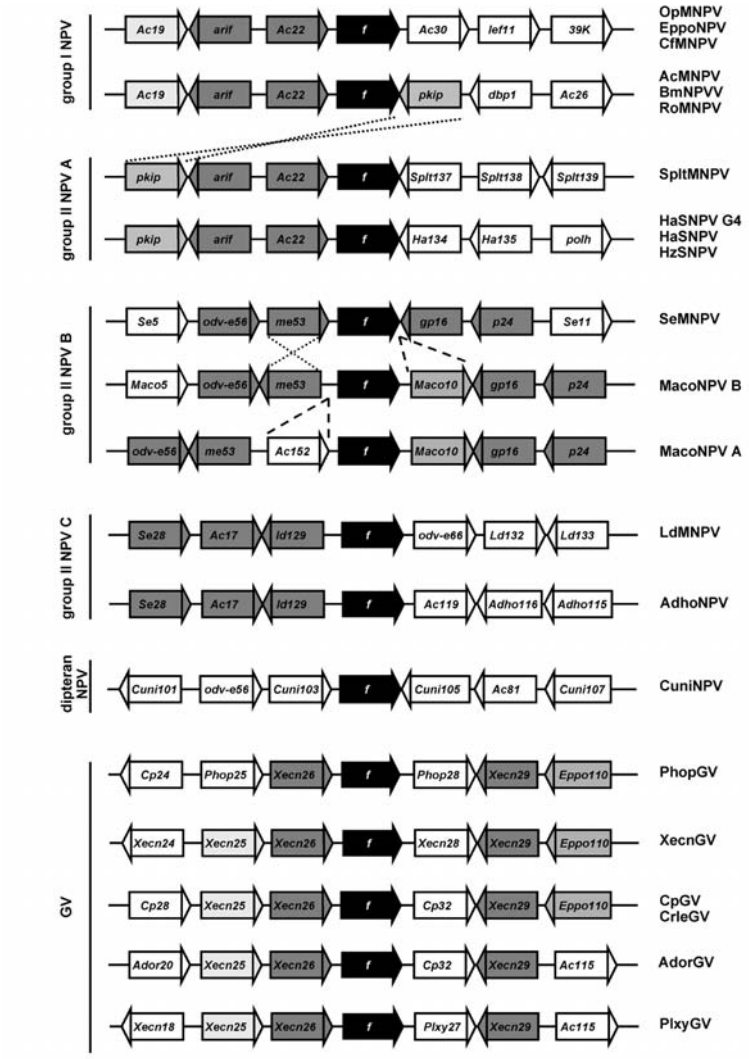

Figure 7.2 Gene arrangements near the baculovirus *f* genes. Based on six genes flanking the *f* gene (black arrow), baculoviruses can be divided in six groups: (i) the group I NPVs, (ii) the SpltMNPV, HaSNPV and HzSNPV (group II NPV A), (iii) SeMNPV and MacoNPV (group II NPV B), (iv) LdMNPV and AdhoNPV (group II NPV C), (v) the dipteran NPV CuniNPV, and (vi) the GVs. Gene homologs found in each member of the group are indicated in dark grey, gene homologs found in two or more members of each group are indicated in light gray and those that are unique around the *f* gene are indicated in white. Arrows show the orientation of the open reading frames. Dashed and dotted lines indicate gene insertion and gene relocation respectively.

Receptor binding and host range

In general host and tissue tropism of viruses is often determined by a physical interaction between a virion and its cognate cell surface receptor. These receptors are normal occurring cell surface molecules often involved in maintaining the physiological state of cells. The viral host range differs among baculoviruses species. For instance AcMNPV is able to infect more than 70 insect species (Adams and McClintock, 1991), while SeMNPV infects only a single insect species, the beet armyworm *Spodoptera exigua* (Onstad, 2003). However, these observed differences in host range cannot be directly related to their envelope fusion proteins. BmNPV, which is closely related to

AcMNPV, can only infect nine insect species (Onstad, 2003), although their GP64 fusion proteins are 94% identical. Despite its narrow host range SeMNPV is capable to transduce a variety of non-permissive cells originating from different insect species (Yanase *et al*., 1998). Baculovirus host range seems to correlate with the absence or presence of specific genes such as the LdMNPV host range factor-1 (*hrf-1*) (*Thiem et al.*, 1996) or cell specific anti-apoptotic genes such as *p35* (Clem *et al*., 1991) and *iap* (Crook *et al*., 1993), hence the search for the function of genes that are unique to a specific baculovirus (IJkel *et al*., 2001; Long *et al*., 2003). Thus, viral entry and host range of baculoviruses should be considered as two separate issues in the viral life cycle.

GP64 and F are not only involved in the fusion process, they are also involved in the attachment of the virion to the target cell (Hefferon *et al*., 1999; Chapter 3) The insect cell surface receptor(s) for baculoviruses are enigmatic (Chapter 1). Competition experiments between AcMNPV and LdMNPV have shown that those viruses use different receptors (Wickham *et al*., 1992). Preliminary results with *gp64*-null AcMNPV virus pseudotyped with either HaSNPV or SeMNPV F showed that these viruses are vital and could compete with each other but not with *gp64*-positive AcMNPV virus (Fig. 7.3) (Westenberg and Uijtdewilligen, unpublished data). This implies that GP64 interacts with cell surface receptors different from those used by F and thereby perhaps giving gp64 positive baculoviruses an infection advantage by means of stronger attachment kinetics.

To experimental address this possibility an *f*-null SeMNPV virus was made and pseudotyped with AcMNPV GP64 (Chapter 5). However, unlike the pseudotyping of AcMNPV with heterologous *f* genes, infectious BVs were not obtained for SeMNPV. GP64 alone seems unable to trigger the budding of SeMNPV BV. The cytoplasmic tail

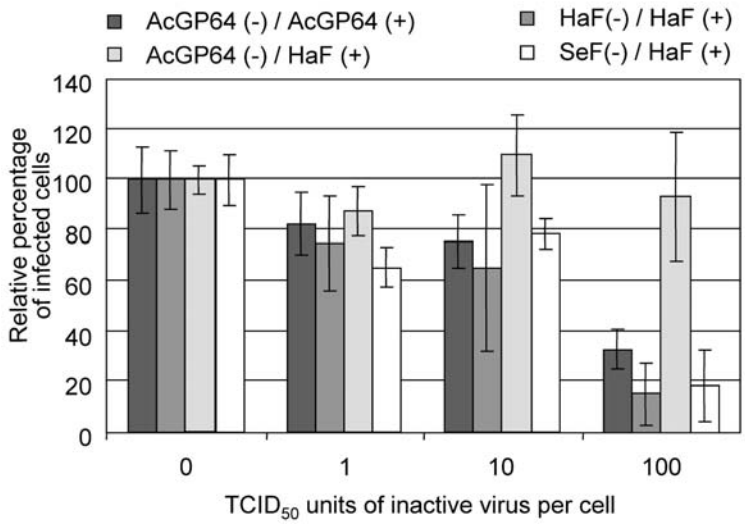

Figure 7.3 Competition assay for baculovirus BV attachment to insect cells. To determine whether F and GP64 interact with different cell surface receptors on Sf21 cells, psoralen-inactivated pseudotyped *gp64*-null AcMNPV BV (-) was used as competitor for binding of BV of active pseudotyped *gp64*-null AcMNPV (+). Sf21 cells were preincubated with increasing concentrations of psoralen-treated virus at 4° C for 1 h, then active virus was added with a m.o.i. of 1 $TCID_{50}$ units/cell and allowed to bind for 1.5 h at 4° C. Cells were washed three times in medium, and infection was allowed for 24 h at 27° C. Infected cells were made visible by GUS-staining and counted.

domain (CTD) of GP64 (3-8 amino acids) is relatively short compared to that of F proteins of group II NPVs (54-78 amino acids). Possibly, the CTD of GP64 is missing an assembly signal specifically required for insertion into SeMNPV BV. To test this possibility an *f*-null SeMNPV virus can be pseudotyped with a chimeric fusion protein consisting of the ectodomain of GP64 and the CTD of SeMNPV F. Such an approach was the basis for the incorporation of the Human immunodeficiency virus (HIV) Env protein in the Vesicular stomatitis virus (VSV) (Owens and Rose, 1993). A successful acquisition of GP64 might also increase its biological infectivity of SeMNPV and therefore a good approach to develop this virus as an effective and more efficient biological control agent to combat insect pests.

Mammalian cells seemed to have a surface receptor for AcMNPV since this virus is able to transduce these cells (Kost and Condreay, 2002). However, a *gp64*-null AcMNPV virus pseudotyped with the SeMNPV F protein was not able to transduce pig (LLC-PK1) or baby hamster kidney cells (BHK-21), while *gp64* positive AcMNPV virus was able to do so (M. Westenberg and P. Uijtdewilligen, unpublished data). This would imply that mammalian cells or at least BHK-21 and LLC-PK1 cells do not posses a cell surface receptor for the SeMNPV F protein. The lack of an appropriate receptor in mammalian cells is in support of the safety of group II NPVs for a biocontrol or protein expression purposes.

The observation that baculovirus fusion proteins are involved in the virion attachment to target cells is not an unusual phenomenon for viral fusion proteins. Similar

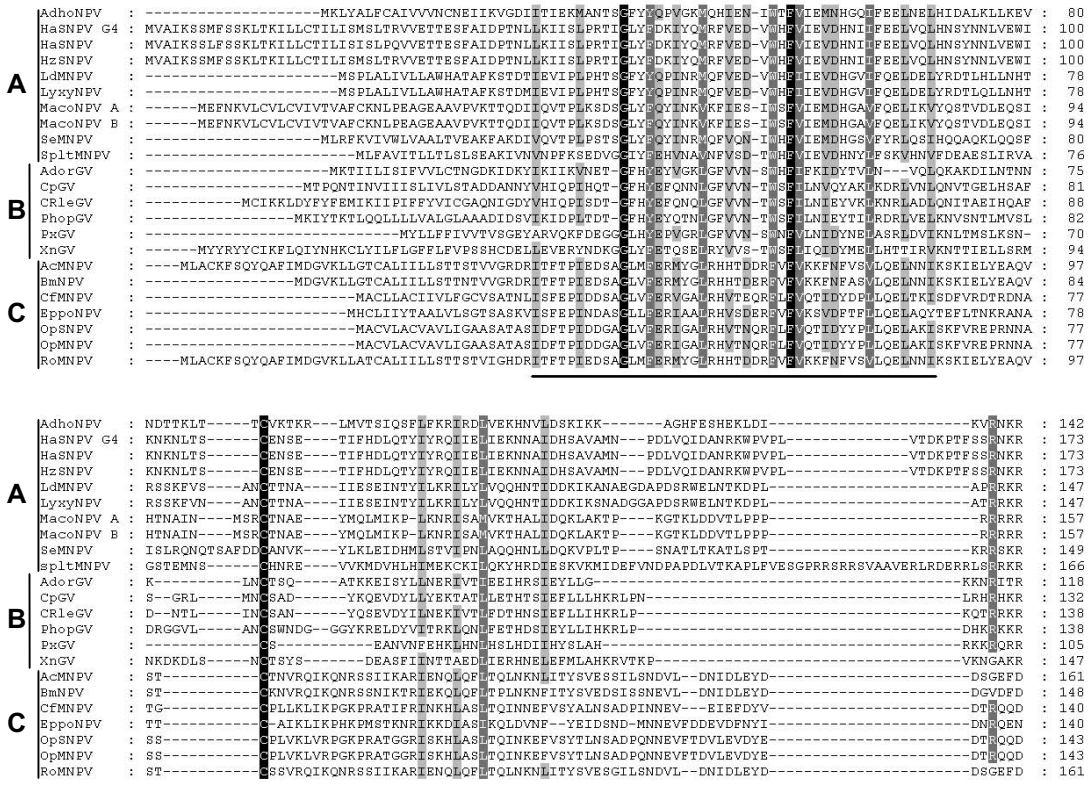

Figure 7.4 Sequence alignment of the F_2 protein fragment of group II NPVs (A) and GVs (B) with the corresponding sequence of the F protein homolog of group I NPVs (C). Black and dark grey shaded amino acids are 100% identical and similar, respectively. Those light grey shaded are similar in 80 to 100% of the F homologs. The underlined sequences are well conserved.

situations exist for Influenza virus HA (Wiley and Skehel, 1987), HIV GP160 (Weis, 1992), Avian sarcoma and leukosis virus Pr95 (Dorner and Coffin, 1986), and the coronavirus S protein (Bosch *et al.,* 2003; Cavanagh, 1995). These viral fusion proteins are also synthesized as a single chain precursor and are subsequently posttranslationally cleaved in the secretory pathway into a C-terminal transmembrane-anchored domain and in an N-terminal domain. These domains remain either covalently or noncovalently associated after cleavage. For these viruses the N-terminal domain of the protein harbours the binding site for the attachment to the receptor (Willey and Skehel, 1987; Weis, 1992; Donner and Coffin, 1986; Taguchi, 1995). It is therefore well possible that the F_2 fragment of the baculovirus F protein also encompasses receptor binding capacity. To experimentally address this a soluble F_2 protein alone should be able to bind to the cell surface of insect cells. The F_2 fragment of the group II NPVs and GVs have amino acid similarities in common with the N-terminus of the F homolog of group I NPVs (Fig. 7.4). It may be that the F homolog of group I NPVs can still function as (co)receptor binding protein, but has lost its fusion ability. This may also explain why an *f*-null AcMNPV virus causes a time-to-death that is longer than that of wildtype AcMNPV virus (Lung *et al*., 2003). A second binding protein may shift the equilibrium between attachment and detachment to the side of attachment, thereby increasing the change of infection (Wickham *et al.,* 1990).

F protein expression

Sequence analysis of the 5' UTR of the OpMNPV and LdMNPV *f* mRNAs revealed that the transcripts originate from both an early (C-A-G/T-T) and a late (G/A/T-T-A-A-G) baculovirus motif in the promoter region (Pearson *et al.,* 2002b). Those motifs were also noted for the *f* genes in AcMNPV, HaSNPV, SeMNPV, SpltMNPV (Pearson *et al.,* 2002b), but experimental evidence for the use of these promoter elements is not yet available. The baculovirus *gp64* genes (group I NPV) are transcribed from an early and a late promoter. To explain the requirement for both early and late expression two theories have been put forward (Blissard and Rohrmann, 1989; Volkman, 1997; Washburn *et al*., 2003). Early GP64 expression would allow sufficient time for posttranslational modification necessary to reach the mature glycosylation state of the protein, transportation and integration into the plasma membrane before newly formed nucleocapsids (NCs) arrive at the plasma membrane for budding. Late expression would then sustain GP64 production and allow replenishing of the plasma membrane (Blissard and Rohrmann, 1989). The second hypothesis suggests that early GP64 expression in midgut epithelial cells allowed a portion of the incoming NCs to bypass the nucleus and to bud directly from the basal side of the cell thereby acquiring GP64. In doing so, BVs are able to escape prior to the host defense mechanism of the midgut epithelial cells causing cell sloughing (Volkman, 1997; Washburn *et al*., 2003). These hypotheses are also applicable for the F protein, there its gene has "early" and "late" transcription start sites. However the "bypass' hypothesis is difficult to keep for GVs and SNPVs, because their ODVs contain a single NC and therefore midgut cells will be mainly invaded by a single NC. The group I NPV BVs have an advantage by having the F protein as part of their BV (Lung *et al*., 2003), therefore it is plausible for those viruses to exploit a similar

expression profile for F and GP64, so that both proteins can simultaneously be incorporated in BVs. Obvious is the lack of both early and late baculovirus consensus promoter elements in the *f* genes of all GVs analyzed so far (Hayakawa *et al*., 1999; Hashimoto *et al.*, 2000; Luque *et al*., 2001; Wormleaton *et al.,* 2003; Lange and Jehle, 2003). Probably, GVs use different promoter elements for the transcription of their *f* genes.

