Structure-Function Relationship of the Baculovirus Envelope Fusion Protein F

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Dit onderzoek is uitgevoerd binnen de C.T. de Wit onderzoekschool 'Production Ecology and Resource Conservation'.

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. M. J. Kropff
in het openbaar te verdedigen
op maandag 19 maart 2007
des namiddags te half twee in de Aula

The study presented in this thesis was carried out at the Laboratory of Virology, Wageningen University, The Netherlands, in co-operation with the Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, P.R. China.

Long, G. (2007)

Structure-function relationship of the baculovirus envelope fusion protein F
Thesis Wageningen University – with references – with summary in Dutch
ISBN 90-8504-639-4
Subject headings: baculovirus, envelope fusion protein, mutational analysis, HearNPV, cell entry

To Xiaoyu and my parents.

Abbreviations

	General
Ac23	Translation product of the AcMNPV ORF23 (F homolog)
Acgp64	AcMNPV major envelope glycoprotein
BV	Budded virus
C3 fucos	α -1,3-linked core fucose
CTD	Cytoplasmic tail domain
CNX/CRT	Calnexin/Calreticulin
EFP	Envelope fusion protein
EGS	Ethylene Glycolbis-succinimidylsuccinate
ER	Endoplasmic reticulum
F	Group II NPV envelope fusion protein
F_1	C-terminal cleavage fragment of F
F_2	N-terminal cleavage fragment of F
FP	Fusion peptide
GV	Granulovirus
HA	Influenza virus hemagglutinin
HaF	HearNPV F protein (Ha133)
HPLC	High performance liquid chromatography
HR	Heptad repeat
Ld130	LdMNPV F protein
LTR	Long terminal repeat
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
m.o.i.	Multiplicity of infection
MNPV	Multiple Nucleopolyhedrovirus
MS	Mass spectrometry
NC	Nucleocapsid
NPV	Nucleopolyhedrovirus
nt	Nucleotide
OB	Occlusion body
ODV	Occlusion derived virus
Op21	Translational product of the OpMNPV ORF21 (F homolog)
ORF	Open reading frame
PDI	Protein-disulfide isomerase
PIF	*per os* infectivity factor
Se8	SeMNPV F protein
SNPV	Single Nucleopolyhedrovirus
SP	Signal peptide
$TCID_{50}$	Tissue culture infective dose of 50%
TM	Transmembrane domain

	Viruses
AcMNPV	*Autographa californica* MNPV
AdhoNPV	*Adoxophyes honmai* NPV
AdorGV	*Adoxophyes orana* GV
AgseNPV	*Agrotis segetum* NPV
AgseGV	*Agrotis segetum* GV
AngeMNPV	*Anticarsia gemmatalis* NPV
BmNPV	*Bombyx mori* NPV
CfMNPV	*Choristoneura fumiferana* MNPV
ChchNPV	*Chrysodeixis chalcites* NPV
ChocGV	*Choristoneura occidentalis* GV
CpGV	*Cydia pomonella* GV
CrleGV	*Cryptophlebia leucotreta* GV
Cuninpv	*Culex nigripalpus* NPV
EcobNPV	*Ectopis obliqua* NPV
EppoNPV	*Epiphyas postvittana* NPV
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HearNPV	*Helicoverpa amigera* NPV
HIV	Human immunodeficiency virus
HycuNPV	*Hyphantria cunea* NPV
HzSNPV	*Helicoverpa zea* SNPV
LdMNPV	*Lymantria dispar* MNPV
MacoNPV	*Mamestra configurata* NPV
MHV	Mouse hepatitis virus
NeleNPV	*Neodiprion lecontei* NPV
NeseNPV	*Neodiprion sertifer* NPV
OpMNPV	*Orgyia pseudotsugata* MNPV
PhopGV	*Phthorimaea operculella* GV
PlxyGV	*Plutella xylostella* GV
PlxyNPV	*Plutella xylostella* NPV
RoMNPV	*Rachiplusia ou* MNPV
RSV	Rous sarcoma virus
SARS-CoV	Severe acute respiratory syndrome associated coronavirus
SeMNPV	*Spodoptera exigua* MNPV
SpltMNPV	*Spodoptera litura* MNPV
SV5	Simian virus 5
TBEV	Tick-borne encephalitis virus
TnSNPV	*Trichoplusia ni* SNPV
VSV	Vesicular stomatitis virus
XecnGV	*Xestia c-nigrum* GV

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Chapter 1

General Introduction

Chapter 1

General Introduction

Initial interactions between viruses and host are very important for the outcome of infection. Strategies to prevent these interactions form the basis for interventions, such as the development of vaccines and antivirals. The first interaction of a virus with a target cell is through the attachment of the virus with receptors on cellular membranes followed by uptake and replication. Structures on the surface of animal viruses are important in this interaction. The enveloped viruses usually have glycoproteins on the surface of the virion and these are directly involved in the initial interactions with host cells. The major envelope glycoprotein of baculoviruses is a Class I fusion protein and this is a unique feature for DNA viruses, as only RNA viruses have such a protein on the virion envelope. Most enveloped DNA viruses (herpesvirus, poxvirus) have other types of glycoproteins and enter cells by a different mode. This thesis is devoted to the baculoviruses class I fusion protein F and the role of the various domains of this protein in the infection process, such as entry into insect cells and assembly into virions.

INTRODUCTION TO THE BACULOVIRUSES

Taxonomy and structure

Baculoviruses often cause a fatal disease and initiate an epidemic. The baculoviruses are a family (*Baculoviridae*) of large, enveloped viruses, with circular covalently closed double-stranded DNA genomes ranging in size from 80 to 180 kilobase pairs (kbp) (Theilmann *et al.*, 2005). The virions are occluded into large proteinacious bodies, so called occlusion bodies (OBs). They infect the larval stages of arthropods, mainly insects of the orders Lepidoptera, Hymenoptera and Diptera (Federici, 1999). Baculoviruses have been identified in over 800 different insect species so far. Most baculoviruses have limited host ranges and infect one or a few closely related insect species from a single order, even restricted to a single insect host (Adams and McClintock 1991, Federici, 1997). Based upon the distinct morphology of OB, the *Baculoviridae* can be taxonomically divided into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Theilmann *et al.*, 2005) (Fig. 1). NPVs produce polyhedron-shaped proteinacious OBs, named polyhedra that contain multiple virions within each OB. GVs are characterized by smaller ovicylindrical-shaped OBs, named granules that contain only a single virion. NPVs have two morphotypes, SNPV and MNPV, depending on the single (S) or multiple (M) encapsulation of the nucleocapsid in the envelope of a virion which is embedded in OBs. According to phylogenetic analysis of several conserved genes (the polyhedrin/granulin, late essential factor-2 [*lef-2*] and DNA polymerase [*pol*]) and intact genomes, the NPVs can be further subdivided into group I and group II NPV (Zanotto *et al.*, 1993, Bulach *et al.*, 1999, Herniou *et al.*, 2001). To date 35 baculovirus genomes have been sequenced in their entirety (Table 1).

Baculovirus virions appear in two distinct forms (Fig. 1): the occlusion derived virions (ODV) or the non-occluded virions form assembled at the cell surface named budded virus (BV). While both ODVs and BVs contain NCs with similar morphology, they differ in the lipid and protein composition of their envelope. In the infection cycle these two phenotypes play distinct roles. ODVs are infectious only to midgut columnar epithelial cells and are responsible for the initiation of the horizontal transmission of baculoviruses from insect to inset. BVs are highly infectious for insect cells and are responsible for the transmission of virus from cell to cell *in vivo* (in the insect) and in cell culture.

Table 1 Baculoviruses which complete genome sequences have been determined.

Group	Species	Abbreviation	Reference
I NPVs	*Autographa californica* MNPV	AcMNPV	Ayres *et al*., 1994
	Anticarsia gemmatalis MNPV	AngeMNPV	de Castro Oliveira *et al*.,2006
	Bombyx mori NPV	BmNPV	Gomi *et al*., 1999
	Choristoneura fumiferana MNPV	CfMNPV	de Jong *et al*., 2005
	Choristoneura fumiferana MNPV(D)	CfDefNPV	Lauzon *et al*., 2005
	Epiphyas postvittana NPV	EppoNPV	Hyink *et al*., 2002
	Hyphantria cunea NPV	HycuNPV	Ikeda *et al*., 2006
	Orgyia pseudotsugata MNPV	OpMNPV	Ahrens *et al*., 1997
	Plutella xylostella MNPV	PlxyMNPV	NC_008349
	Rachiplusia ou MNPV	RoMNPV	Harrison *et al*., 2003
II NPVs	*Adoxophyes honmai* NPV	AdhoNPV	Nakai *et al*., 2003
	Agrotis segetum NPV	AgseNPV	Jakubowska *et al*., 2006
	Chrysodeixis chalcites NPV	ChchNPV	van Oers *et al*., 2005
	Clanis bilineata NPV	ClbiNPV	NC_8293
	Ecotropis obliqua NPV	EoNPV	Ma *et al*., 2006
	Helicoverpa amigera NPV	HearNPV	Chen *et al*., 2001
	Helicoverpa zea SNPV	HzSNPV	Chen *et al*., 2002
	Mamestra configurata NPV A	MacoNPV A	Li *et al*., 2002a
	Mamestra configurata NPV B	MacoNPV B	Li *et al*., 2002b
	Lymantria dispar MNPV	LdMNPV	Kuzio *et al*., 1999
	Leucania separata NPV	LeseNPV	AY_394490
	Spodoptera exigua MNPV	SeMNPV	IJkel *et al*., 1999
	Spodoptera litura MNPV	SpltNPV	Pang *et al*., 2001
	Trichoplusia ni SNPV	TnSNPV	Willis *et al*., 2005
GVs	*Adoxophyes orana* GV	AdorGV	Wormleaton *et al*., 2003
	Agrotis segetum GV	AgseGV	NC_005839
	Choristoneura occidentalis GV	ChocGV	Escasa *et al.,* 2006
	Cryptophlebia leucotreta GV	CrleGV	Lange *et al*., 2003
	Cydia pomonella GV	CpGV	Luque *et al*., 2001
	Plutella xylostella GV	PlxyGV	Hashimoto *et al*., 2000
	Phthorimaea operculella GV	PhopGV	NC_004062
	Xestia C-nigrum GV	XecnGV	Hayakawa *et al*., 1999
Dipteran NPVs	*Culex nigripalpus* NPV	CuniNPV	Afonso *et al*., 2001
Hymenopteran NPVs	*Neodiprion lecontei* NPV	NeleNPV	Lauzon *et al*., 2004
	Neodiprion sertifer NPV	NeseNPV	Garcia-Maruniak *et al*., 2004

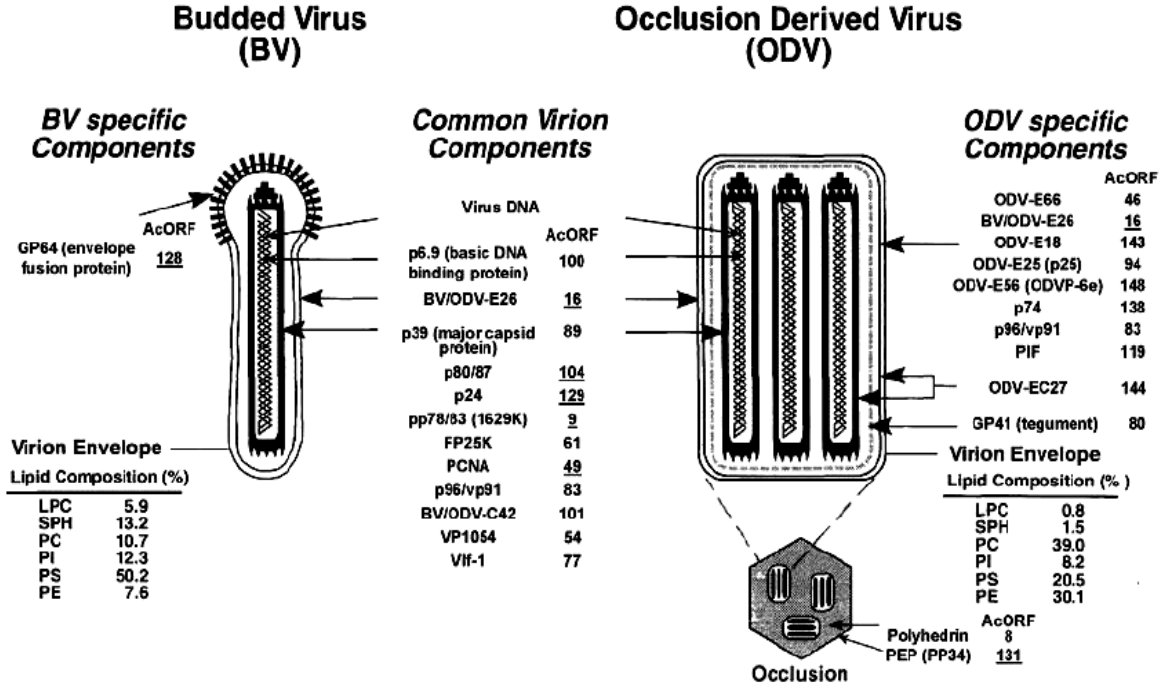

Figure 1. Schematic composition of the budded virus (BV) and occlusion derived virus (ODV) (adapted from Blissard, 1996 and Braunagel *et al.*, 2003). In this figure the ODV structure represent the multiple-capsid nucleopolyhedrovirus (MNPV). BV and ODV specific proteins are indicated at the left and the right, respectively. Proteins common for both phenotypes are indicated in the center. The corresponding AcMNPV open reading frame (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 is derived from isolated virions of AcMNPV-infected Sf9 cells (Braunagel and Summers, 1994). LPC (lysophoshatidylcholine), SPH (sphingomyelin), PC (phosphatidylcholine), PI (phosphatidylinositol), PS (phosphatidylserine), PE (phosphatidylethanolamine).