The SeMNPV F protein seems to be a BV specific protein, as it was not detected in ODVs (Chapter 2 and 3). This was also observed for the group I NPV OpMNPV F protein homolog (Pearson *et al.,* 2001). However, screening of an AcMNPV expression library with ODV specific antibodies, revealed that the AcMNPV F protein was also a component of the ODVs (Braunagel *et al.,* 2003). This result was confirmed by using antiserum directed against the F protein, revealing the presence of a 75 kDa ODV protein and to a larger BV protein. Probably late or very late in the baculovirus infection cycle a shift in the translocation of the F protein from plasma membrane to nucleus occurs. The smaller size of the F protein in ODVs might be due to a rerouting in the cytoplasm glycosylation pathway towards the nuclear transport route upon progression of the viral infection. The presence of the F protein in AcMNPV ODVs could only be detected in the AcMNPV ODVs by western analysis when $HgCl_2$ was used to inhibit proteases during ODV purification. So, it cannot fully be excluded that also the SeMNPV and OpMNPV F proteins are not structural related to ODVs, as $HgCl_2$ was not included during ODV purification. The possible role of the F protein in ODVs remains elusive. Perhaps it also has a positive effect on the ODV attachment to the midgut epithelial cells.

Classification of baculovirus fusion proteins

All viral envelope fusion proteins that have been studied to date belong to the type I transmembrane proteins. GP64 as well as F contain an N-terminal signal peptide (SP), a large ectodomain, a membrane anchored domain (TM) and a relative small C-terminal cytoplasmic tail domain (CTD) (Fig. 1.3 and 7.5). For the F protein those domains are mainly based on computer predictions, but there is compelling evidence that the N-terminal signal peptide is active, since the first 16 N-terminal amino acid sequences of LdMNPV F were not present in the mature protein (Pearson *et al.*, 2002a).

At least two classes of viral fusion proteins can be distinguished (Hernandez *et al*., 1996). Class I is represented by orthomyxo-, retro-, paramyxo-, corona- and filoviruses. Their fusion proteins mature by proteolytic cleavage of a precursor protein, yielding a membrane-anchored subunit with an amino-terminal or amino-proximal fusion peptide. Receptor binding or low pH results in the formation of a characteristic trimeric postfusion structure with a triple-stranded coiled coil at its core (Skehel and Wiley, 1998). Representatives of class II viral fusion proteins include flavi- and alphaviruses. Their fusion proteins are not proteolytically cleaved and have internal rather than amino-terminal fusion peptides. They are synthesized as a complex with a second membrane-associated glycoprotein, and the activation of the fusogenic potential involves the cleavage of this accessory protein (Kielian *et al*., 2000).

Classification of GP64 along these lines seems to be rather unclear. It consists of a homotrimer (Oomens *et al*., 1995) with a predicted triple-stranded coiled coil (Kingsley *et al*., 1999), but it contains an internal fusion peptide, because it is not posttranslational cleaved (Oomens *et al*., 1995). However, posttranslational cleavage is not always a prerequisite for the maturation of class I fusion proteins as shown for Ebola virus GP (Neumann *et al*., 2002). It could be envisaged to classify GP64, together with for instance the VSV G protein, into a third class (Yao *et al.,* 2003). Both proteins harbour an internal fusion domain, are not posttranslationally cleaved to activate their fusiogenic potential, and appeared to be independent on other proteins for their function (Fredericksen and Whitt, 1995; Monsma and Blissard, 1995; Zang and Ghosh 1994). Furthermore they lack one (GP64) or both (VSV G) heptad repeats (HR), which are characteristic for class I fusion proteins (Singh *et al*., 1999).

The F-type of fusion protein turns out to be a typical class I viral fusion protein. Its fusion capability is activated upon a posttranslational cleavage (Chapter 3), it has a putative N-terminal fusion peptide (Chapter 6; Pearson *et al*., 2002a; Pearson and Rohrmann, 2003) and consist of two regions in the membrane anchored domain F_1 with a 4,3 hydrophobic heptad repeat, HR-N and HR-C (Fig. 7.5A).

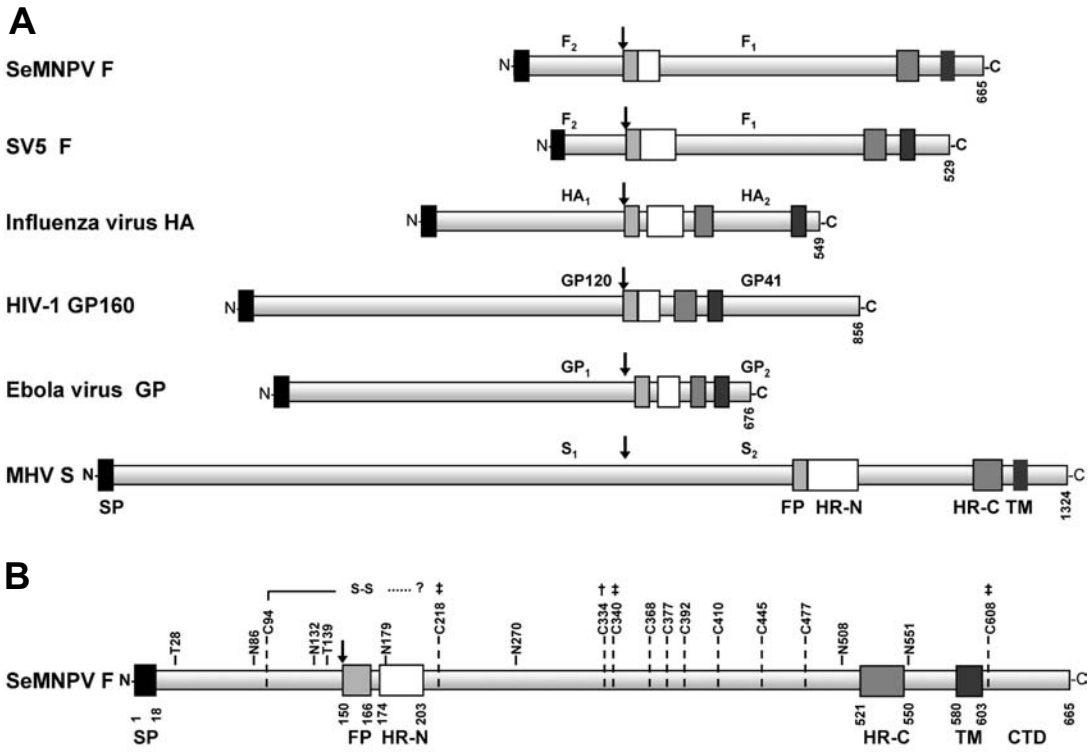

Figure 7.5 Schematic representation of class I viral fusion proteins. A) The relative position of the signal peptide (SP), fusion peptide (FP), N-terminal heptad repeat (HR-N), C-terminal heptad repeat (HR-C) and transmembrane domain (TM) in the class I viral fusion proteins from viruses of six different families are presented: SeMNPV F (*Baculoviridae*), SV5 F (*Paramyxoviridae*), influenza virus HA (*Orthomyxoviridae*), HIV-1 GP160 (*Retroviridae*), Ebola virus (*Filoviridae*) and MHV (*Coronaviridae*). B) Enlargement of the schematic presentation of the SeMNPV F protein. CTD (cytoplasmic tail domain), S-S (disulfide bridge between C94 of F_2 and an unknown (S-S·····?) cysteine residue of F_1). Arginine (N) residues possibly used for N-glycosylation, threonine (T) residues possibly used for O-glycosylation and cysteine (C) residues conserved among baculoviruses (‡not in CuniNPV, †not in CuniNPV and CfMNPV) are indicated. The posttranslational cleavage sites are indicated by arrows.

The posttranslational cleavage fragments F_1 and F_2 of the SeMNPV F protein are covalently associated by a disulfide-linkage, similar to the fusion proteins of influenza viruses, herpesviruses, paramyxoviruses and filoviruses (Lazarowitz et al., 1971; Meyer et al., 1990; Scheid and Choppin, 1977; Volchkov et al., 1998). This in contrast to the fusion proteins of coronaviruses and retroviruses, where the protein fragments remains noncovalently associated after cleavage (Sturman *et al*., 1985; Weiss *et al*., 1990). The F_2 fragment of SeMNPV contains only one cysteine (C94), which is conserved among the baculovirus F protein homologs and therefore most likely involved in the linkage (Fig. 7.5B). In contrast, The SeMNPV F_1 fragments contains fifteen cysteine residues, of which six are conserved among all baculovirus F proteins (Fig. 7.5B). Mass-spectrometric analysis of peptides derived from Cyanogen bromide (CNBr)-treated SeMNPV F protein should reveal which of the conserved cysteine residues in the F_1 fragment forms the disulfide-link with the F_2 fragment. Furthermore, this analysis will also divulge which cysteine residues are used for internal disulfide-links.

Structural studies of viral fusion proteins of Moloney murine leukemia virus (Fass *et al.,* 1996), HIV (Chan *et al.,* 1997), Ebola virus (Weissenhorn *et al*., 1998), Simian virus 5 (SV5) (Baker *et al*., 1999), HRSV (Zhao *et al*., 2000) and Influenza virus (Chen *et al*., 1999) revealed that the HR regions form six-helically bundled structures during the fusion process. The six-helical bundles consist of a homotrimeric coiled coil of HR-N domains with exposed hydrophobic grooves, in which the HR-C domains are packed in an antiparrallel manner. This conformation brings the N-terminal fusion peptide, when inserted into the plasma membrane into close proximity of the transmembrane domain, thereby facilitating close apposition of the viral and cellular membrane. This apposition is followed by fusion of the two membranes (Eckert and Kim, 2001). Compared with other class I fusion proteins the distance between the two HRs is the largest by baculoviruses (Bosch *et al.,* 2003; Fig 7.5A).

Biophysical studies of synthetic peptides corresponding to the HR regions will provide further evidence that the predicted HRs in the SeMNPV F protein will lead to a similar structural architecture to those do in other fusion proteins. The helical contents of the peptides can be verified by circular dichroism and three-dimensional structure analyses can reveal whether the F protein HRs form a six-helically bundled structure. Furthermore, it is of interest to see, whether these peptides can also inhibit viral infection, as has been shown to be the case for peptides derived from HR regions of retrovirus (Sia *et al.,* 2002; He *et al*., 2003; Markosyan *et al*., 2002), paramyxovirus (Russell *et al*., 2001; Wang *et al*., 2003; Yao and Compans, 1996; Young *et al*., 1999; Yu *et al*., 2002) and recently coronavirus (Bosch *et al*., 2003) fusion proteins. This inhibition is supposedly provoked by binding of the peptides to the non-corresponding HR in the fusion protein before or during the fusion process, thereby interfering the necessary conformational changes of the fusion protein. These changes are absolutely required in moving the viral and cellular membrane in close proximity of each other.

Another feature of class I viral fusion proteins is the formation of a higher oligomeric structure. Biochemical evidence suggested that many viral fusion proteins occur either in trimeric or tetrameric forms. X-ray crystal structure and NMR structure analysis of the fusion proteins of Influenza virus (Wilson *et al*., 1981), SV5 (Baker *et al*.,

1999), NDV (Chen *et al*., 2001) and HIV (Chan *et al*., 1997; Weissenhorn *et al*., 1997; Caffrey *et al*., 1998) have shown conclusively that these fusion proteins are trimeric. Several studies indicated that without proper folding into a trimeric state the fusion proteins generally do not reach the cell surface (Doms *et al*., 1993). One of the outcomes of these studies is that glycosylation of the fusion proteins is extremely important for the proper folding and transport of the protein. For influenza virus HA five or more N-linked glycans (seven putative N-linked glycosylation sites have been identified by computer analysis) are needed for intracellular transport (Gallagher *et al*., 1992; Roberts *et al*., 1993). In the case of HIV GP41 at least two of the four or five potential glycosylation sites have to be glycosylated in order to be efficient transported to the cell membrane (Fenouillet *et al*., 1993; Fenouillet and Jones, 1995; Perrin *et al*., 1998). For the membrane anchored subunit of the SV5 F protein removal of any of the four glycosylation sites resulted in either moderate to severe delay in intracellular transport or instability of the F protein (Bagai and Lamb, 1995).

The SeMNPV F protein contains putatively two O-glycosylation and two N-glycosylation sites in the F_2 fragment and four N-glycosylation sites in the F_1 fragment (Fig 7.5B). Preliminary experiments with periodic acid Schiff's reagent indicated that the F_1 and F_2 fragment of SeMNPV as well as those from HaSNPV seemed to be glycosylated (G. Long, personal communication). Mutagenesis should reveal which of the potential glycosylations sites are used and what their importance is for oligomerization, transport of the protein to the plasma membrane and the fusion process.

Baculovirus F protein fusion model

Based on the similarities of the baculovirus F proteins with several vertebrate class I viral fusion proteins a model based on the stalk-pore fusion model proposed for influenza virus HA (Cross *et al.,* 2001a) can be made in which the F protein guides the entry of the BV genome into insect cells (Fig. 7.6). Upon attachment of BVs to the surface of insect cells the F_2 fragments of the trimeric F proteins, which are formed by the covalent interaction of the HR-N domains, bind to the enigmatic cell surface receptors (Fig. 7.6B). The binding probably causes a conformational change in the receptors, which triggers the endocytotic pathway for NC uptake by a mechanism that is not fully understood. The receptors and the attached BV are taken up in a clatherin-coated pit. The coated vesicles are rapidly stripped through the action of uncoating enzymes (Schmid, 1992) and will subsequently fuse with "early" endosomes. Acidification of the endosome through membrane-bound vacuolar H+-ATPase (Mellman *et al.,* 1986) will then mediate a change in the conformation of the F protein. In this process, the fusion peptide dissociates from its sequestered environment in the metasatable structure at neutral pH and projects as an α-helix towards the receptor binding-domain where it inserts in the endosomal membrane probably perpendicularly (Fig. 7.6C). Upon further conformational changes the F protein reaches its stable state in which the HR-C domains are packed antiparallel in the hydrophobic grooves of the trimeric coiled coil formed by the HR-N domains (Fig. 7.6D). This conformational change will lead to the bending of the endosmal and viral membrane. The outer lipid bilayer

leaflets will be pulled together to form a highly bent stalk arrangement leading to the so-called hemifusion intermediate structure (Fig. 7.6E). The bending tension of the F molecules will finally lead to the formation of the fusion pore through which the viral NCs can enter the cytoplasm of the insect cells.