Infection cycle

In the environment OBs are commonly found on plant surfaces and in the soil and are taken up by insect larvae. Upon ingestion the OBs dissolve in the alkaline environment of the larval midgut, releasing the ODVs. After direct membrane fusion of the ODV envelope with the microvilli of the midgut epithelial cells, the virions are transported in a cytoskeleton-dependent transport manner and through the nuclear pore enter the nucleus, where gene expression, DNA replication and assembly of progeny NCs take place. In the early phase of infection, newly formed NCs are transported back into the cell membrane where the BV assembles and the budding through the basal membrane occurs. The BVs subsequently spread the infection to neighboring cells *in vivo*, causing systematic infection. In the late phase of infection, the NCs are assembled in a *de novo* formed envelope to give ODVs. The ODVs are subsequently occluded in a matrix of the polyhedron protein leading to the formation of OBs. At the end of the infection the OBs are released upon cell rupture and insect liquefaction and they are able to initiate a new round of infection.

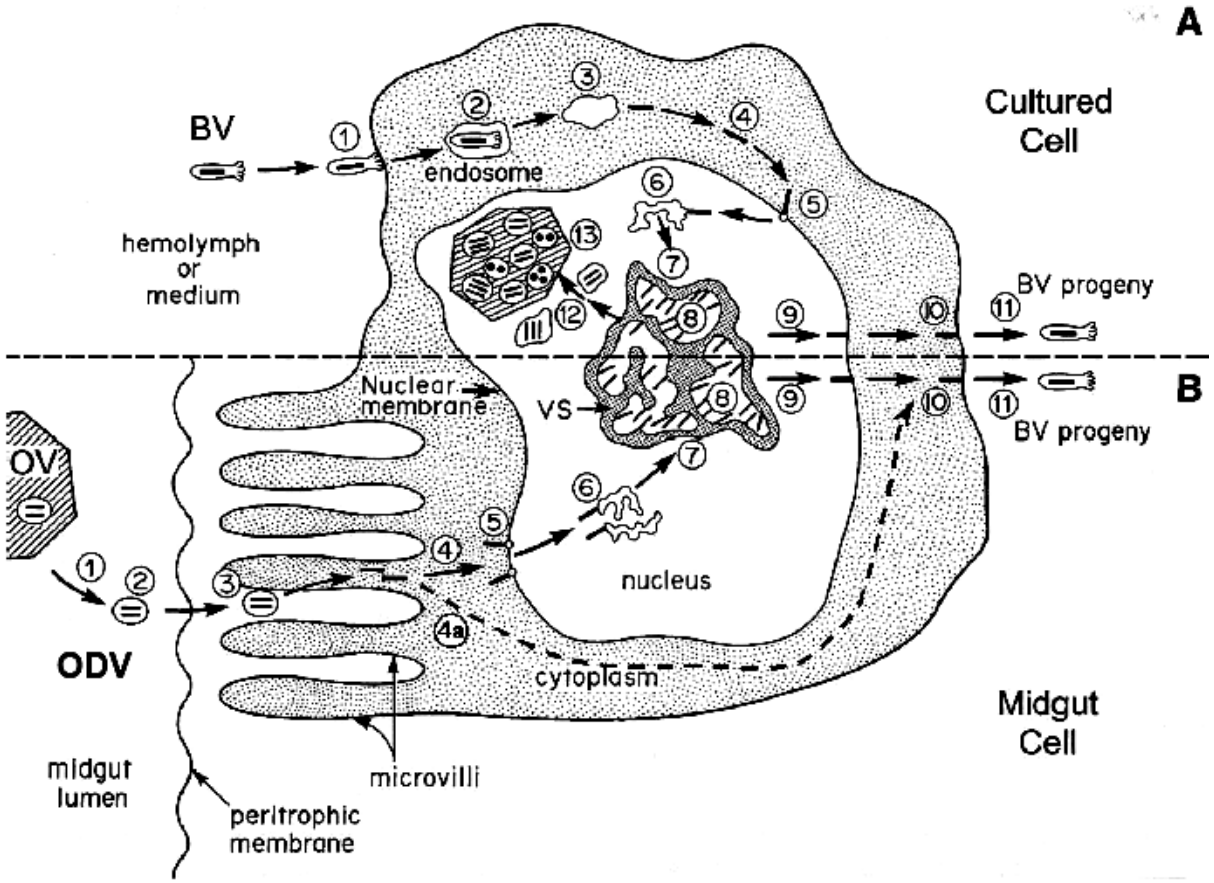

Figure 2. The baculovirus infection cycle (adapted from Miller, 1996 and Westenberg, 2004). (A) In the first step the budded virus (BV) attaches to the cell surface of cultured cells in vitro or cells other than midgut epithelial cells in vivo (1), and enters by absorptive endocytosis (2). The endosomal and viral membrane fuse upon acidification of the late endosome, releasing the nucleocapsid (NC) into cytoplasm (3). The NC was transported to the nucleus in a cytoskeleton dependent manner (4), and enters through 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 virogenic stroma (VS). During the late phase of infection the newly formed NCs leave the nucleus (9) and travel to the plasma membrane (10) where the virus budding occurs (11). During the very late phase, the NCs are enveloped (12) and embedded in a polyhedrin protein matrix to form OBs (13). (B) Infection of the midgut epithelial cells starts with the uptake of OBs (1), which dissolve upon the alkaline environment of the midgut lumen, to liberate 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 cytoplasm through direct fusion (4). The remaining events are similar to that of the BV infection described above, except that little OBs are formed in the columnar cells of the midgut epithelium. Further it is thought that a part of the incoming NCs may bypass the nucleus and transfer directly to the basolateral membrane to bud from the cell (4a).