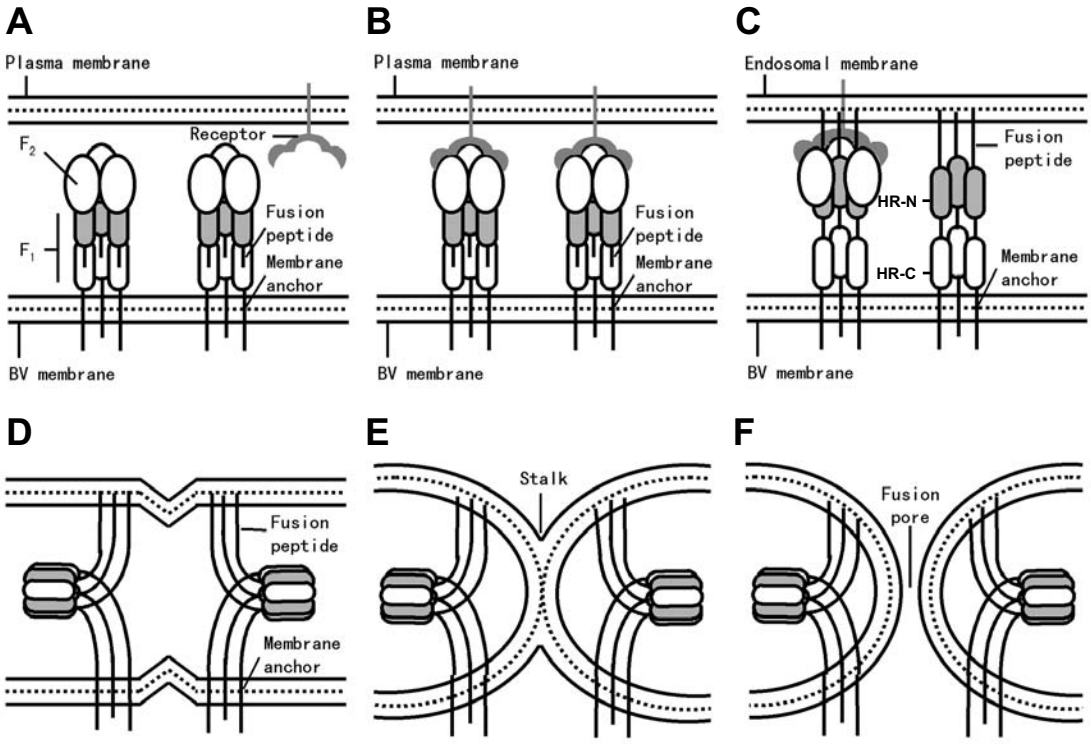

Figure 7.6 F protein model for baculovirus entry into insect cells. A) BVs with trimeric F proteins in the metastable conformation will come in close contact to the insect cell plasma membrane. B) The F_2 fragments of the F protein will bind to the cell surface receptor, which triggers the endocytotic uptake of the virus. C) Acidification of the endosome will mediate a conformational change in which the N-terminal fusion peptide projects to the head of the protein and inserts in the endosomal membrane. D) Upon further conformational changes the F protein reaches its stable state in which the HR-C domains are packed antiparallel in the hydrophobic grooves formed by the trimeric HR-N domains. E) This will lead to the bending of the endosmal and viral membrane. D) The outer lipid bilayer leaflets will be pulled together to form a highly bent stalk arrangement leading to the so-called hemifusion intermediate structure. F) The bending will finally lead to the formation of the fusion pore through which the viral NCs enters the cytoplasm of the insect cell.

Final remarks and baculovirus F protein research prospective

The discovery and the functional analysis of the baculovirus envelope fusion protein F described in this thesis has given a novel insight in baculovirus entry into (insects) cells. However, most of these insight are based on comparisons with other class I viral fusion proteins, in particular with those of vertebrate viruses. Further research on baculovirus F proteins should reveal, if these comparisons are justified. A three-dimensional structure of a baculovirus F protein could be compared to available structures of influenza virus HA (Wilson *et al*., 1981), HIV GP160 (Chan *et al*., 1997; Weissenhorn *et al*., 1997; Caffrey *et al*., 1998) and NDV F (Chen *et al*., 2001) to reveal novel and unique features. Especially the possible structural relatedness with fusion

proteins of the taxonomically distant paramyxoviruses may provide a functional clue in that fusion proteins may have originated from an ancestral fusion protein.

The role of the F protein in the group II NPV as fusion protein and receptor binding protein seems to be clear (Chapter 2 and 4). However, whether this is also the case for the F protein in granuloviruses is not clear. Syncytium formation of insect cells expressing the PlxyGV F protein were unsuccessful and *gp64*-null AcMNPV virions pseudotyped with this protein were not infectious (Chapter 2 and 4). So, research in this area should further focus on the differences between the group II NPV and GV F proteins. Perhaps, granuloviruses need a second protein that works in conjunction with the F protein to gain entry in insect cells or maybe the surface receptor is not present on the insect cells used for the pseudotyping experiments and the syncytium formation assays. Therefore it is better to explore the use of the established *Plutella xylostella* cell line (Goodman *et al.,* 2001) in such experiments.

Finally, further research on the baculovirus entry mechanism has to be performed to understand the baculovirus-host interaction. In particular the determination of the insect cell receptor for BVs and the investigation of the role of the F_2 fragment in the binding to this receptor will be of considerable interest. Not only the F_2 fragment of the group II NPVs and the GVs should be explored as possible viral binding domain, but also the corresponding domain in the remnant F protein of the group I NPVs to investigate the general role of F as receptor binding protein in baculovirus BVs.

SUMMARY

The *Baculoviridae* are a family of large double stranded DNA viruses that are exclusively pathogenic to arthropods. Baculoviruses have been studied i) with the aim to develop alternatives to chemical pest control, ii) for their application as an eukaryotic expression system to express heterologous proteins, and recently iii) as gene delivery vehicle in gene therapy. Baculoviruses cluster into two distinct genera on the basis of the occlusion body (OB) morphology: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). The NPVs are further subdivided into a group I and a group II, based on the phylogeny of some of their genes. A feature, shared by all baculoviruses, is the production of two viral phenotypes during the infection cycle: the occlusion derived virus (ODV), which is responsible for the horizontal transmission of the virus in insect populations, and the budded virus (BV), which is responsible for the spread of the virus within the host and in cultured insect cells. A NPV infection starts with the uptake of OBs, which dissolve in the midgut, liberating numerous ODV particles. The nucleocapsid (NC) enters the midgut epithelial cells after fusion of the ODV envelope with the cellular membrane. Then the NCs are transported to the nucleus, where transcription, replication and assembly of progeny NCs occur. The progeny NCs are translocated to the plasma membrane, where they acquire an envelope containing, at least in group I NPVs, the virus encoded major envelope glycoprotein GP64. After budding from the plasma membrane the progeny BV is responsible for the systemic infection of the insect beyond the initially infected midgut epithelium and enters other insect cells by the mechanism of receptor mediated endocytosis. The acidification of the endosome triggers the GP64-mediated fusion of the viral and endosomal membrane. The NCs are thereby released in the cytoplasm and transported to the nucleus. Determination of the genome sequence of the group II NPVs *Lymantria dispar* (Ld)MNPV and *Spodoptera exigua* (Se)MNPV led to new insights with regard to BV envelope structure. Neither LdMNPV nor SeMNPV contains a *gp64* gene. Instead, group II NPVs, or at least LdMNPV and SeMNPV, possess another BV envelope (glyco)protein that has a function analogous to GP64.

First it was investigated whether the SeMNPV BV enters the cell by direct membrane fusion or by the endocytotic pathway (**chapter 2**). The use of a lysosomothrophic reagent inhibited SeMNPV BVs infection, indicating that the virus enters by the endocytotic pathway. The major envelope protein of the SeMNPV BVs was sequenced and this appeared to be a C-terminal cleavage fragment of the primary translation product of ORF 8 in the SeMNPV genome. The *Se8* gene product as well as its homolog (Ld130) was shown to be localized at the plasma membrane. Syncytium formation assays showed that these proteins alone were sufficient to mediate pH-dependent membrane fusion and were therefore named the fusion (F) protein. The fact that F was detected in BVs as a smaller fragment derived from a larger proprotein, suggested that upon maturation F was posttranslationally cleaved. Such cleavage is a general mechanism, found among vertebrate viruses to activate their fusion proteins. The occurrence of a conserved proprotein convertase cleavage site in the primary

translation product of SeMNPV F as well as LdMNPV F suggested the possible involvement of a cellular furin in the cleavage reaction. The use of a specific inhibitor indicated that furin is indeed involved in the cleavage of SeMNPV F and that this cleavage is required for the fusiogenic activity of the protein (**chapter 3**). This was further confirmed by demonstrating that a mutant F protein with a dysfunctional cleavage site did not mediate membrane fusion in a syncytium formation assay. By means of Western analysis it was shown that F is a BV specific protein and that both its N-terminal (F_2) and C-terminal (F_1) cleavage product is present in BVs and presumably covalently linked by a disulfide-bridge.

As far as its function as fusion protein, all results indicated that F was functionally analogous to GP64. To investigate whether this analogy also held with respect to receptor binding and virion budding, an AcMNPV pseudotyping system was developed (**chapter 4**). A *gp64*-deficient AcMNPV bacmid was successfully pseudotyped with SeMNPV and LdMNPV F proteins, as infectious BVs were obtained upon transfection of the bacmids into insect cells. This pseudotyping also demonstrated once more that F has to be cleaved to release its fusiogenic capabilities, since an F protein with a dysfunctional cleavage site was not able to rescue the loss of the *gp64* gene. Western analysis showed that the mutated protein was incorporated in BVs, but in its uncleaved (F_0) conformation. The meantime available genome sequences of *Xestia c-nigrum* (Xecn)GV, *Plutella xylostella* (Plxy)GV and *Cydia pomonella* (Cp)GV revealed that also these granuloviruses do not contain a *gp64* gene. Therefore it was also examined whether the F protein homolog of PlxyGV was capable to pseudotype the *gp64*-null AcMNPV mutant. However, no infectious BVs were produced upon transfection of the bacmid in insect cells, although the PlxyGV F protein was demonstrated in the produced BVs.

In addition to a *gp64* gene group I NPVs have a remnant *f* gene in their genome. GP64 homologs are also found on two tick-transmitted Orthomyxoviruses, Thogoto virus and Dhori virus. This suggests that an ancestral group I NPV may have acquired the *gp64* gene either from their host or from another virus, thereby acquiring a selective advantage in replication and obviating the need for a functional F protein. To experimentally address this hypothesis a SeMNPV *f*-null mutant was pseudotyped with GP64 (**chapter 5**). However, no infectious BVs could be obtained in this experiment, indicating that the *f* gene is essential for viral propagation of SeMNPV. Reinsertion of the *f* gene of SeMNPV restored the wild-type phenotype of SeMNPV. These results suggest that F from group II NPVs might contain additional essential functions in producing infectious BVs. These functions are essential in group II NPVs, but not in group I NPVs.

The biological significance of the posttranslational cleavage of SeMNPV F was further investigated (**chapter 6**). In fusion proteins of vertebrate viruses this cleavage occurs just in front of a hydrophobic, called “fusion peptide”. As the N-terminus of SeMNPV F_1 possesses common features to these vertebrate viral fusion peptides this sequence was studied by mutational analysis. SeMNPV F proteins with single point mutations at conserved positions as well as with a complete deletion of the fusion peptide domain were analyzed in the baculovirus pseudotyping system. Two of the F mutants, including the deletion mutant, were unable to rescue the propagation defect of

the *gp64*-null bacmid, while other mutants were significantly reduced in their viral infectivity or viral propagation in cell culture. These results indicate that N-terminus of SeMNPV F_1 is most likely the “fusion peptide” involved in the viral entry mechanism.

Finally the results obtained during the reported PhD study are discussed (**chapter 7**) with respect to baculovirus entry, evolution and taxonomy.

SAMENVATTING

Baculovirussen zijn grote membraanvirussen met een circulair dubbelstrengig DNA-genoom, die uitsluitend voorkomen bij geleedpotigen, met name insecten. Onderzoek aan baculovirussen richtte zich in eerste instantie op de ontwikkeling van het virus als bio-insecticide ter bestrijding van plaaginsecten. Later is men baculovirussen ook gaan bestuderen en toepassen als genvectorsysteem voor de productie van heterologe eiwitten, terwijl zeer recentelijk ook onderzoek gestart is om te bekijken in hoeverre baculovirussen als “delivery” vector geschikt zijn voor toepassing in de humane gentherapie. Op grond van de tijdens het infectieproces gevormde kerninsluitsels worden baculovirussen taxonomisch ingedeeld in twee genera: de *kernpolyedervirussen* (Engels: *nucleopolyhedrovirus*, NPV) en de *granulovirussen* (GV). De NPV’s worden op basis van genetische verschillen onderscheiden in groep I- en groep II-NPV’s. Een unieke eigenschap van baculovirussen is dat zij tijdens de infectiecyclus twee verschillende type deeltjes kunnen vormen: ODV (“occlusion derived” virus), betrokken bij de verspreiding van het virus in gastheerpopulaties en BV (“budded” virus), verantwoordelijk voor de verspreiding van het virus binnen de gastheer. De BV’s zijn tevens infectieus voor insectencelcultures.

Het infectieproces van een NPV begint met de opname van polyeders door insectenlarven via het voedsel. Onder invloed van de alkalische pH van de middendarm vallen de polyeders uiteen, waardoor de ODV-deeltjes vrijkomen. Deze kunnen nu het peritrofe membraan van de darm passeren en vervolgens aan de middendarm epitheelcellen binden. Na fusie van de ODV-membraan met de celmembraan bereiken de virale nucleocapsiden (NC’s) het cytoplasma. Vervolgens worden ze naar de celkern getransporteerd, alwaar transcriptie, replicatie en vorming van nieuwe NC’s plaatsvindt. Nieuw gevormde NC’s worden naar de celmembraan getransporteerd en verkrijgen tijdens het afsnoeringsproces een membraan, die in geval van groep I-NPV’s onder meer het virale glycoproteïne GP64 bevat. Aldus komen de BV’s in het hemolymf van het insect terecht. Deze BV’s veroorzaken secundaire infecties elders in het insect door middel van een proces dat receptor-gestuurde endocytose wordt genoemd. Bij dit proces speelt bij de groep I-NPV’s het GP64-eiwit een sleutelrol. Dit eiwit zorgt ervoor dat de virale en endosomale membranen na het verzuren van het endosoom met elkaar kunnen fuseren. Het idee dat dit het enige envelop-fusie-eiwit van baculovirus BV zou zijn, moest drastisch bijgesteld worden toen de genoomsequenties van de groep II-NPV’s, *Lymantria dispar* (Ld)MNPV en *Spodoptera exigua* Se(MNPV), bekend werden. Geen van deze virussen bleek een *gp64*-gen te bezitten. Centraal in het onderzoek, beschreven in dit proefschrift, staat dan ook de vraag hoe deze groep II-NPV’s de gastheercel kan binnendringen en welk viraal eiwit(ten) betrokken zou(den) zijn bij binding en membraanfusie.

Daartoe werd in de eerste plaats onderzocht of SeMNPV-BV’s, net zoals AcMNPV, cellen binnendringen via endocytose (**hoofdstuk 2**). Omdat ammoniumchloride, dat verzuring van het endosoom voorkomt, het binnendringen van BV’s in de cel blokkeerde, werden aanwijzingen verkregen dat SeMNPV inderdaad via

endocytose de gastheercel binnenkomt. Vervolgens werd het meest voorkomende eiwit in de SeMNPV-BV's nader onderzocht. Dit 60 kDa-eiwit bleek een C-terminaal fragment te zijn van het primaire translatieproduct van het open leesraam "ORF8" in het SeMNPV-genoom. Expressie van dit open leesraam (Se8) en dat van de LdMNPV-homoloog (Ld130) toonde aan dat deze eiwitten voornamelijk voorkomen in de celmembraan. Nader onderzoek wees uit dat zij geheel zelfstandig in staat zijn om bij lage pH cellen met elkaar te doen fuseren. Derhalve werden deze eiwitten fusie- of, kortweg, F-eiwitten genoemd.

Het feit dat F als een fragment van een groter pro-eiwit voorkwam, deed vermoeden dat dit eiwit na synthese een klieving had ondergaan. Een dergelijke klieving is inderdaad een bij vertebratenvirussen algemeen voorkomend mechanisme om hun fusie-eiwit te activeren. Een mogelijke pro-eiwit convertase-knipplaats in zowel SeMNPV-F als LdMNPV-F deed vermoeden dat bij deze virussen het cellulaire furine betrokken is bij de klieving. Door gebruik te maken van een speciale remmer kon aangetoond worden dat furine inderdaad betrokken is bij de klieving en bovendien dat klieving essentieel is voor activering van F (**hoofdstuk 3**). Het belang van de klieving werd verder bevestigd door mutagenese van de klievingsplaats, resulterend in een F eiwit dat niet meer in staat bleek om cellen met elkaar te laten fuseren. Verder werd aangetoond dat F een BV-specifiek eiwit is en dat de N-terminale (F_2) en C-terminale (F_1) klievingsproducten covalent met elkaar verbonden blijven door middel van een zwavelbrug.

De tot dusver behaalde resultaten impliceerden dat F een functionele analoog zou kunnen zijn van GP64, tenminste waar het de functie van fusie-eiwit betreft. Dit betekende echter nog niet dat F ook betrokken is bij receptorbinding en essentieel is voor assemblage van de BV. Om te onderzoeken of F ook deze twee functies bezit, werd een AcMNPV-pseudotyperingssysteem ontwikkeld (**hoofdstuk 4**). De *f*-genen van SeMNPV en LdMNPV werden in een infectieuze AcMNPV kloon (bacmide) met een gedeleteerd *gp64*-gen (*gp64*-null-bacmide) geplaatst. Transfectie van deze bacmides in insectencellen resulteerde in de productie van infectieuze BV's, terwijl controle-transfecties met het *gp64*-null-bacmide geen infectieuze BV's opleverde. Plaatsing van een SeMNPV-*f*-gen met een gemuteerde furine-knipplaats in de *gp64*-null-bacmide, leverde, zoals verwacht, eveneens geen infectieuze virusdeeltjes op, maar wel BV's met een ongekliefd F-eiwit (F_0). Hiermee werd onomstotelijk aangetoond dat het F-eiwit gekliefd moet worden om te kunnen functioneren als fusie-eiwit. In de loop van het onderzoek waren ook de volledige genoomsequenties van drie granulovirussen bekend geworden, te weten die van *Xestia c-nigrum* (Xecn)GV, *Plutella xylostella* (Plxy)GV en *Cydia pomonella* (Cp)GV. Ook deze baculovirussen blijken, net zoals de groep II-NPV's, geen *gp64*-gen te bevatten. Daarom werd ook geprobeerd om AcMNPV te pseudotyperen met het F-eiwit van PlxyGV. Echter, transfectie van het bacmide in insectencellen leverde geen infectieuze virusdeeltjes op, hetgeen erop duidt dat de PlxyGV-F-homoloog niet als zodanig werkt of dat het pseudotyperingssysteem niet geschikt is.

Groep I-NPV's bezitten naast een *gp64*-gen ook een onfunctioneel *f*-gen. GP64-homologen zijn ook aangetroffen in de door teken overdraagbare orthomyxovirussen

(Thogoto virus en Dhori virus). Al deze bevindingen wijzen erop dat de groep I-NPV's wellicht het *gp64*-gen hebben verkregen van hun gastheer of van een ander virus, waarna er onder invloed van een positieve selectiedruk geen behoefte meer was aan een functioneel F-eiwit. Om deze hypothese te toetsen werd getracht SeMNPV te pseudotyperen met GP64 (**hoofdstuk 5**). Transfectie met een SeMNPV-bacmide met een gedeleteerd *f*-gen resulteerde niet in productie van infectieuze BV's. Hieruit blijkt dat het *f* gen een essentieel gen is voor de productie van BV's van SeMNPV. Terugplaatsing van het SeMNPV-*f*-gen zorgde voor opheffing van het defect, terwijl dit defect niet kon worden opgeheven door het *gp64*-gen te plaatsen in het virale genoom van SeMNPV. Deze resultaten suggereren dat F van groep II-NPV's een extra functie bezit voor de formatie van BV's, waarin het GP64 niet kan voorzien.

In de fusie-eiwitten van diverse vertebratenvirussen vindt de post-translationele digestie plaats net voorafgaand aan een hydrofoob domein, dat ook wel het "fusiepeptide" wordt genoemd. Dit peptide speelt een cruciale rol bij het uiteindelijke bij elkaar brengen en fuseren van virus- en celmembraan. De N-terminus van SeMNPV-F_1 bezit verscheidende overeenkomsten met de virale fusiepeptiden van vertebratenvirussen. De functionaliteit van dit domein werd onderzocht door middel van mutatieanalyse, waarbij opnieuw gebruik gemaakt werd van het AcMNPV-(bacmide)-pseudotyperingssysteem (**hoofdstuk 6**). Twee van de mutanten, waaronder een mutant waarin het gehele hydrofobe domein van F_0 was gedeleteerd, waren niet in staat om infectieuze BV's te produceren, terwijl de andere aminozuur-specifieke mutanten een gereduceerde titer of een vertraagde verspreiding lieten zien. Deze resultaten wijzen erop dat het hydrofobe domein in F_0 een grote rol speelt bij het binnentreden van het virus in de cel, vermoedelijk als "fusiepeptide".

Tenslotte worden de belangrijkste uitkomsten van het in dit proefschrift beschreven promotieonderzoek tegen het licht gehouden in relatie tot het baculovirale infectieproces, de taxonomie en de evolutie (**hoofdstuk 7**).

REFERENCES

Adams, J. R., and J. T. McClintock. 1991. Nuclear polyhedrosis viruses of insects, p. 87-204. *In* J. R. Adams, and J. R. Bonami (ed.), Atlas of invertebrate viruses. CRC Press, Boca Raton, FL.

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, P. W. Li, R. A. Hoskins, R. F. Galle, R. A. George, S. E. Lewis, S. Richards, M. Ashburner, S. N. Henderson, G. G. Sutton, J. R. Wortman, M. D. Yandell, Q. Zhang, L. X. Chen, R. C. Brandon, Y. H. Rogers, R. G. Blazej, M. Champe, B. D. Pfeiffer, K. H. Wan, C. Doyle, E. G. Baxter, G. Helt, C. R. Nelson, G. L. Gabor, J. F. Abril, A. Agbayani, H. J. An, C. Andrews-Pfannkoch, D. Baldwin, R. M. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E. M. Beasley, K. Y. Beeson, P. V. Benos, B. P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M. R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K. C. Burtis, D. A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J. M. Cherry, S. Cawley, C. Dahlke, L. B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A. D. Mays, I. Dew, S. M. Dietz, K. Dodson, L. E. Doup, M. Downes, S. Dugan-Rocha, B. C. Dunkov, P. Dunn, K. J. Durbin, C. C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A. E. Gabrielian, N. S. Garg, W. M. Gelbart, K. Glasser, A. Glodek, F. Gong, J. H. Gorrell, Z. Gu, P. Guan, M. Harris, N. L. Harris, D. Harvey, T. J. Heiman, J. R. Hernandez, J. Houck, D. Hostin, K. A. Houston, T. J. Howland, M. H. Wei, C. Ibegwam, M. Jalali, F. Kalush, G. H. Karpen, Z. Ke, J. A. Kennison, K. A. Ketchum, B. E. Kimmel, C. D. Kodira, C. Kraft , S. Kravitz, D. Kulp, Z. Lai, P. Lasko, Y. Lei, A. A. Levitsky, J. Li, Z. Li, Y. Liang, X. Lin, X. Liu, B. Mattei, T. C. McIntosh, M. P. McLeod, D. McPherson, G. Merkulov, N. V. Milshina, C. Mobarry, J. Morris, A. Moshrefi, S. M. Mount, M. Moy, B. Murphy, L. Murphy, D. M. Muzny, D. L. Nelson, D. R. Nelson, K. A. Nelson, K. Nixon, D. R., Nusskern, J. M. Pacleb, M. Palazzolo, G. S. Pittman, S. Pan, J. Pollard, V. Puri, M. G. Reese, K. Reinert, K. Remington, R. D. Saunders, F. Scheeler, H. Shen, B. C. Shue, I. Siden-Kiamos, M. Simpson, M. P. Skupski, T. Smith, E. Spier, A. C. Spradling, M. Stapleton, R. Strong, E. Sun, R. Svirskas, C. Tector, R. Turner, E. Venter, A. H. Wang, X. Wang, Z. Y. Wang, D. A. Wassarman, G. M. Weinstock, J. Weissenbach, S. M. Williams, T. Woodage, K. C. Worley, D. Wu, S. Yang, Q. A. Yao, J. Ye, R. F. Yeh, J. S. Zaveri, M. Zhan, G. Zhang, Q. Zhao, L. Zheng, X. H. Zheng, F. N. Zhong, W. Zhong, X. Zhou, S. Zhu, X. Zhu, H. O. Smith, R. A. Gibbs, E. W. Myers, G. M. Rubin, and J. C. Venter. 2000. The genome sequence of *Drosophila melanogaster*. Science **287:**2185-2195.

Afonso, C. L., E. R. Tulman, Z. Lu, C. A. Balinsky, B. A. Moser, J. J. Becnel, D. L. Rock, and G. F. Kutish. 2001. Genome sequence of a baculovirus pathogenic *for Culex nigripalpus*. J Virol **75:**11157-11165.

Arends, H. M., and J. A. Jehle. 2002. Homologous recombination between the inverted terminal repeats of defective transposon TCp3.2 causes an inversion in the genome of *Cydia pomonella* granulovirus. J Gen Virol **83:**1573-8.

Ahrens, C. H., R. L. Russell, C. J. Funk, J. T. Evans, S. H. Harwood, and G. F. Rohrmann. 1997. The sequence of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. Virology **229:**381-399.

Ahrens, C. H., R. L. Russell, C. J. Funk, J. T. Evans, S. H. Harwood, and G. F. Rohrmann. 1997. The sequence of the *Orgyia pseudotsugata* multinucleocapsid polyhedrosis virus genome. Virology **229:**381-399.

Ali, A., and D. P. Nayak. 2000. Assembly of Sendai virus: M protein interacts with F and HN proteins and with the cytoplasmic tail and transmembrane domain of F protein. Virology **276:**289-303.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.

Ausabel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1994. Current protocols in molecular biology. Greene and Wiley InterScience, New York, NY.

Ayres, M. D., S. C. Howard, J. Kuzio, M. Lopez-Ferber, and R. D. Possee. 1994. The complete

DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology **202:**586-605.

Bagai, S., and R. A. Lamb. 1995. Individual roles of N-linked oligosaccharide chains in intracellular transport of the paramyxovirus SV5 fusion protein. Virology **209:**250-256.

Bairoch, A., P. Buchner, and K. Hofmann. 1997. The PROSITE database; its status in 1997. Nucleic Acids Research **25:**217-221.

Baker, K. A., R. E. Dutch, R. A. Lamb, and T. S. Jardetzky. 1999. Structural basis for paramyxovirus-mediated membrane fusion. Mol Cell **3:**309-319.

Barsoum, J. 1999. Concentration of recombinant baculovirus by cation-exchange chromatography. Biotechniques **26:**834-840.

Barsoum, J., R. Brown, M. McKee, and F. M. Boyce. 1997. Efficient transduction of mammalian cells by a recombinant baculovirus having the Vesicular stomatitis virus G glycoprotein. Hum Gene Ther **8:**2011-2018.

Becnel, J. J., S. E. White, and A. M. Shapiro. 2003. *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV) infections in adult mosquitoes and possible mechanisms for dispersal. J. Invertebr Pathol **83**:181-183.

Bideshi, D. K., and B. A. Federici. 2000. The *Trichoplusia ni* granulovirus helicase is unable to support replication of *Autographa californica* multicapsid nucleopolyhedrovirus in cells and larvae of *T.ni*. J Gen Virol. **81:**1593-1599.

Bilsel, P., M. R. Castrucci, and Y. Kawaoka. 1993. Mutations in the cytoplasmic tail of influenza A virus neuraminidase affect incorporation into virions. J Virol **67:**6762-6767.

Black, B. C., L. A. Brennan, P. M. Dierks, and I. E. Gard. 1997. Commerialization of baculoviral insecticides, p. 341-387. *In* L. K. Miller (ed.), The baculoviruses. Plenum Press, New York, NY.

Blinov, V. M., V. V. Gutorov, N. G. Holodilov, A. A. Iljichev, V. A. Karginov, N. N. Mikrjukov, V. A. Mordvinov, I. V. Nikonov, N. A. Petrov, I. H. Urmanov, and S. K. Vasilenko. 1984. Nucleotide sequence of the *Galleria mellonella* nuclear polyhedrosis virus origin of DNA replication. FEBS Lett **167:**254-258.

Blissard, G. W. 1996. Baculovirus-insect cell interactions. Cytotechnology **20:**73-93.

Blissard, G. W., and G. F. Rohrmann. 1989. Location, sequence, transcriptional mapping, and temporal expression of the *gp64* envelope glycoprotein gene of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology **170:**537-555.

Blissard, G. W., and G. F. Rohrmann. 1990. Baculovirus diversity and molecular biology. Annu Rev Entomol **35:**127-155.

Blissard, G. W., and G. F. Rohrmann. 1991. Baculovirus gp64 gene expression: analysis of sequences modulating early transcription and transactivating by IE1. J Virol. **65:**5820-5827.

Blissard, G. W., and J. R. Wenz. 1992. Baculovirus GP64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. J. Virol. **66:**6829-6835.

Blissard, G. W., B. C. Black, N. Crook, B. A. Keddie, R. Possee, G. Rohrmann, and D. A. Theilmann. 2000. Family *Baculoviridae*, p. 195-202. *In* C. M. van Regenmortel, D. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), Virus taxonomy classification and nomenclature of viruses : seventh report of the International Committee on Taxonomy of Viruses Taxonomy of Viruses. Academic Press, San Diego, Wien New York.

Blumenthal, R., D. P. Sarkar, S. Durell, D. E. Howard, and S. J. Morris. 1996. Dilation of the influenza hemagglutinin fusion pore revealed by the kinetics of individual cell-cell fusion events. J Cell Biol **35:**63-71.

Bosch, B. J., R. van der Zee, C. A. de Haan, and P. J. Rottier. 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J Virol **77:**8801-8811.

Boyce, F. M., and N. L. Bucher. 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc Natl Acad Sci U S A **93:**2348-2352.

Boycott, R., H. D. Klenk, and M. Ohuchi. 1994. Cell tropism of influenza virus mediated by hemagglutinin activation at the stage of virus entry. Virology **203:**313-319.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248-254.

Braunagel, S. C., and M. D. Summers. 1994. *Autographa californica* nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. Virology **202:**315-328.

Braunagel, S. C., and M. D. Summers. 1994. *Autographa californica* nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. Virology **202:**315-328.

Braunagel, S. C., W. K. Russell, G. Rosas-Acosta, D. H. Russell, and M. D. Summers. 2003. Determination of the protein composition of the occlusion-derived virus of *Autographa californica* nucleopolyhedrovirus. Proc Natl Acad Sci U S A **100:**9797-9802.

Briggs, J. A., T. Wilk, and S. D. Fuller. 2003. Do lipid rafts mediate virus assembly and pseudotyping? J Gen Virol **84:**757-768.