The BVs are responsible for the spread of the virus beyond the initially infected midgut epithelial cells. After binding by the major envelope fusion protein to a yet enigmatic cell surface receptor, BVs enter tissue cells or cultured cells through a process called absorptive endocytosis (Volkman and Goldsmith, 1985; Blissard and Wenz, 1992; Hefferon *et al.*, 1999). Acidification of the late endosome triggers the low-pH dependent fusion of the viral envelope and endosomal membrane (Blissard and Wenz, 1992; Pearson *et al.*, 2000). Then the NCs are

released and transported into the nucleus to start viral transcription and DNA replication. Progeny nucleocapsids then travel from the nucleus to the cell membrane, where they acquire an envelope prior to budding into the extracellular space to initiate a new round of virus replication. The role of the baculovirus major envelope fusion protein in virus entry and virus egress is the major theme of this thesis.

BACULOVIRUS IMPORTANCE

Baculovirus as bioinsecticide

Baculoviruses are best known as biological pest control agents in agriculture, forestry and horticulture. They have several natural advantages as biological pesticides, they are naturally occurring pathogens that are highly specific to insects and closely related arthropods. Baculoviruses are also safe in terms of harm against vertebrates. Baculovirus infection causes cessation of feeding and has the potential to influence host population dynamics. Baculoviruses have been used against insect pests in forests since the 1930s (Bird and Burk, 1961). Many baculoviruses have been registered as biological pesticides and have been successfully used as safe and effective bio-insecticides world wide (Black *et al.*, 1997; Hunter-Fujita *et al.*, 1998; Moscardi, 1999; Vail *et al.*, 1999; Copping and Menn, 2000; Lacey *et al.*, 2001). However, the sometimes low infectivity and generally slow killing speed limit the further advance of practical applications of baculoviruses. Therefore, biotechnological approaches, including the expression of insect hormones and heterologous toxins by genetic modification of baculoviruses (see Inceoglu *et al.*, 2006, for review) have been successfully used to increase the insecticidal properties of baculoviruses.

Baculovirus as expression vector

There is a major need for high quality proteins for pharmaceutical applications, such as therapeutics and vaccines in veterinary and human medicine. These proteins often need to be near authentic, both biochemically (post-translational modifications) as well as conformationally, in order to be functional. Therefore higher eukaryotes such as mammals as biosource are preferred (e.g. vaccine production). Also high amounts of good quality proteins are required and need to be isolated from biological material. Biotechnological production process using animal cells may help to produce these proteins and the baculovirus-insect cell expression system is one of those (Yin *et al.*, 2007). In baculovirus-infected insects / insect cells polyhedrin is made in copious amounts and replacement of this gene by a foreign gene often results in the high level expression of recombinant proteins (Summers, 2006). Proteins expressed in insect cells not only provide high amounts of recombinant protein, but also in the correct conformation and with the proper posttranslational modifications (Van Oers, 2006). However, the glycosylation in insect cells is different from mammalian cells in that complex glycosylation is not carried out in insect-derived biosystems (Jarvis, 2003; Fig. 3) and this may influence the biological properties of the pertinent glycoproteins when produced in insect cells. The baculovirus-insect cell expression system is now widely available through commerce and has become a mainstream system to produce recombinant proteins. The first commercial veterinary vaccine, against hog cholera virus (Intervet International) dates from 2003 and the first human vaccine, against cervical carcinoma caused by human papilloma virus 16 and 18 (Cervarix, GlaxoSmithKline), will be on sale in 2007.

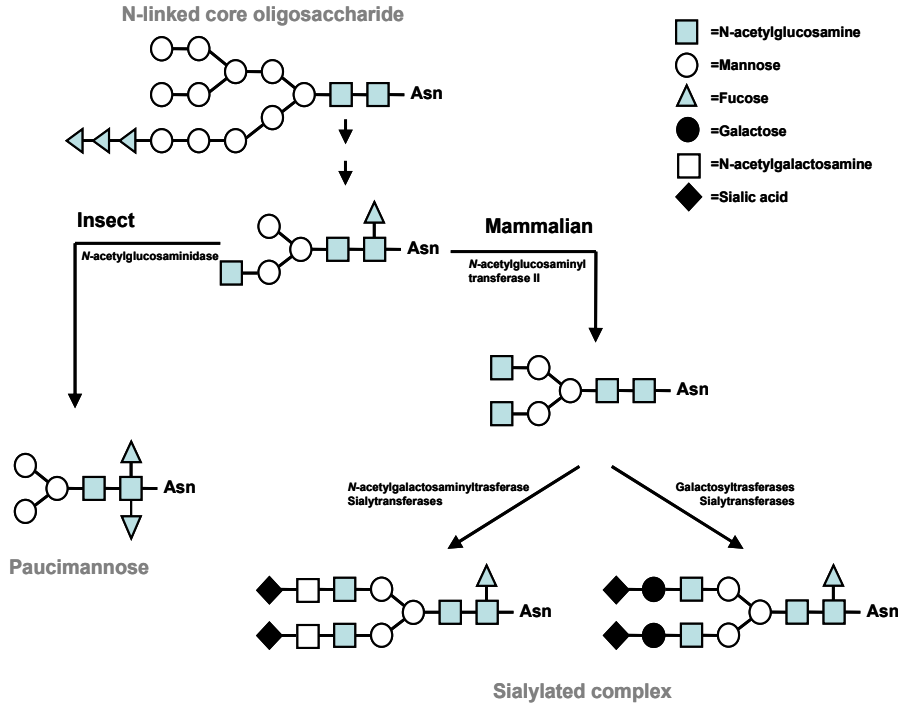

Figure 3. Overview of N-linked glycosylation pathway in lepidopteran insect cells and mammalian cells. The processing pathways in both insect and mammalian cells produce a common intermediate N-linked glycan, GlcNAcMan3GlcNAc2C6Fuc. The major insect cell end product, paucimannose with core fucose (Man3GlcNAc2C3/C6Fuc), is produced by further trimming of the intermediate, while the major mammalian-cell end products, sialylated complex, are produced by elongation of the common intermediate (adapted from Jarvis, 2003).