Broer, R., J. G. Heldens, E. A. van Strien, D. Zuidema, and J. M. Vlak. 1998. Specificity of multiple homologous genomic regions in *Spodoptera exigua* nucleopolyhedrovirus DNA replication. J Gen Virol **79:**1563-1572.

Bulach, D. M., C. A. Kumar, A. Zaia, B. F. Liang, and D. E. Tribe. 1999. Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. J Invertebr Pathol **73:**59-73.

Caballero, P., D. Zuidema, C. Santiago Alvarez, and J. M. Vlak. 1992. Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. Biocontrol Sci Technol **2:**145-157.

Caffrey, M., M. Cai, J. Kaufman, S. J. Stahl, P. T. Wingfield, D. G. Covell, A. M. Gronenborn, and G. M. Clore. 1998. Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. EMBO J **17:**4572-4584.

Carstens, E. B., S. T. Tjia, and W. Doerfler. 1979. Infection of *Spodoptera frugiperda* cells with *Autographa californica* Nuclear Polyhedrosis virus. I. Synthesis of intracellular proteins after virus infection. Virology **99:**368-398.

Carstens, E. B., S. T. Tjia, and W. Doerfler. 1979. Infection *of Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus: Synthesis of intracellular proteins after virus infection. Virology **99:**368-398.

Cavanagh, D. 1995. The coronavirus surface glycoprotein. Plenum Press, New York, NY.

Chan, D. C., D. Fass, J. M. Berger, and P. S. Kim. 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89:**263-273.

Charlton, C. A., and L. E. Volkman. 1993. Penetration of *Autographa californica* nuclear polyhedrosis virus nucleocapsids into IPLB Sf 21 cells induces actin cable formation. Virology **197:**245-254.

Chen, J., J. J. Skehel, and D. C. Wiley. 1999. N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA(2) subunit to form an N cap that terminates the triple-stranded coiled coil. Proc Natl Acad Sci U S A **96:**8967-8972.

Chen, L., J. J. Gorman, J. McKimm Breschkin, L. J. Lawrence, P. A. Tulloch, B. J. Smith, P. M. Colman, and M. C. Lawrence. 2001. The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. Structure **9:**255-266.

Chen, X., F. F. J. IJkel, R. Tarchini, X. Sun, H. Sandbrink, H. Wang, S. Peters, D. Zuidema, R. Klein Lankhorst, J. M. Vlak and Z. Hu. 2001. The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. J. Gen. Virol. **82:**241-257.

Chen, X., W. F. J. IJkel, C. Dominy, P. M. de Andrade Zanotto, Y. Hashimoto, O. Faktor, T. Hayakawa, C. H. Wang, A. Premkumar, S. Mathavan, P. J. Krell, Z. Hu, and J. M. Vlak. 1999. Identification, sequence and phylogeny of the *lef-2* gene of *Helicoverpa armigera* single-nucleocapsid baculovirus. Virus Res*.* **65:**21-32.

Chen, X., Z. Hu, J. A. Jehle, Y. Zhang, and J. M. Vlak. 1997. Analysis of the *ecdysteroid UDP-glucosyltransferase* gene of *Heliothis armigera* single-nucleocapsid baculovirus. Virus Genes **15:**219-225.

Cheshenko, N., N. Krougliak, R. C. Eisensmith, and V. A. Krougliak. 2001. A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. Gene Ther **8:**846-854.

Cieplik, M., H. D. Klenk, and W. Garten. 1998. Identification and characterization of *Spodoptera frugiperda* furin: A thermostable subtilisin-like endoprotease. Biol Chem **379:**1433-1440.

Clem, R. J., M. Fechheimer, and L. K. Miller. 1991. Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science **254:**1388-1390.

Colman, P. M., and M. C. Lawrence. 2003. The structural biology of type I viral membrane fusion.

Nat Rev Mol Cell Biol **4:**309-319.

Crook, N. E., R. J. Clem, and L. K. Miller. 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol **67:**2168-2174.

Cross., K. J., L. M. Burleigh, and D. A. Steinhauer. 2001a. Mechanisms of cell entry by influenza virus. Exp Rev Mol Med 6 augustus, http://www-ermm.cbcu.cam.ac.uk/01003453h.htm.

Cross, K. J., S. A. Wharton, J. J. Skehel, D. C. Wiley, and D. A. Steinhauer. 2001b. Studies on influenza haemagglutinin fusion peptide mutants generated by reverse genetics. EMBO J **20:**4432-4442.

Danieli, T., S. L. Pelletier, Y. L. Henis, and J. M. White. 1996. Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers. J Cell Biol. **133:**559-69.

Davies, C. R., L. D. Jones, and P. A. Nuttall. 1986. Experimental studies on the transmission cycle of Thogoto virus, a candidate orthomyxovirus, in *Rhipicephalus appendiculatus*. Am J Trop Med Hyg **35:**1256-1262.

Davis, S. J. and R. D. Vierstra. 1996. Soluble derivatives of green fluorescent protein (GFP) for use in *Arabidopsis thaliana*. Weeds World **3:**43-48.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12:**387-395.

Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius. 1993. Folding and assembly of viral membrane proteins. Virology **193:**545-562.

Dorner, A. J., and J. M. Coffin. 1986. Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85. Cell **45:**365-374.

Duisit, G., S. Saleun, S. Douthe, J. Barsoum, G. Chadeuf, and P. Moullier. 1999. Baculovirus vector requires electrostatic interactions including heparan sulfate for efficient gene transfer in mammalian cells. J Gene Med **1:**93-102.

Dutch, R. E., T. S. Jardetzky, and R. A. Lamb. 2000. Virus membrane fusion proteins: biological machines that undergo a metamorphosis. Biosci Rep **20:**597-612.

Eckert, D. M., and P. S. Kim. 2001. Mechanisms of viral membrane fusion and its inhibition. Annu Rev Biochem **70:**777-810.

Edwards, R. A., L. H. Keller, and D. M. Schifferli. 1998. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene **207:**149-157.

Eisenberg, D. 1984 Three-dimensional structure of membrane and surface proteins. Annu Rev Biochem **53:**595-623.

Fabian, P., J. Murvai, Z. Hatsagi, K. Vlahovicek, H. Hegyi, and S. Pongor. 1997. The SBASE protein domain library, release 5.0: a collection of annotated protein sequence segments. Nucleic Acids Res **25:**240-243.

Fass, D., S. C. Harrison, and P. S. Kim. 1996. Retrovirus envelope domain at 1.7 angstrom resolution. Nat Struct Biol **3:**465-469.

Faulkner, P., J. Kuzio, G. V. Williams, and J. A. Wilson. 1997. Analysis of p74, a PDV envelope protein of *Autographa californica* nucleopolyhedrovirus required for occlusion body infectivity in vivo. J Gen Virol **78:**3091-3100.

Federici, B. A. 1997. Baculovirus pathogenesis, p. 33-59. *In* L. K. Miller (ed.), The baculoviruses. Plenum Press, New York, NY.

Federici, B. A., and J. V. Maddox. 1996. Host specificity in microbe-insect interactions. Bioscience **46:**410-421.

Federici, B. A., and R. H. Hice. 1997. Organization and molecular characterization of genes in the polyhedrin region of the *Anagrapha falcifera* multinucleocapsid NPV. Arch Virol **142:**333-348.

Fenouillet, E., and I. M. Jones. 1995. The glycosylation of Human immunodeficiency virus type 1 transmembrane glycoprotein (gp41) is important for the efficient intracellular transport of the envelope precursor gp160. J Gen Virol **76:**1509-1514.

Fenouillet, E., I. Jones, B. Powell, D. Schmitt, M. P. Kieny, and J. C. Gluckman. 1993. Functional role of the glycan cluster of the Human immunodeficiency virus type 1 transmembrane glycoprotein (gp41) ectodomain. J Virol **67:**150-160.

Flipsen, J. T. M. 1995. Systemic infection of *Spodoptera exigua* larvae using a marked *Autographa californica* nuclear polyhdrosis virus recombinant. *In* Pathogenesis induced by (recombinant) baculovirus in insects, p. 51-64. PhD thesis, Wageningen University.

Flipsen, J. T. M., J. W. M. Martens, M. M. Van Oers, J. M. Vlak, and J. W. M. Van Lent. 1995.

Passage of *Autographa californica* nuclear polyhedrosis virus through the midgut epithelium of *Spodoptera exigua* larvae. Virology **208:**328-335.

Fraser, M. J., G. E. Smith, and M. D. Summers. 1983. Acquisition of host cell DNA sequences by baculoviruses; relationships between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis virus. J Viro. **47:**287-300.

Fredericksen, B. L., and M. A. Whitt. 1995. Vesicular stomatitis virus glycoprotein mutations that affect membrane fusion activity and abolish virus infectivity. J Virol **69:**1435-1443.

Freedman-Faulstich, E. Z., and F. J. Fuller. 1990. Nucleotide sequence of the tick-borne, orthomyxo-like Dhori/Indian/1313/61 virus envelope gene. Virology **175:**10-18.

Friesen, P. D. 1997. Regulation of baculovirus early gene expression, p. 141-170. *In* L. K. Miller (ed.), The baculoviruses. Plenum Press, New York, NY.

Friesen, P. D., and L. K. Miller. 2001. Insect viruses, p. 599-628. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, M. A. Martin, R. A. Lamb, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott-Raven Publishers, Philadelphia, PA..

Friesen, P. D., and M. S. Nissen. 1990. Gene organization and transcription of TED, a lepidopteran retrotransposon integrated within the baculovirus genome. Mol Cell Biol **10:**3067-3077.

Friesen, P. D., W. C. Rice, D. W. Miller, and L. K. Miller. 1986. Bidirectional transcription from a solo long terminal repeat of the retrotransposon TED: symmetrical RNA start sites. Mol Cell Biol **6:**1599-1607.

Gallagher, P. J., J. M. Henneberry, J. F. Sambrook, and M. J. Gething. 1992. Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus. J Virol **66:**7136-7145.

Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol **120:**97-120.

Garten, W., A. Stieneke, E. Shaw, P. Wikstrom, and H. D. Klenk. 1989. Inhibition of proteolytic activation of influenza virus hemagglutinin by specific peptidyl chloroalkyl ketones. Virology **172:**25-31.

Gelernter, W. D., and B. A. Federici. 1986. Isolation, identification, and determination of virulence of a nuclear polyhedrosis virus from the beet armyworm, *Spodoptera exigua* (*Lepidoptera*: *Noctuidae*). Environ Entomol **15:**240-245.

Gelernter, W. D., and B. A. Federici. 1986. Isolation, identification and determination of virulence of a nuclear polyhedrosis virus from the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). Environ. Entomol. **15:**240-245.

Ghosh, S., M. K. Parvez, K. Banerjee, S. K. Sarin, and S. E. Hasnain. 2002. Baculovirus as mammalian cell expression vector for gene therapy: an emerging strategy. Mol Ther **6:**5-11.

Goldstein, N. I., and A. H. McIntosh. 1980. Glycoproteins of nuclear polyhedrosis viruses. Arch Virol **64:**119-126.

Goodman, C. L., G. N. El Sayed, A. H. McIntosh, J. J. Grasela, and B. Stiles. 2001. Establishment and characterization of insect cell lines from 10 lepidopteran species. In Vitro Cell Dev Biol Anim. **37:**367-73.

Gomi, S., K. Majima, and S. Maeda. 1999. Sequence analysis of the genome *of Bombyx mori* nucleopolyhedrovirus. J Gen Virol **80:**1323-1337.

Gotoh, B., Y. Ohnishi, N. M. Inocencio, E. Esaki, K. Nakayama, P. J. Barr, G. Thomas, and Y. Nagai. 1992. Mammalian subtilisin-related proteinases in cleavage activation of the paramyxovirus fusion glycoprotein: superiority of furin/PACE to PC2 or PC1/PC3. J Virol **66:**6391-6397.

Granados, R. R. 1978. Early events in the infection of *Heliothis zea* midgut cells by a baculovirus. Virology **90:**170-174.

Granados, R. R., and K. A. Lawler. 1981. In *vivo* pathway of *Autographa californica* baculovirus invasion and infection. Virology **108:**297-308.

Gray, W.R. 1997. Disulfide bonds between cysteine residues, p. 165-186. *In* T. E. Creighton (ed.), Protein structure: a pratical approach. Oxford University Press, Oxford, NY.

Gross, C. H., G. M. Wolgamot, R. L. Russell, M. N. Pearson, and G. F. Rohrmann. 1993. A baculovirus encoded 16-kDa glycoprotein localizes near the nuclear membrane of infected cells. Virology **192:**386-390.

Guarino, L. A., B. Xu, J. Jin, and W. Dong. 1998. A virus-encoded RNA polymerase purified from

baculovirus-infected cells. J Virol **72:**7985-7991.

Guinea, R., and L. Carrasco. 1995. Requirement for vacuolar proton-ATPase activity during entry of influenza virus into cells. J Virol **69:**2306-2312.

Haas-Stapleton, E. J., J. O. Washburn, and L. E. Volkman. 2002. AcMNPV p74 mediates ODV binding to the midgut epithelium of *Heliothis virescens*. Abstract of the 21st Annual Meeting of the American Society for Virology, Lexington, KY, p97.

Hallenberger, S., V. Bosch, H. Angliker, E. Shaw, H. D. Klenk, and W. Garten. 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature **360:**358-361.

Hara, K., M. Funakoshi, and T. Kawarabata. 1995. A cloned cell line of *Spodoptera exigua* has a highly increased susceptibility to the *Spodoptera exigua* nuclear polyhedrosis virus. Can. J. Microbiol. **41:**1111-1116.

Harrison, R. L., and B. C. Bonning. 2003. Comparative analysis of the genomes of *Rachiplusia ou* and *Autographa californica* multiple nucleopolyhedroviruses. J Gen Virol **84:**1827-1842.

Hashimoto, Y., T. Hayakawa, Y. Ueno, T. Fujita, Y. Sano, and T. Matsumoto. 2000. Sequence analysis of the *Plutella xylostella* granulovirus genome. Virology **275:**358-372.

Hayakawa, T., G. F. Rohrmann, and Y. Hashimoto. 2000. Patterns of genome organization and content in lepidopteran baculoviruses. Virology **278:**1-12.

Hayakawa, T., R. Ko, K. Okano, S. I. Seong, C. Goto, and S. Maeda. 1999. Sequence analysis of the *Xestia c-nigrum* granulovirus genome. Virology **262:**277-297.

He, Y., R. Vassell, M. Zaitseva, N. Nguyen, Z. Yang, Y. Weng, and C. D. Weiss. 2003. Peptides trap the Human immunodeficiency virus type 1 envelope glycoprotein fusion intermediate at two sites. J Virol **77:**1666-1671.

Hefferon, K. L., A. G. Oomens, S. A. Monsma, C. M. Finnerty, and G. W. Blissard. 1999. Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. Virology **258:**455-468.

Helenius, A., M. Marsh, and J. White. 1982. Inhibition of Semliki forest virus penetration by lysosomotropic weak bases. J Gen Virol **58:**47-61.

Hernandez, L. D., L. R. Hoffman, T. G. Wolfsberg, and J. M. White. 1996. Virus-cell and cell-cell fusion. Annu Rev Cell Dev Biol **12:**627-661.

Herniou, E. A., J. A. Olszewski, J. S. Cory, and D. R. O'Reilly. 2003. The genome sequence and evolution of baculoviruses. Annu Rev Entomol **48:**211-234.

Herniou, E. A., T. Luque, X. Chen, J. M. Vlak, D. Winstanley, J. S. Cory, and D. R. O'Reilly. 2001. Use of whole-genome sequence data to infer baculovirus phylogeny. J Virol **75:**8117-8126.

Hess, R. T., and L. A. Falcon. 1977. Observation on the interaction of baculoviruses with the plasma membrane. J Gen Virol **36:**525-530.

Hill, J. E., and P. Faulkner. 1994. Identification of the *gp67* gene of a baculovirus pathogenic to the spruce budworm, *Choristoneura fumiferana* multinucleocapsid nuclear polyhedrosis virus. J Gen Virol **75:**1811-1813.

Hofmann, C., V. Sandig, G. Jennings, M. Rudolph, P. Schlag, and M. Strauss. 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc Natl Acad Sci U S A **92:**10099-10103.

Hohmann, A. W., and P. Faulkner. 1983. Monoclonal antibodies to baculovirus structural proteins: determination of specificities by Western blot analysis. Virology **125:**432-444.

Holt, R. A., G. M. Subramanian, A. Halpern, G. G. Sutton, R. Charlab, D. R. Nusskern, P. Wincker, A. G. Clark, J. M. Ribeiro, R. Wides, S. L. Salzberg, B. Loftus, M. Yandell, W. H. Majoros, D. B. Rusch, Z. Lai, C. L. Kraft, J. F. Abril, V. Anthouard, P. Arensburger, P. W. Atkinson, H. Baden, V. de Berardinis, D. Baldwin, V. Benes, J. Biedler, C. Blass, R. Bolanos, D. Boscus, M. Barnstead, S. Cai, A. Center, K. Chaturverdi, G. K. Christophides, M. A. Chrystal, M. Clamp, A. Cravchik, V. Curwen, A. Dana, A. Delcher, I. Dew, C. A. Evans, M. Flanigan, A. Grundschober-Freimoser, L. Friedli, Z. Gu, P. Guan, R. Guigo, M. E. Hillenmeyer, S. L. Hladun, J. R. Hogan, Y. S. Hong, J. Hoover, O. Jaillon, Z. Ke, C. Kodira, E. Kokoza, A. Koutsos, I. Letunic, A. Levitsky, Y. Liang, J. J. Lin, N. F. Lobo, J. R. Lopez, J. A. Malek, T. C. McIntosh, S. Meister, J. Miller, C. Mobarry, E. Mongin, S. D. Murphy, D. A. O'Brochta, C. Pfannkoch, R. Qi, M. A. Regier, K. Remington, H. Shao, M. V. Sharakhova, C. D. Sitter, J. Shetty, T. J. Smith, R. Strong, J. Sun, D. Thomasova, L. Q. Ton, P. Topalis, Z. Tu, M. F. Unger, B. Walenz, A. Wang, J. Wang, M. Wang, X. Wang, K. J. Woodford, J. R. Wortman, M. Wu, A. Yao, E. M. Zdobnov,

H. Zhang, Q. Zhao, S. Zhao, S. C. Zhu, I. Zhimulev, M. Coluzzi, A. Della Torre, C. W. Roth, C. Louis, F. Kalush, R. J. Mural, E. W. Myers, M. D. Adams, H. O. Smith, S. Broder, M. J. Gardner, C. M. Fraser E. Birney, P. Bork, P. T. Brey J. C. Weissenbach, F. C. Kafatos, F. H. Collins, and S. L. Hoffman. 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. Science **298:**129-149.

Horth, M., B. Lambrecht, M. C. Khim, F. Bex, C. Thiriart, J. M. Ruysschaert, A. Burny, and R. Brasseur. 1991. Theoretical and functional analysis of the SIV fusion peptide. EMBO J **10:**2747-2755.

Horton, H. M., and J. P. Burand. 1993. Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. J Virol **67:**1860-1868.

Horvath, C. M., and R. A. Lamb. 1992. Studies on the fusion peptide of a paramyxovirus fusion glycoprotein: roles of conserved residues in cell fusion. J Virol **66:**2443-2455.

Hosaka, M., M. Nagahama, W. S. Kim, T. Watanabe, K. Hatsuzawa, J. Ikemizu, K. Murakami, and K. Nakayama. 1991. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. J Biol Chem **266:**12127-12130.

Hu, Z. H., B. M. Arif, F. Jin, J. W. Martens, X. W. Chen, J. S. Sun, D. Zuidema, R. W. Goldbach, and J. M. Vlak. 1998. Distinct gene arrangement in the *Buzura suppressaria* single-nucleocapsid nucleopolyhedrovirus genome. J Gen Virol **79:**2841-2851.

Huh, N. E., and R. F. Weaver. 1990. Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. J Gen Virol **71:**195-201.

Hyink, O., R. A. Dellow, M. J. Olsen, K. M. Caradoc-Davies, K. Drake, E. A. Herniou, J. S. Cory, D. R. O'Reilly, and V. K. Ward. 2002. Whole genome analysis of *the Epiphyas postvittana* nucleopolyhedrovirus. J Gen Virol **83:**957-971.

IJkel, W. F., E. A. Van Strien, J. G. Heldens, R. Broer, D. Zuidema, R. W. Goldbach, and J. M. Vlak. 1999. Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. J. Gen. Virol. **80:**3289-3304.

IJkel, W. F., M. Westenberg, R. W. Goldbach, G. W. Blissard, J. M. Vlak, and D. Zuidema. 2000. A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. Virology **275:**30-41.

IJkel, W. F., R-J Lebbink, M. L. Op den Brouw, R. W. Goldbach, J. M. Vlak, and D. Zuidema. 2001. Identification of a novel occlusion derived virus-specific protein in *Spodoptera exigua* multicapsid nucleopolyhedrosisvirus. Virology **284:**170-181.

Ito, H., S. Watanabe, A. Sanchez, M. A. Whitt, and Y. Kawaoka. 1999. Mutational analysis of the putative fusion domain of Ebola virus glycoprotein. J Virol **73:**8907-8912.

Jarvis, D. L. 2003. Developing baculovirus-insect cell expression systems for humanized recombinant glycoprotein production. Virology **310:**1-7.

Jarvis, D. L., L. Wills, G. Burow, and D. A. Bohlmeyer. 1998. Mutational analysis of the N-linked glycans on *Autographa californica* nucleopolyhedrovirus gp64. J Virol **72:**9459-9469.

Jones, J. S., and R. Risser. 1993. Cell fusion induced by the Murine leukemia virus envelope glycoprotein. J Virol **67:**67-74.

Keddie, B. A., and L. E. Volkman. 1985. Infectivity difference between the two phenotypes of *Autographa californica* nuclear polyhedrosis virus: Importance of tyhe 64K envelope protein. J Gen Virol **66:**1195-1200.

Keddie, B. A., G. W. Aponte, L. E. Volkman. 1989. The pathway of infection of *Autographa californica* nuclear polyhedrosis virus in an insect host. Science **243:**1728-1730.

Kielian, M., P. K. Chatterjee, D. L. Gibbons, and Y. E. Lu. 2000. Specific roles for lipids in virus fusion and exit. Examples from the alphaviruses. Subcell Biochem **34:**409-455.

Kikhno, I., S. Gutierrez, L. Croizier, G. Croizier, and M. L. Ferber. 2002. Characterization of *pif*, a gene required for the per os infectivity *of Spodoptera littoralis* nucleopolyhedrovirus. J Gen Virol **83:**3013-3022.

Kingsley, D. H., A. Behbahani, A. Rashtian, G. W. Blissard, and J. Zimmerberg. 1999. A discrete stage of baculovirus GP64-mediated membrane fusion. Mol Biol Cell **10:**4191-4200.

Klenk, H. D., and W. Garten. 1994. Activation of viral spike proteins by host proteases, p. 241-280. *In* E. Wimmer (ed.), Cellular receptors for animal viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn. 1975. Activation of influenza A viruses by trypsin

treatment. Virology **68:**426-439.
Kost, T. A., and J. P. Condreay. 2002. Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol **20:**173-180.
Kumar, M., B. P. Bradow, and J. Zimmerberg. 2003. Large-Scale Production of pseudotyped lentiviral vectors using baculovirus GP64. Hum Gene Ther **14:**67-77.
Kuzio, J., M. N. Pearson, S. H. Harwood, C. J. Funk, J. T. Evans, J. M. Slavicek, and G. F. Rohrmann. 1999. Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. Virology **253:**17-34.
Kuzio, J., R. Jaques, and P. Faulkner. 1989. Identification of *p74*, a gene essential for virulence of baculovirus occlusion bodies. Virology **173:**759-763.
Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J Mol Biol **157:**105-132.
Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680-685.
Lamb, R. A. 1993. Paramyxovirus fusion: A hypothesis for changes. Virology **197:**1-11.
Landau, N. R., K. A. Page, and D. R. Littman. 1991. Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J Virol **65:**162-169.
Lange, M., and J. A. Jehle. 2003. The genome of the *Cryptophlebia leucotreta* granulovirus. Virology **317:**220-236.
Lanier, L. M., and L. E. Volkman. 1998. Actin binding and nucleation by *Autographa california* M nucleopolyhedrovirus. Virology **243:**167-177.
Lauzon, H. A. M., C. Lucarotti, P. J. Krell, and B. M. Arif. 2003. Gene organization and content of the *Neodiprion lecontei* NPV genome. Abstract of the 36th Annual Meeting of the Society for Invertebrate Phatology, Burlington, V.T, p. 77.
Lazarowitz, S. G., and P. W. Choppin. 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology **68:**440-454.
Lazarowitz, S. G., R. W. Compans, and P. W. Choppin. 1971. Influenza virus structural and non-structural proteins in infected cells and their plasma membrane. Virology **46:**830-843.
Leikina, E., H. O. Onaran, and J. Zimmerberg. 1992. Acidic pH induces fusion of cells infected with baculovirus to form syncytia. FEBS Lett. **304:**221-224.
Lenard, K., and D. K. Miller. 1982. Uncoating of enveloped viruses. Cell **28:**5-6.
Levy-Mintz, P., and M. Kielian. 1991. Mutagenesis of the putative fusion domain of the Semliki forest virus spike protein. J Virol **65:**4292-4300.
Li, L., C. Donly, Q. Li, L. G. Willis, B. A. Keddie, M. A. Erlandson, and D. A. Theilmann. 2002. Identification and genomic analysis of a second species of nucleopolyhedrovirus isolated from *Mamestra configurata*. Virology **297:**226-244.
Li, Q., C. Donly, L. Li, L. G. Willis, D. A. Theilmann, and M. Erlandson. 2002. Sequence and organization of the *Mamestra configurata* nucleopolyhedrovirus genome. Virology **294:**106-121.
Long, G., X. Chen, D. Peters, J. M. Vlak, Z. Hu. 2003. Open reading frame 122 of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus encodes a novel structural protein of ocllusion derived virions. J Gen Virol **84:**151-121.
Lu, A., and L. K. Miller. 1997. Regulation of baculovirus late and very late gene expression, p. 193-216. *In* L. K. Miller (ed.), The baculoviruses. Plenum Press, New York, NY.
Luckow, V. A. 1991. Cloning and expression of heterologous genes in insect cells with baculovirus vectors, p. 97-152. *In* A. Prokop, R. K. Bajpaj, and C. Ho (ed.), Recombinant DNA technology and applications. McGraw-Hill, New York, NY.
Luckow, V. A., S. C. Lee, G. F. Barry, and P. O. Olins. 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. **67:**4566-4579.
Lung, O. Y., M. Cruz-Alvarez, and G. W. Blissard. 2003. Ac23, an envelope fusion protein homolog in the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus, is a viral pathogenicity factor. J Virol **77:**328-339.
Lung, O., and G. W. Blissard. 2003. Comparitive analysis of baculovirus envelope fusion protein and a F homolog of *D. melanogaster*. Abstract of the 36th Annual Meeting of the Society for Invertebrate Phatology, Burlington, V.T, p. 96.
Lung, O., M. Westenberg, J. M. Vlak, D. Zuidema, and G. W. Blissard. 2002. Pseudotyping

Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from group II NPVs are functionally analogous to AcMNPV GP64. J Virol **76:**5729-5736.

Luque, T., R. Finch, N. Crook, D. R. O'Reilly, and D. Winstanley. 2001. The complete sequence of the *Cydia pomonella* granulovirus genome. J Gen Virol **82:**2531-2547.

Maeda, S., S. G. Kamita, and A. Kondo. 1993. Host range expansion of *Autographa californica* nuclear polyhedrosis virus (NPV) following recombination of a 0.6-kilobase-pair DNA fragment originating from *Bombyx mori* NPV. J Virol **67:**6234-6238.

Maeda, T., and S. Ohnishi. 1980. Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. FEBS Lett **122:**283-287.

Malik, H. S., S. Henikoff, and T. H. Eickbush. 2000. Poised for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. Genome Res **10:**1307-1318.

Mangor, J. T., S. A. Monsma, M. C. Johnson, and G. W. Blissard. 2001. A GP64-null baculovirus pseudotyped with vesicular stomatitis virus G protein. J. Virol. **75:**2544-2556.

Markosyan, R. M., X. Ma, M. Lu, F. S. Cohen, and G. B. Melikyan. 2002. The mechanism of inhibition of HIV-1 env-mediated cell-cell fusion by recombinant cores of gp41 ectodomain. Virology **302:**174-184.

Markovic, I., H. Pulyaeva, A. Sokoloff, and L. V. Chernomordik. 1998. Membrane fusion mediated by baculovirus gp64 involves assembly of stable gp64 trimers into multiprotein aggregates. J Cell Biol **143:**1155-1166.

Marks, H., M. Mennens, J. M. Vlak, and M. C. Van Hulten. 2003. Transcriptional analysis of the white spot syndrome virus major virion protein genes. J Gen Virol **84:**1517-1523.

Martin, I. I., J. Ruysschaert, and R. M. Epand. 1999. Role of the N-terminal peptides of viral envelope proteins in membrane fusion. Adv Drug Deliv Rev **38:**233-255.

Mebatsion, T., M. Konig, and K. K. Conzelmann. 1996. Budding of Rabies virus particles in the absence of the spike glycoprotein. Cell **84:**941-951.

Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and exocytic pathways. Annu Rev Biochem **55:** 663-700.

Meyer, H., Y. Masuho, and M. Mach. 1990. The gp116 of the gp58/116 complex of human cytomegalovirus represents the amino-terminal part of the precursor molecule and contains a neutralizing epitope. J Gen Virol **71:**2443-2450.

Miller, D. W., and L. K. Miller. 1982. A virus mutant with an insertion of a copia-like transposable element. Nature **299:**562-564.

Miller, L. K. 1996. Insect viruses, p. 33-59. *In* B. N. Fields, D. M. Knipe., and P. M. Howley. (ed.), Fields Virology. Lippincott-Ravem Publishers, New York, NY.

Misseri, Y., G. Labesse, A. Bucheton, and C. Terzian. 2003. Comparative sequence analysis and predictions for the envelope glycoproteins of insect endogenous retroviruses. Trends Microbiol **11:**253-256.

Monsma, S. A., A. G. Oomens, and G. W. Blissard. 1996. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J Virol **70:**4607-4616.

Monsma, S. A., and G. W. Blissard. 1995. Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein. J Virol **69:**2583-2595.

Morrison, T. G. 2003. Structure and function of a paramyxovirus fusion protein. Biochim Biophys Acta **1614:**73-84.

Morse, M. A., A. C. Marriott, and P. A. Nuttall. 1992. The glycoprotein of Thogoto virus (a tick-borne orthomyxo-like virus) is related to the baculovirus glycoprotein GP64. Virology **186:**640-646.