Glycobiology studies indicate that insect cell lines derived from dipterans and lepidopterans (Hsieh and Robbins, 1984; Parker *et al.*, 1991; Marchal *et al.*, 1999) could produce similar lipid linked oligosaccharides as the N-linked glycan precursor (Glc3Man9GlcNAc2, where Glc is glucose, Man is mannose, GlcNAc is N-acetylglucosamine, and the subscripts refer to the numbers of each residue in the oligosaccharide) from mammalian cells (Fig. 3). These oligosaccharides are transferred to nascent polypeptides at the asparagine (Asn) residue in the consensus sequence Asn-X-Ser/Thr (where X is any amino acid other than proline) (Burda and Aebi, 1999). This glycan core structure is then trimmed predominantly to oligomannose or paucimannose N-linked glycans on native glycoproteins produced in insect cells (Williams *et al.*, 1991; Kubelka *et al.*, 1994; Park *et al.*, 1999; Fabini *et al.*, 2001; Kim *et al.*, 2003). Compared to the N-linked glycosylation pathway in mammalian cells, insect cells do not possess the capability to elongate the trimmed N-linked glycans to produce complex N-linked glycans with terminal galactose and/or sialic acid residues (Harrison and Jarvis 2006; Jarvis 2003). Thus the eukaryotic proteins produced in baculovirus infected insect cells are modified mostly with oligomannose or paucimannose N-linked glycans containing no galactose or sialic acids (Kuroda *et al.*, 1990; Lopez *et al.*, 1997; Takahashi *et al.*, 1999; Hollister *et al.*, 2002; Ding *et al.*, 2003; Wendeler *et al.*, 2003). In many different ways terminal sialic acids play a critical role for glycoproteins (Traving and Schauer, 1998). Therefore glycoproteins produced by the baculovirus-insect cell expression system are not always optimal for clinical applications. Recent efforts were invested to "humanize" the baculovirus-insect cell expression system by

introducing mammalian genes encoding N-linked glycosylation activities (N-acetylglucosaminyl transferase II, N-acetylgalactosaminyl transferase, galactosyl transferase and sialyl transferase, Fig. 3) that are absent in the parental insect cell lines. The "humanized" cells produce a complex type of N-linked glycans with terminal sialic acid residues (Jarvis *et al.*, 2001; Aumiller *et al.*, 2003). The presence of core α-1,3-linked (C3) fucose (see Fig. 3) is another potential problem for therapeutic usage of insect cell derived glycoproteins, because the C3 fucose is a potentially allergenic epitope on N-linked glycans in mammalian species (Prenner *et al.*, 1992; Tretter *et al.*, 1993; Wilson *et al.*, 1998). Selection of suitable cell lines and genetic modification can be used to overcome this impediment (Harrison and Jarvis, 2006).

Baculovirus as gene delivery vector

In the mid 1990s it was reported that recombinant baculovirus carrying a cytomegalovirus promoter-controlled luciferase gene cassette (Hofmann *et al.*, 1995) or a *Rous sarcoma virus* (RSV) long terminal repeat (LTR) promoter-controlled *β*-galactosidase gene cassette (Boyce and Bucher, 1996) efficiently expressed the reporter genes in primary human hepatocytes and a variety of non-hepatic human cell lines. These pioneering findings paved the way for use of baculoviruses as gene delivery vectors for mammalian cells. Since then the list of cells permissive to recombinant baculovirus transduction has rapidly expanded (Hu, 2006). As a gene therapy vector baculovirus possess many advantages. Baculoviruses do not replicate in transduced mammalian cells (Hofmann *et al.*, 1995; Kost and Condreay, 2002) and possess the capacity for accommodating large DNA inserts. Adenovirus genome sequences (35kbp) for example were successfully inserted into baculovirus vector to produce recombinant and defective adenovirus vectors (Cheshenko *et al.*, 2001; Sollerbrant *et al.*, 2001). Baculovirus transduction is nontoxic to mammalian cells and does not impair cell growth even at a very high multiplicity of infection (Hofmann, *et al.*, 1995; Gao *et al.*, 2002; Ho *et al.*, 2004). The research of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) was impeded by the lack of permissive cell culture systems until the recent advances in subgenomic replicon system (Lohmann *et al.*, 1999; Blight *et al.*, 2000). This impediment was successfully overcome using recombinant baculovirus vectors delivering genomes of HBV and HCV into hepatoma cell lines (Delaney and Isom, 1998; Fipaldini *et al.*, 1999). RNA interference (Fire *et al.*, 1998) has become a well accepted means to study gene functions by knock down the expression of interested gene products. A recent report using engineered baculovirus expressing hairpin RNA from the U6 promoter to reduce expression of lamin A/C RNA and protein in multiple human cell lines (Nicholson *et al.*, 2005) demonstrated the capability for baculovirus to effectively deliver small interfering RNAs.

The baculoviruses can accommodate large insertions of foreign DNA and can cause little observable cytopathic effect in mammalian cells, which makes these viruses attractive vectors for *in vivo* applications. However, *in vivo* application of baculoviruses in humans is more challenging as the viruses can be rapidly inactivated by serum complement (Hofmann and Strauss, 1998; Hoare *et al.*, 2005). Research efforts have been invested to cope with this obstacle. It has been reported that a soluble form of complement receptor type 1 (sCR1) (Hofmann *et al.*, 1999) and a surface-displayed decay-accelerating factor (DAF) (Huser *et al.*, 2001) would protect baculovirus from inactivation *in vivo*. In this regard it has been shown that baculoviruses pseudotyped with Vesicular Stomatitis Virus (VSV)-G protein are more resistant to inactivation (Barsoum *et al.*, 1997; Tani *et al.*, 2003).

With a good bio-safety profile from its inability to replicate in mammalian cells, baculovirus still contains risks for gene therapy applications. Baculovirus sequences were found to integrate into the genome of cultured mammalian cells in an illegitimate mode (Merrihew *et al.*, 2001), thus baculovirus gene delivery could be potentially dangerous because of the possible integration at an undesired position in the chromosome. Some studies demonstrated that viral delivery resulted in signs of inflammation, possibly because of the induction of inflammatory cytokines produced in response to the virus (Abe *et al.*, 2003). Thus defining cytotoxic and immunogenic effects of baculovirus is very important for future applications of baculovirus as gene delivery vectors for therapeutic purpose.