Moscardi, F. 1999. Assessment of the application of baculoviruses for control of *Lepidoptera*. Annu Rev Entomol **44:**257-289.

Muñoz, D., J. I. Castillejo, and P. Caballero. 1998. Naturally occurring deletion mutants are parasitic genotypes in a wild-type nucleopolyhedrovirus population of *Spodoptera exigua*. Appl Environ Microbiol **64:**4372-4377.

Muyrers, J. P., Y. Zhang, G. Testa, and A. F. Stewart. 1999. Rapid modification of bacterial artificial chromosomes by ET-recombination. Nucleic Acids Res **27:**1555-1557.

Nakai M., C. Goto, W. Kang, M. Shikata, T. Luque and Y. Kunimi.2003. Genome sequence and organization of a nucleopolyhedrovirus isolated from the smaller tea tortrix, *Adoxophyes honmai.* Virology **316:**171-183

Nakayama, K. 1997. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. J Biochem **327:**625-635.

Neumann, G., H. Feldmann, S. Watanabe, I. Lukashevich, and Y. Kawaoka. 2002. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. J Virol **76:**406-410.

Nicholas, K. B., H. B. Nicholas Jr, and D. W. Deerfield. 1997. GeneDoc: analysis and visualization of genetic variation. EMBNET News **4:**1-4.

Nishimura, T., D. Favre, M. Durrenberger, M. R. Michel, I. Ichihara, and H. Koblet. 1994. Nuclear export of recombinant baculovirus nucleocapsids through small pore or nuclear-pore-like structure in Sf9 cells. Okajimas Folia Anat Jpn **71:**83-97.

Ohkawa, T., A. R. Rowe, and L. E. Volkman. 2002. Identification of six Autographa californica multicapsid nucleopolyhedrovirus early genes that mediate nuclear localization of G-actin. J Virol **76:**12281-12289.

Onstad, D. W. 2003, posting date. EDWIP: Ecological Database of the World's Insect Pathogens. Champaign, Illinois: Illinois Natural History Survey, http://insectweb.inhs.uiuc.edu/Pathogens/EDWIP/index.html

Oomens, A. G. P. 1999a. Characterization of the *lef7-gp64-p74* locus of *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus. *In* Functional and structural analysis of GP64, the major envelope glycoprotein of the budded virus phenotype of *Autographa californica* and *Orgyia pseudotsugata* multicapsid nucleopolyhedroviruses, p. 103-122. PhD thesis, Wageningen University.

Oomens, A. G. P. 1999b. Functional and structural analysis of GP64 the major glycoprotein of the Budded Virus phenotype of *Autographa californica* and *Orgyia pseudotsugata* Multicapsid Nucleopolyhedroviruses. PhD thesis, Wageningen University.

Oomens, A. G., and G. W. Blissard. 1999. Requirement for GP64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. Virology **254:**297-314.

Oomens, A. G., S. A. Monsma, and G. W. Blissard. 1995. The baculovirus GP64 envelope fusion protein: synthesis, oligomerization, and processing. Virology **209:**592-603.

O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1992. Baculovirus expression vectors: a laboratory manual. W. H. Freeman and Co, New York, NY.

Ortmann, D., M. Ohuchi, H. Angliker, E. Shaw, W. Garten, H. D. and Klenk. 1994. Proteolytic cleavage of wild type and mutants of the F protein of human parainfluenza virus type 3 by two subtilisin-like endoproteases, furin and Kex2. J Virol 68:2772-2776.

Owen, K. E., and R. J. Kuhn. 1997. Alphavirus budding is dependent on the interaction between the nucleocapsid and hydrophobic amino acids on the cytoplasmic domain of the E2 envelope glycoprotein. Virology **230:**187-196.

Owens, R. J., and J. K. Rose. 1993. Cytoplasmic domain requirement for incorporation of a foreign envelope protein into vesicular stomatitis virus. J Virol **67:**360-365.

Ozers, M. S., and P. D. Friesen. 1996. The *env*-like open reading frame of the baculovirus-integrated retrotransposon TED encodes a retrovirus-like envelope protein. Virology **226:**252-259.

Pang, Y., J. Yu, L. Wang, X. Hu, W. Bao, G. Li, C. Chen, H. Han, S. Hu, and H. Yang. 2001. Sequence analysis of the *Spodoptera litura* multicapsid nucleopolyhedrovirus genome. Virology **287:**391-404.

Park, C. G., M. Y. Jung, Y. Choi, and G. M. Winslow. 1995. Proteolytic processing is required for viral superantigen activity. J Exp Med **181:**1899-1904.

Pearson, M. N., and G. F. Rohrmann. 2003. Biophysical analysis of the LdMNPV F protein type fusion protein. Abstract of the Anual Meeting of the American Society of Virology, Davis, C.A, p.191.

Pearson, M. N., C. Groten, and G. F. Rohrmann. 2000. Identification of *the Lymantria dispar* nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the *Baculoviridae*. J Virol **74:**6126-6131.

Pearson, M. N., R. L. Russell, and G. F. Rohrmann. 2001. Characterization of a baculovirus-encoded protein that is associated with infected-cell membranes and budded virions. Virology **291:**22-31.

Pearson, M. N., R. L. Russell, and G. F. Rohrmann. 2002a. Functional analysis of a conserved region of the baculovirus envelope fusion protein, LD130. Virology **304:**81-88.

Pearson, M. N., R. L. Russell, and G. F. Rohrmann. 2002b. Transcriptional mapping of two genes

encoding baculovirus envelope-associated proteins. J Gen Virol **83:**937-943.

Pearson, W. R. 1990. Rapid and sensitive sequence comparison with FASTP and FASTA. Methods Enzymol **183:**63-98.

Pelisson, A., L. Teysset, F. Chalvet, A. Kim, N. Prud'homme, C. Terzian, and A. Bucheton. 1997. About the origin of retroviruses and the co-evolution of the gypsy retrovirus with the Drosophila flamenco host gene. Genetica **100:**29-37.

Perrin, C., E. Fenouillet, and I. M. Jones. 1998. Role of gp41 glycosylation sites in the biological activity of Human immunodeficiency virus type 1 envelope glycoprotein. Virology **242:**338-345.

Pieroni, L., and N. La Monica. 2001. Towards the use of baculovirus as a gene therapy vector. Curr Opin Mol Ther **3:**464-467.

Pijlman, G. P., A. J. P. Pruijssers, and J. M. Vlak 2003. Identification of pif-2, a third conserved baculovirus gene required for per os infection of insects. J Gen Virol **84:**2041-2049.

Pijlman, G. P., J. C. Dortmans, A. M. Vermeesch, K. Yang, D. E. Martens, R. W. Goldbach, and J. M. Vlak. 2002. Pivotal role of the non-hr origin of DNA replication in the genesis of defective interfering baculoviruses. J Virol **76:**5605-5611.

Plonsky, I., and J. Zimmerberg. 1996. The initial fusion pore induced by baculovirus GP64 is large and forms quickly. J Cell Biol **135:**1831-1839.

Plonsky, I., M. S. Cho, A. G. P. Oomens, G. W. Blissard, and J. Zimmerberg. 1999. An analysis of the role of the target membrane on the Gp64-induced fusion pore. Virology **253:**65-76.

Richt, J. A., T. Fürbringer, A. Koch, I. Pfeuffer., C. Herden, I. Bause-Niedrig, and W. Garten. 1998. Processing of the Borna disease virus glycoprotein gp94 by the sutilisin-like endoprotease furin. J Virol **72:**4528-4533.

Riegel, C. I., and J. M. Slavicek. 1997. Characterization of the replication cycle of the *Lymantria dispar* nuclear polyhedrosis virus. Virus Res **51:**9-17.

Riegel, C. I., C. Lanner-Herrera, and J. M. Slavicek. 1994. Identification and characterization of the *ecdysteroid UDP-glucosyltransferase* gene of the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. J. Gen. Virol. **75:**829-838.

Roberts, P. C., W. Garten, and H. D. Klenk. 1993. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. J Virol **67:**3048-3060.

Robison, C. S., and M. A. Whitt. 2000. The membrane-proximal stem region of vesicular stomatitis virus G protein confers efficient virus assembly. J Virol **74:**2239-2246.

Rohrmann, G. F., and P. A. Karplus. 2001. Relatedness of baculovirus and gypsy retrotransposon envelope proteins. BMC Evol Biol **1:**1.

Rost, B. 1996. PHD: predicting one-dimensional protein structure by profile based neural networks. Methods Enzymol **266:**525-539.

Russell, C. J., T. S. Jardetzky, and R. A. Lamb. 2001. Membrane fusion machines of paramyxoviruses: capture of intermediates of fusion. EMBO J **20:**4024-4034.

Schied, A., and P. W. Choppin. 1977. Two disulfide-linked polypeptide chains constitute the active F protein of paramyxoviruses. Virology **80:**54-66.

Schmid, S. L. 1992. The mechanism of receptor-mediated endocytosis: more questions than answers. BioEssays **14:** 589-596.

Schneider-Schaulies, J. 2000. Cellular receptors for viruses: links to tropism and pathogenesis. J Gen Virol **81:**1413-1429.

Sharrocks, A. D., and P. E. Shaw. 1992. Improved primer design for PCR-based, site-directed mutagenesis. Nucleic Acids Res **20:**1147.

Shirata, N., M. Ikeda, K. Kamiya, S. Kawamura, Y. Kunimi, and M. Kobayashi. 1999. Replication of nucleopolyhedroviruses of *Autographa californica* (*Lepidoptera* : *Noctuidae*), *Bombyx mori* (*Lepidoptera* : *Bombycidae*), *Hyphantria cunea* (*Lepidoptera* : *Arctiidae*), and *Spodoptera exigua* (*Lepidoptera* : *Noctuidae*) in four lepidopteran cell lines. Appl Entomol Zool **34:**507-516.

Shizuya, H., B. Birren, U. J. Kim, V. Mancino, T. Slepak, Y. Tachiiri, and M. Simon. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proc Natl Acad Sci U S A **89:**8794-8797.

Sia, S. K., P. A. Carr, A. G. Cochran, V. N. Malashkevich, and P. S. Kim. 2002. Short constrained peptides that inhibit HIV-1 entry. Proc Natl Acad Sci U S A **99:**14664-14669.

Singh, M., B. Berger, and P. S. Kim. 1999. LearnCoil-VMF: computational evidence for coiled-coil-like motifs in many viral membrane-fusion proteins. J Mol Biol **290:**1031-1041.

Skehel, J. J., and D. C. Wiley. 1998. Coiled coils in both intracellular vesicle and viral membrane

fusion. Cell **95:**871-874.

Skehel, J. J., and D. C. Wiley. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem **69:**531-569.

Smith, G. E., and M. D. Summers. 1978. Analysis of baculovirus genomes with restriction endonucleases. Virology **89:**517-527.

Smits, P. H., and J. M. Vlak. 1988. Quantitative and qualitative aspects in the production of a nuclear polyhedrosis virus in *Spodoptera exigua* larvae. Ann Appl Biol **112:**249-257.

Song, C., S. R. Dubay, and E. Hunter. 2003. A tyrosine motif in the cytoplasmic domain of Mason-pfizer monkey virus is essential for the incorporation of glycoprotein into virions. J Virol **77:**5192-5200.

Song, S. U., T. Gerasimova, M. Kurkulos, J. D. Boeke, and V. G. Corces. 1994. An env-like protein encoded by a *Drosophila* retroelement: evidence that gypsy is an infectious retrovirus. Genes Dev **8:**2046-2057.

Steinhauer, D. A. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:**1-20.

Stieneke-Gröber, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten. 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J. **11:**2407-2414.

Sturman, L. S., C. S. Ricard, and K. V. Holmes. 1985. Proteolytic cleavage of the E2 glycoprotein of murine coronavirus activation of cell-fusing activity of virions by tripsin and seperation of two different 90K cleavage fragments. J Virol 56:904-911.

Summers, M. D. 1977. Characterization of shrimp baculovirus. EPA Ecological Reaseach Series, EPA, Gulf Breeze, FL.

Taguchi, F. 1995. The S2 subunit of the murine coronavirus spike protein is not involved in receptor binding. J Virol **69:**7260-7263.

Tanada, Y., R. T. Hess, and E. M. Omi. 1975. Invasion of a nuclear polyhedrosis virus in midgut of the armyworm, *Pseudaletia unipuncta*, and the enhancement of a synergistic enzyme. J Invertebr Pathol **26:**99-104.

Tanda, S., J. L. Mullor, and V. G. Corces. 1994. The *Drosophila* tom retrotransposon encodes an envelope protein. Mol Cell Biol **14:**5392-5401.

Terzian, C., A. Pelisson, and A. Bucheton. 2001. Evolution and phylogeny of insect endogenous retroviruses. BMC Evol Biol **1:**3.

Thiem, S. M., and L. K. Miller. 1990. Differential gene expression mediated by late, very late and hybrid baculovirus promoters. Gene **91:**87-94.

Thiem, S. M., X. Du, M. E. Quentin, and M. M. Berner. 1996. Identification of baculovirus gene that promotes Autographa californica nuclear polyhedrosis virus replication in a nonpermissive insect cell line. J Virol **70:**2221-2229.

van Beek, N. A. M., and P. R. Hughes. 1998. The response time of insect larvae infected with recombinant baculoviruses. J Invertebr Pathol **72:**338-347.

van Loo, N. D., E. Fortunati, E. Ehlert, M. Rabelink, F. Grosveld, and B. J. Scholte. 2001. Baculovirus infection of nondividing mammalian cells: Mechanisms of entry and nuclear transport of capsids. J Virol **75:**961-970.

van Regenmortel, C. M., D. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner. 2000. Virus taxonomy classification and nomenclature of viruses : seventh report of the International Committee on Taxonomy of Viruses Taxonomy of Viruses. Academic Press, San Diego, Wien New York.

Vaughn, J. L., R. H. Goodwin, G. J. Tompkins, and P. McCawley. 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (*Lepidoptera*; *Noctuidae*). In Vitro **13:**213-217.

Veronese, F. D., A. L. DeVico, T. D. Copeland, S. Oroszlan, R. C. Gallo, M. G. Sarngadharan. 1985. Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. Science **229**:1402-1405.

Vey, M., M. Orlich, S. Adler, H. D. Klenk, R. Rott, and W. Garten. 1992. Hemagglutinin activation of pathogenic avian influenza viruses of serotype H7 requires the protease recognition motif R-X-K/R-R. Virology **188:**408-413.

Vey, M., W. Schäfer, B. Reis, R. Ohuchi, W. Britt, W. Garten, H. D. Klenk, and K. Radsak. 1995. Proteolytic processing of Human cytomegalovirus glycoprotein B (gpUL55) is mediated by the

human endoprotease furin. Virology **206:**746-749.

Vey, M., W. Schäfer, S. Berghöfer, H. D. Klenk, and W. Garten. 1994. Maturation of the *trans*-Golgi network protease furin: compartmentalization of propetide removal, substrate cleavage, and COOH-terminal truncation. J Cell Biol **127:**1829-1842.

Volchkov, V. E., H. Feldmann, A. V. Volchkova, and H. D. Klenk. 1998. Processing of the Ebola virus glycoprotein by the proprotein convertase furin. Proc Natl Acad Sci U S A **95:**5762-5767.

Volkman, L. E. 1986. The 64K envelope protein of budded Autographa californica nuclear polyhedrosis virus. Curr Top Microbiol Immunol **131:**103-118.

Volkman, L. E. 1997. Nucleopolyhedrovirus interactions with their insect hosts. Adv Virus Res **48:**313-348.

Volkman, L. E., and M. D. Summers. 1977. *Autographa californica* nuclear polyhedrosis virus: Comparitive infectivity of the occluded, alkali-liberated, and nonoccluded forms. J Invertebr Pathol **30:**102-103.

Volkman, L. E., and P. A. Goldsmith. 1984. Budded *Autographa californica* NPV 64K protein: further biochemical analysis and effects of postimmunoprecipitation sample preparation conditions. Virology **139:**295-302.

Volkman, L. E., and P. A. Goldsmith. 1985. Mechanism of neutralization of budded *Autograpa californica* nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. Virology **143**:185-195.

Volkman, L. E., G. W. Blissard, P. Friesen, B. A. Keddie, R. Possee, and D. A. Theilmann. 1995. Family *Baculoviridae*, p. 104-113. *In* F. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial. A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers (ed.), Virus taxonomy : classification and nomenclature of viruses : sixth report of the International Committee on Taxonomy of Viruses. Springer, Wien New York.

Volkman, L. E., P. A. Goldsmith, R. T. Hess, and P. Faulkner. 1984. Neutralization of budded *Autographa californica* NPV by a monoclonal antibody: Identification of the target antigen. Virology **133:**354-362.

Wang, E., X. Sun, Y. Qian, L. Zhao, P. Tien, and G. F. Gao. 2003. Both heptad repeats of Human respiratory syncytial virus fusion protein are potent inhibitors of viral fusion. Biochem Biophys Res Commun **302:**469-475.

Wang, K., C. Boysen, H. Shizuya, M. I. Simon, and L. Hood. 1997a. Complete nucleotide sequence of two generations of a bacterial artificial chromosome cloning vector. Biotechniques **23:**992-994.

Wang, P., D. A. Hammer, and R. R. Granados. 1997b. Binding and fusion of *Autographa californica* nuclear polyhedrosis virus. J Gen Virol **63:**1393-1399.

Washburn, J. O., D. Trudeau, J. F. Wong, and L. E. Volkman. 2003. Early pathogenesis of Autographa californica multiple nucleopolyhedrovirus and Helicoverpa zea single nucleopolyhedrovirus in Heliothis virescens: a comparison of the 'M' and 'S' strategies for establishing fatal infection. J Gen Virol **84:**343-351.

Watanabe, M., A. Hirano, S. Stenglein, J. Nelson, G. Thomas, and T. C. Wong. 1995. Engineered serine protease inhibitor prevents furin-catalyzed activation of the fusion glycoprotein and production of infectious Measles virus. J. Virol. **69:**3206-3210.

Weis, R. 1992. Cellular receptors and viral glycoproteins involved in retrovirus entry, p. 1-108. *In* L. Levy (ed.), The Retroviruses, vol. 2. Plenum Press, New York, NY.

Weiss, C. D., J. A. Levy, and J. M. White. 1990. Oligomeric organization of gp120 on infectious human immunodeficiency virus type I particles. J Virol **64:**5674-5677.

Weissenhorn, W., A. Carfi, K. H. Lee, J. J. Skehel, and D. C. Wiley. 1998. Crystal structure of the Ebola virus membrane fusion subunit, GP2, from the envelope glycoprotein ectodomain. Mol Cell **2:**605-616.

Weissenhorn, W., A. Dessen, S. C. Harrison, J. J. Skehel, and D. C. Wiley. 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature **387:**426-430.

Westenberg, M., H. Wang, W. F. IJkel, R. W. Goldbach, J. M. Vlak, and D. Zuidema. 2002. Furin is involved in baculovirus envelope fusion protein activation. J Virol **76:**178-184.

White, J. M. 1990. Viral and cellular membrane fusion proteins. Annu Rev Physiol **52:**675-697.

White, J. M. 1992. Membrane fusion. Science **258:**917-924.

Whitford, M., S. Stewart, J. Kuzio, and P. Faulkner. 1989. Identification and sequence analysis of a gene encoding *gp67*, an abundant envelope glycoprotein of the baculovirus *Autographa*

californica nuclear polyhedrosis virus. J Virol **63:**1393-1399.

Whittaker, G. R., and A. Helenius. 1998. Nuclear import and export of viruses and virus genomes. Virology **246:**1-23.

Wickham, T. J., M. L. Shuler, D. A. Hammer, R. R. Granados, and H. A. Wood. 1992. Equilibrium and kinetic analysis of *Autographa californica* nuclear polyhedrosis virus attachment to different insect cell lines. J Gen Virol **73:**3185-3194.

Wickham, T. J., R. R. Granados, H. A. Wood, D. A. Hammer, and M. L. Shuler. 1990. General analysis of receptor-mediated viral attachment to cell surfaces. Biophys J **58:**1501-1516.

Wiley, D. C., and J. J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu Rev Biochem **56:**365-394.

Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature **289:**366-373.

Wimmer, E. 1994. Cellular Receptors for Animal viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wormleaton, S., J. Kuzio, and D. Winstanley. 2003. The complete sequence of the *Adoxophyes orana* granulovirus genome. Virology **311:**350-365.

Yanase, T., C. Yasunaga, and T. Kawarabata. 1998. Replication of *Spodoptera exigua* nucleopolyhedrovirus in permissive and non-permissive lepidopteran cell lines. Acta Virol **42:**293-298.

Yao, Q., and R. W. Compans. 1996. Peptides corresponding to the heptad repeat sequence of human parainfluenza virus fusion protein are potent inhibitors of virus infection. Virology **223:**103-112.

Young, J. K., D. Li, M. C. Abramowitz, and T. G. Morrison. 1999. Interaction of peptides with sequences from the Newcastle disease virus fusion protein heptad repeat regions. J Virol **73:**5945-5956.

Yu, M., E. Wang, Y. Liu, D. Cao, N. Jin, C. W. Zhang, M. Bartlam, Z. Rao, P. Tien, and G. F. Gao. 2002. Six-helix bundle assembly and characterization of heptad repeat regions from the F protein of Newcastle disease virus. J Gen Virol **83:**623-629.

Zanotto, P. M., B. D. Kessing, and J. E. Maruniak. 1993. Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. J Invertebr Pathol **62:**147-164.

Zhang, L., and H. P. Ghosh. 1994. Characterization of the putative fusogenic domain in Vesicular stomatitis virus glycoprotein G. J Virol **68:**2186-2193.

Zhang, P., B. Yang, Y. Pang, and D. M. Su. 2001. Cloning and Expression of SpltMNPV Sl136 Gene and Functions of the Expressed Product. Acta Biochim Biophys Sin **33:**531-536.

Zhang, S. X., Y. Han, and G. W. Blissard. 2003. Palmitoylation of the *Autographa californica* multicapsid nucleopolyhedrovirus envelope glycoprotein GP64: mapping, functional studies, and lipid rafts. J Virol **77:**6265-6273.

Zhao, H., B. Lindqvist, H. Garoff, C. H. von Bonsdorff, and P. Liljestrom. 1994. A tyrosine-based motif in the cytoplasmic domain of the alphavirus envelope protein is essential for budding. EMBO J **13:**4204-4211.

Zhao, X., M. Singh, V. N. Malashkevich, and P. S. Kim. 2000. Structural characterization of the human respiratory syncytial virus fusion protein core Structural characterization of the human respiratory syncytial virus fusion protein core. Proc Natl Acad Sci U S A **97:**14172-14177.

NAWOORD

Verstoken van Nederlandse kranten werd ik in Australië gelukkig toch op de hoogte gehouden van de Nederlandse arbeidsmarkt. Zo kreeg ik eind 1998 per e-mail een advertentie toegestuurd, waarin een AIO werd gevraagd voor het onderzoek naar de karakterisatie van de insectencel receptor voor baculovirussen. Dit leek mij wel een zeer interessant onderzoek en een sollicitatiebrief was dan ook snel geschreven. Nadat ik te horen had gekregen dat ik zou worden aangenomen, ben ik in juni 1999 met veel enthousiasme begonnen aan dit onderzoek. Als ik terug kijk na de afgelopen vier jaar moet ik concluderen dat het jaren zijn geweest met veel ‘ups’ maar ook regelmatig wat ‘downs’. Soms zat ik weken te ploeteren in het lab en wou echt niks lukken. Maar natuurlijk was het niet altijd kommer en kwel, want anders was dit proefschrift nooit ontstaan. Daarvoor wil ik speciaal Douwe bedanken. Hij slaagde er iedere keer weer in mij met zeer goede discussies te motiveren om met veel plezier het lab in te duiken. Maar ook Just en Rob hebben, doormiddel van hun goede ideeën voor het onderzoek, hier zeker een grote bijdrage in gehad. Mijn onderzoek kreeg al snel een zeer grote wending toen er een nieuw baculovirus fusie-eiwit werd ontdekt. Na het vullen van zo’n 1000 gele en 200 blauwe pipetpuntenbakjes ligt er dan ook geen proefschrift klaar over een cellulaire receptor voor baculovirussen, maar over een baculovirus eiwit dat aan die receptor bindt.

Zonder de hulp van een aantal personen had dit proefschrift er waarschijnlijk anders uitgezien en zijn een aantal bedankjes naar mijn idee wel op zijn plaats. Allereerst Wilfred voor het ophelderen van de genoomsequentie van SeMNPV, anders hadden we samen nooit een nieuw baculovirus fusie-eiwit ontdekt. Verder heb ik prettig mogen samenwerken met een tweetal chinezen. Iedere keer wanneer er een Chinese gastmedewerker op ons baculo-lab kwam werken was er weer verwarring over hun namen. Dus, Hualin en Long of misschien wel Wang en Gang, bedankt voor jullie samenwerking. Natuurlijk mag ik Magda, Angela en Els niet vergeten, want hun stonden altijd klaar om een flesje met HzAM-1, Hz2E5, Se301, SeUCR1, SeIZD2109, Sf21, Sf9 of $Sf9^{Op1D}$ cellen af te staan. Verder stonden Jan en Joop, waarvan de laatste helaas mijn promotie niet meer mee mocht maken, altijd klaar om me te helpen met het maken van EM opnames, wanneer ik weer eens op een andere manier getracht had SeMNPV “budded virussen” op te zuiveren. Mijn speciale dank gaat uit naar Garry en Oliver aan de overkant van de grote plas. Met hen heb ik dikwijls overleg gehad over de voortgang van het onderzoek, hetgeen heeft geresulteerd in wederzijdse en gezamenlijke publicaties.

Natuurlijk heb ik niet alleen maar zitten pipetteren. Zo heb ik met veel plezier vele studenten tijdens diverse practica in aanraking mogen laten komen met het fenomeen virus. Verder kijk ik met veel plezier terug naar het begeleiden van studenten tijdens hun afstudeervak. De komst van Juliette, Maurice, Frank, Fotios en Peter was ook een goede motivatie om mijn labtafel weer eens op te ruimen, daar mijn pipetten, chemicaliën en proeven zich dikwijls gestaagd over drie werkplekken uitspreiden.

Ik heb veel tijd doorgebracht op mijn “kamer”. Daar werden de meeste ideeën uitgewerkt en de artikelen geschreven. Ik heb veel AIO’s zien verhuizen naar een andere kamer. Daarentegen heb ik vier jaar op dezelfde kamer mogen vertoeven waar iedere keer weer een frisse wind door heen blies bij het verkrijgen van een nieuwe kamergenoot. Wilfred, Mariëlle, Sehaam, Xinwen, Juliette, Gorben en Salva bedankt voor jullie gezelschap en discussies. Gorben wil ik nog even in het bijzonder bedanken. Ons onderzoek leek in eerste instantie weinig met elkaar te maken, maar gaande weg

konden we steeds meer van elkaars onderzoek profiteren. Onder het genot van vele disco classics werden dan ook nieuwe ideeën geboren, die niet altijd met wetenschap te maken hadden. De discussies over “keiharde radijspulp” en het idee om 13 februari tot nationale feestdag (inmiddels internationale feestdag) te promoten, zal ik niet snel vergeten. In oktober 2003 ben ik dan toch nog verhuisd naar een andere kamer en ben ik begonnen als postdoc op het whispo-lab met twee AIO’s aan mijn zijde. Jeroen en Hendrik ik vindt het dan ook erg leuk dat jullie mij ook tijdens mijn promotie ter zijde willen staan.

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CURRICULUM VITAE

Marcel Westenberg werd geboren op 15 april 1974 te Rhenen. Hij behaalde zijn MAVO diploma aan de Whilhelmina MAVO te Rhenen in 1990, en achtereenvolgens zijn HAVO en VWO diploma in 1992 en 1994 aan het Christelijke lyceum Veenendaal (CLV) te Veenendaal. In datzelfde jaar werd aangevangen met de studie Bioprocestechnologie, met als specialisatie dierlijke celtechnologie, aan de Landbouwuniversiteit Wageningen, alwaar hij in juli 1999 met lof afstudeerde. Tijdens de doctoraalfase heeft hij twee afstudeeronderzoeken verricht in de virologie. De eerste aan de Landbouwuniversiteit Wageningen, waar hij de meest voorkomende virale eiwitten van het white spot syndrome virus heeft geïdentificeerd onder begeleiding van prof. dr. R.W. Goldbach en prof. dr. J.M. Vlak en de tweede aan het CSIRO - Australian Animal Health Laboratory in Geelong, waar hij het nieuw ontdekte Menangle virus heeft gekarakteriseerd, onderbegeleiding van dr. D. Boyle en dr. B.T. Eaton. Van juni 1999 tot en met september 2003 was hij werkzaam als assistent in opleiding (AIO) bij het laboratorium voor virologie aan de Wageningen Universiteit onder begeleiding van prof. dr. J.M. Vlak, prof. dr. R.W. Goldbach, en dr. D. Zuidema. Het onderzoek dat in deze periode werd uitgevoerd is in dit proefschrift weergegeven. In oktober 2003 begon hij aan een postdoctorale studie aan het white spot syndrome virus, eveneens in het laboratorium van virologie aan de Wageningen Universiteit onder begeleiding van prof. dr. J.M. Vlak en dr. D. Zuidema.

ACCOUNT

Bowden, T. R., M. Westenberg, L. F. Wang, B. T. Eaton, and D. B. Boyle. 2001. Molecular characterization of Menangle virus, a novel paramyxovirus which infects pigs, fruit bats, and humans. *Virology* **283:**358-373.

IJkel, W. F. J., M. Westenberg, R. W. Goldbach, G. W. Blissard, J. M. Vlak, and D. Zuidema. 2000. A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. *Virology* **275:**30-41.

Lung, O., M. Westenberg, J. M. Vlak, D. Zuidema, and G. W. Blissard. 2002. Pseudotyping *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from group II NPVs are functionally analogous to AcMNPV GP64. *J Virol* **76:**5729-5736.

van Hulten, M. C., M. Westenberg, S. D. Goodall, and J. M. Vlak. 2000. Identification of two major virion protein genes of white spot syndrome virus of shrimp. *Virology* **266:**227-236

Westenberg, M, F. Veenman, E. C. Roode, R. W. Goldbach, J. M. Vlak and D. Zuidema. Functional analysis of the putative fusion domain of the baculovirus envelope fusion protein F. *Submitted for publication.*

Westenberg, M., H. Wang, W. F. IJkel, R. W. Goldbach, J. M. Vlak, and D. Zuidema. 2002. Furin is involved in baculovirus envelope fusion protein activation. *J Virol* **76:**178-184.