VIRUS ENTRY AND FUSION PROTEIN

Virus endocytosis

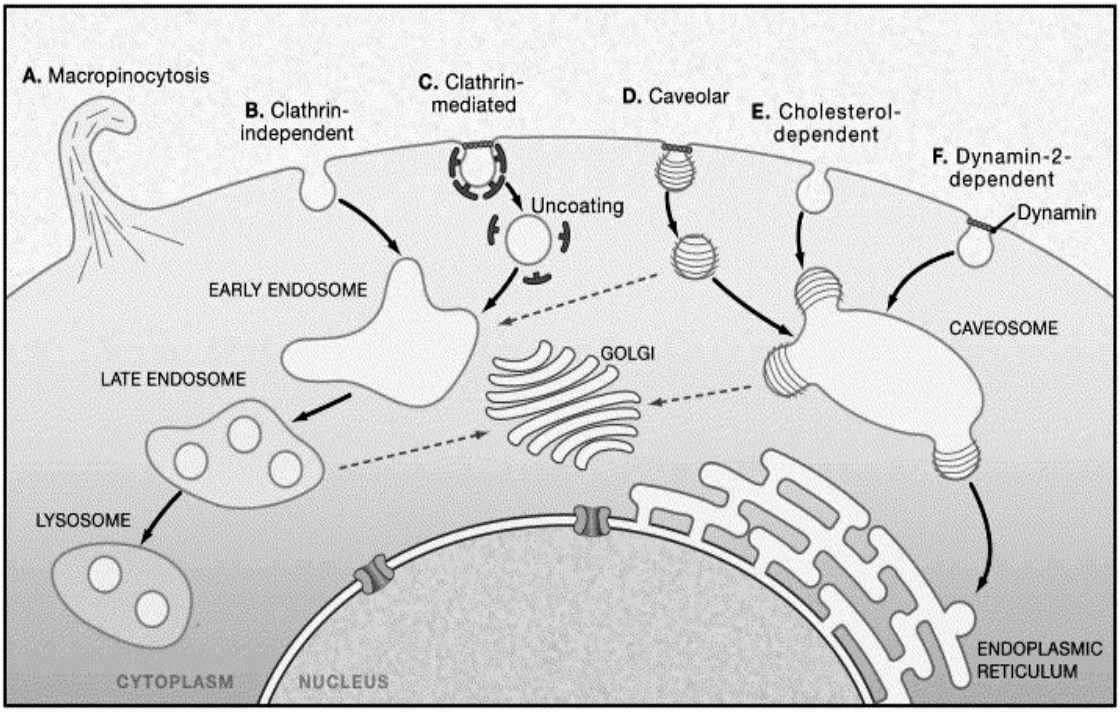

Figure 4. Endocytic Pathways Used by Viruses. In mammalian cells, many different mechanisms are available for the endocytic internalization of virus particles (adapted from Marsh and Helenius, 2006; with permission). Some of these mechanisms, such as clathrin-mediated endocytosis, are ongoing, whereas others, such as caveolae, are ligand and cargo induced. Currently, there is evidence for six pathways. (A) Macropinocytosis is involved in the entry of adenoviruses. (B) A clathrin-independent pathway from the plasma membrane has been shown to exist for influenza virus and arenaviruses. (C) The clathrin-mediated pathway is the most commonly observed uptake pathway for viruses. The viruses are transported via early endosomes to late endosomes and eventually to lysosomes. (D) The caveolar pathway is one of several closely related, cholesterol-dependent pathways that bring viruses including SV40, coxsackie B, mouse polyoma, and Echo 1 to caveosomes, from which many of them continue, by a second vesicle transport step, to the ER. (E) A cholesterol-dependent endocytic pathway devoid of clathrin and caveolin-1, used by polyomavirus and SV40. (F) A pathway similar to (D) except dependent on dynamin-2. It is used by Echo virus 1.

As obligatory intracellular parasites, viruses must enter target cells and deliver their genetic load to allow viral genome replication and progeny production. The first step of virus infection for the virus is to attach to specific receptors on the plasma membrane of target cells via viral surface molecules. This attachment is followed by penetration through membrane fusion between viral envelop and cellular membrane structures (Marsh and Helenius, 2006). Some enveloped viruses, e.g. HIV-1 (*Retroviridae*), SV5 (*Paramyxoviridae*) and HSV-1 (*Herpesviridae*), enter target cells by direct fusion between viral envelope and the plasma membrane at neutral pH. Many other enveloped viruses, e.g. influenza virus (*Orthomyxoviridae*), Ebola virus (*Filoviridae*), Dengue virus (*Flaviviridae*), and also baculoviruses (see below) enter cells via the endocytic pathway. Here the fusion process is activated by a low pH and occurs between viral envelope and the intracellular membrane (endosomal membrane or ER membrane).

Endocytosis appeared to be the primary route of entry into host cells for most members of virus families (Fig. 4). To date multiple endocytic pathways have been found for virus entry, e.g. macropinocytosis for adenoviruses, the clathrin-independent pathway for arenaviruses, the clathrin-mediated pathway for influenza virus, the caveolar pathway for SV40 and the dynamin-2-dependent pathway for Echo virus 1(Fig. 4) (Marsh and Helenius, 2006). The best characterized and most commonly used endocytic pathway is the clathrin-mediated pathway. After receptor binding, virus particles with their receptors are internalized into early and late endosomes. The endosolic low pH environment activates a low-pH dependent fusion process that leads to membrane penetration of the nucleocapsids. For Ebola virus and SARS coronavirus, proteolytic cleavages by acid-dependent endosomal proteases, e.g. cathepsins L and B, of the virus envelope fusion proteins are essential for triggering membrane fusion (Chandran *et al.*, 2005; Simmons *et al.*, 2005; Huang *et al.*, 2006; Schornberg *et al.*, 2006). This cleavage induces a conformational change of the protein leading to exposure of domains important for fusion e.g. the fusion peptide. Another well defined endocytic route used for virus entry, e.g. SV40, is the caveolar pathway (Fig. 4). This is dependent on the presence of cholesterol-rich microdomains (lipid rafts) and activation of the tyrosine-kinase signaling pathway (Pelkmans *et al.*, 2001; Smith *et al.*, 2003; Tsai *et al.*, 2002) The caveolar pathway takes the incoming viruses first to pH-neutral organelles in the cytoplasm named caveosomes, followed by transportation of virus containing vesicles to ER for virus penetration (Pelkmans *et al.*, 2001).

Classification of viral fusion proteins

Enveloped viruses penetrate cellular membranes by fusion mediated by specialized virus envelope fusion proteins. This envelope fusion process can occur either at the plasma membrane or at an intracellular location following virus uptake by endocytic routes (Marsh and Helenius, 2006). To date at least two classes of virus envelope fusion proteins have been defined based on functional and structural criteria (Heinz and Allison 2001; Lescar *et al.*, 2001) (Table 2).

Class I fusion proteins are generally synthesized as precursors that are cleaved by intracellular protease into two subunits that are associated through either a disulfide bond (e.g. influenza HA) or non-covalent interactions (e.g. HIV-1 Env). This proteolytic cleavage is critical for creating the metastable, prefusion conformation of fusion proteins (Chen *et al.*, 1999). Class I fusion proteins exist as relatively long trimeric spikes in both their metastable and post-fusion state. They project perpendicularly to the virus envelope. After receptor

binding and/or low-pH activation, a N-terminal conserved hydrophobic region referred as fusion peptide is exposed, which then inserts into the target membrane. This interaction is followed by a dramatic conformational change of the fusion protein from a metastable state to a highly stable rod-like structure with a central trimeric α-helical coiled coil (also known as 6 helix bundle), then promoting the fusion between virus envelope and cellular membrane (Colman and Lawrence, 2003).

Table 2. Class I vs. Class II membrane fusion proteins

Property	Class I (influenza HA)	Class II (SFV E1)
Type of integral membrane protein	Type I	Type I
Synthesized as	Inactive precursor	Inactive precursor
Exist on virion in	Metastable state	Metastable state
Orientation in virion to membrane	Perpendicular	Parallel
Converted to metastable state by	Proteolytic processing with fusion protein precursor	Proteolytic processing of an associated protein
Predominant secondary structure	α-helix	β-sheet
Heptad repeat	2 or more	No
Number of subunits in fusion protein	2	1
Activated to fusogenic form by	Low pH or receptor binding	Low pH
Conformational change during fusion	Metastable fusion protein trimer to stable fusion protein trimer	Metastable dimer to stable fusion protein trimer
Location of fusion peptide	N-terminal or internal	Internal
Structure of final fusogenic form	Trimers of hairpins(coiled coil)	Trimers of hairpins

The general structure of class II fusion proteins exhibit distinct characteristics from that of class I fusion protein. During biosynthesis class II fusion proteins, e.g. tick-borne encephalitis virus (TBE) E protein, form heterodimers with a second viral membrane glycoprotein, the precursor of the membrane protein (prM). Host cell protease cleaves prM during virus maturation, resulting in a metastable homodimer of the E protein (Allison *et al.*, 1995). This metastable E homodimer lies parallel to the viral membrane. After receptor binding and endocytosis, the endosomal low-pH environment activates a rearrangement of the E protein, allowing the internal fusion loop to expose and insert into the host cell membrane and to form an intermediate trimeric E protein. The membrane fusion is completed by the formation of a final post-fusion homotrimer of E protein (Kielian and Rey, 2005).

Baculovirus entry and fusion protein

Baculovirus entry starts from the ingestion of OBs by host larvae, followed by the dissolution of OBs and liberation of ODVs due to the alkaline pH in the midgut. After penetration of the protective peritrophic membrane bordering the midgut epithelium, ODVs attach to the apical microvilli of columnar cells in order to enter and initiate infection. Enzymatic treatment of columnar cells indicated that a protein receptor is involved in the attachment (Horton and Burand, 1993). To date the only proposed mode of ODV entry into midgut columnar cells is fusion of the ODV envelope with the microvillar membrane, which is clearly documented by a number of electron-microscopic studies (Tanada *et al.*, 1975; Adams and McClintock, 1991; Federici, 1997). An ODV-specific envelope protein P74, one of a few highly conserved proteins essential for *per os* infectivity (PIFs), is found to be an ODV attachment protein that seems to bind to the specific receptor on columnar cells within midgut and facilitates the subsequent fusion process (Kuzio *et al.*, 1989; Faulkner *et al.*, 1997). The entry of ODV provides an unusual opportunity to study receptor binding and membrane fusion in an extreme alkaline environment, as the available paradigm on viral entry and

membrane fusion requires a neutral or acidic environment. Besides the P74, two additional conserved *pif* genes, *pif-1*, *Spodoptera littoralis* NPV *orf*7 (homologous to AcMNPV *orf*119) (Kikhno *et al.*, 2002) and *pif-2*, *Spodeptera exigua* NPV *orf*35 (homologous to AcMNPV *orf*22) (Pijlman *et al.*, 2003), have been described. Deletion of any *pif* gene from the baculovirus blocks *per os* infection, but the BVs are still infectious when injected intrahemocoelically indicating *pifs* are dispensable for BV infectivity (Ohkawa, 1997; Pijlman *et al.*, 2003).

In contrast to ODVs, BVs enter target insect cells through a general mechanism called adsorptive endocytosis (Volkman and Goldsmith, 1985). This entry process is dependent on endosomal low-pH activation. Two types of BV envelope fusion protein were identified in baculoviruses. In group I NPVs (Table 1) a BV-specific envelope fusion protein GP64 is responsible for BV entry, for attachment (Hefferon *et al*., 1999) and for low-pH dependent fusion (Blissard and Wenz 1992). GP64 resides on the apical end of the BV as spikes consisting of phosphorylated, acetylated and N-linked glycosylated disulfide-bridged GP64 homotrimers (Goldstein and McIntosh, 1980; Volkman and Goldsmith, 1985; Oomens *et al.*, 1995). GP64 is also required for BV maturation (Monsma *et al.*, 1996; Oomens and Blissard, 1999).

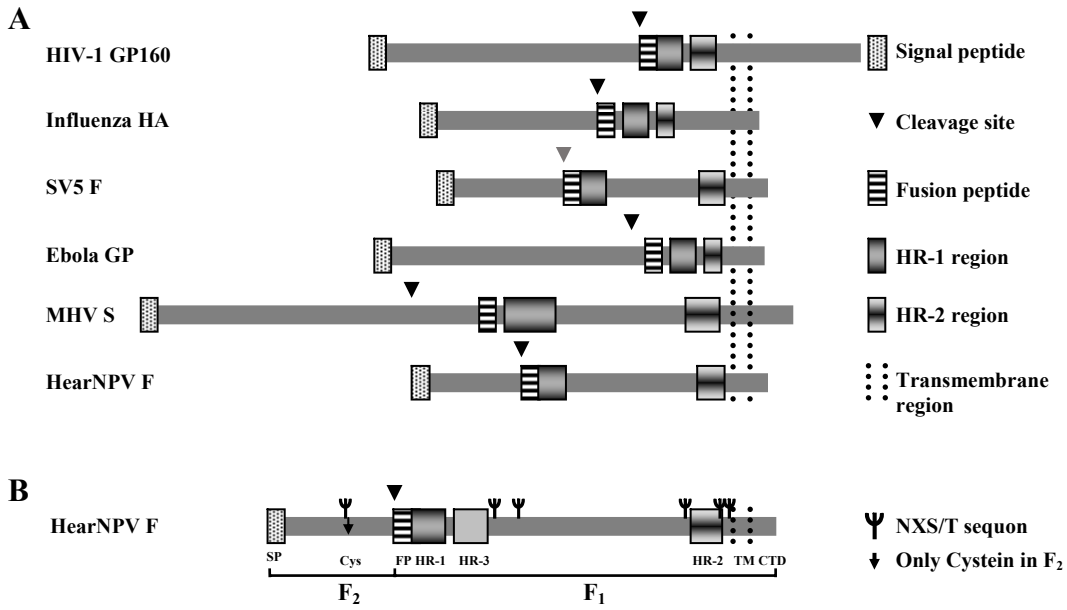

Figure 5. (A) Common architecture of class I virus membrane fusion proteins: HIV-1 GP160 (*Retroviridae*), Influenza HA (*Orthomyxoviridae*), SV5 F (*Paramyxoviridae*), Ebola GP (*Filoviridae*), MHV S (*Coronaviridae*) and HearNPV F (*Baculoviridae*). The relative positions of signal peptide (SP), proteolytic cleavage site, fusion peptide (FP), heptad repeat (HR-1, HR-2) and transmembrane region (TM) are indicated. (B) Schematic representation of structural organization of HearNPV F protein, showing functional elements.

In group II NPVs, a different type of envelope fusion proteins, named F, has been identified (IJkel *et al.*, 2000; Pearson *et al.*, 2000). Baculovirus F has two subunits F_1 and F_2 that are covalently linked by a disulfide bridge. F_2 is located N-terminal of F_1 (Fig. 5).

Genomics study revealed that genes for F proteins are also present in GV probably encoding the putative envelope fusion proteins from GVs. GP64 in BVs of AcMNPV can be substituted by the F proteins from group II NPVs, indicating that F proteins are functional analogs of GP64 (Lung *et al.*, 2002). Unlike the GP64 homologous proteins, baculovirus F proteins share the common architecture of class I viral membrane fusion proteins (Fig. 5; Table 2). Proteolytic cleavage by a furin-like proprotein convertase (protease), cleaving the F precursor F_0 into a small N-terminal fragment (F_2) and a large C-terminal fragment (F_1). This cleavage is essential for F fusogenicity (Westenberg *et al.*, 2002). An N-terminal fusion peptide was identified in LdMNPV and SeMNPV F protein (Pearson et al., 2002; Westenberg *et al.*, 2004). The fusion peptide is located at the N-terminus of the large subunit F_1 (Fig. 5). Multiple heptad repeats (HRs) were predicted in baculovirus F proteins, another hallmark of class I viral envelope fusion proteins. Baculovirus F proteins have a classical transmembrane region (TM) with hydrophobic amino acids and a C-terminal domain (CTD) of variable length among the various baculoviruses. Baculovirus F proteins contain multiple potential glycosylation sites (NXS/T) in both F_1 and F_2 subunits. Little is know about the other domains in the F protein.

OUTLINE OF THIS THESIS

Helicoverpa armigera single nucleopolyhedrovirus (HearNPV), a group II NPV, is a highly infectious and selective cotton bollworm pathogen that was isolated from deceased *H. armigera* larvae in Hubei province of China in 1980 (Zhang, 1989). HearNPV has been adopted for industrial production as a natural insecticide and has been widely applied to control the pest insects in China (Zhang *et al.*, 1995) and in other countries (Jones, 1994). Genomic and phylogenetic analysis showed that HearNPV is a group II NPV and HearNPV open reading frame *Ha133* was identified to encode a putative F protein (Chen *et al.*, 2001). In this thesis, with HearNPV F protein as the object of study, an attempt was made to answer a few of the unsolved fundamental questions related to the functioning of baculovirus F proteins. HearNPV is a classical group II baculovirus, can replicate permissively in cell culture and is amenable to mutational analysis using a bacmid system (Wang *et al.*, 2002). This makes it an appropriate system to study baculovirus F proteins.

An overview of baculoviruses, their infection process and applications is given in **Chapter 1**. To obtain more insight on F protein synthesis and processing, more specifically with regard to N-linked glycosylation and possible oligomerization status, a functional and biochemical analysis of HearNPV F was described in **Chapter 2**. In general, virus envelope proteins are heavily N-linked glycosylated. N-linked glycosylation plays an important role in the function of fusion proteins. Furthermore the N-linked glycans are critical for chaperon binding and proper folding in the endoplasmic reticulum (ER). In **Chapter 3** the importance of the only N-linked glycosylation site in HearNPV F protein F_2 subunit was investigated. It has been documented mainly from immuno- and electronic- microscopic evidence that baculovirus BV enters host cells through absorptive encytosis. The entry mode of baculovirus BVs into mammalian cells is largely unknown. Therefore in **Chapter 4** the functional entry pathway of baculovirus BV into insect and mammalian cells is investigated by using specific endocytosis inhibitors and various protein constructs to dissect the entry process. The cytoplasmic tail domain (CTD) of the virus envelope fusion protein is critical for virus maturation, as the CTD has potential to regulate the intracellular trafficking of the virus envelope fusion protein and budding process by interactions between CTD and nucleocapsid.

Unlike the GP64, baculovirus F proteins possess a much longer CTD. The CTD of GP64 was not essential for functionality (Oomens *et al.*, 1999). In **Chapter 5** the functional role of HearNPV CTD was investigated by two parallel rescue systems, the HearNPV F rescuing system and the AcMNPV pseudotyping system. Heptad repeats (HRs) are symbolic marks of class I fusion proteins. Multiple HRs were predicted in the HearNPV F protein. HR-1, immediately downstream of the putative fusion peptide, is the only one recognized by Learn-Coil VMF, a software specialized for the prediction of HRs in virus envelope fusion protein. To obtain more insight in the mechanism of baculovirus F protein mediated fusion and the role of HR, we investigated the impact of the HR1 on F protein's biosynthesis and function using site-directed mutagenesis in **Chapter 6**. In **Chapter 7** the results from this thesis research integrated with recent research advances in virus endocytosis, membrane fusion and virus egress are discussed in the light of baculovirus entry, biosynthesis and BV maturation.

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Chapter 2

Function, Oligomerization and N-linked Glycosylation of the *Helicoverpa armigera* SNPV Envelope Fusion Protein

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This chapter was published as J. Gen. Virol. 87:839-846 (2006).

SUMMARY

In the family *Baculoviridae* two distinct envelope fusion proteins are identified in budded virions (BV). GP64 is the major envelope fusion protein of group I nucleopolyhedrovirus (NPV) BVs. An unrelated type of envelope fusion protein, named F, is encoded by group II NPVs. The genome of *Helicoverpa armigera* (Hear) NPV, a group II NPV of the single nucleocpasid or S type, also encodes an F-like protein: open reading frame 133 (*Ha133*). We demonstrated by N-terminal sequencing of the major 59 kDa protein present in HearNPV BV, that this protein is one of the two F subunits: F_1 (transmembrane subunit of 59 kDa) and F_2 (surface subunit of 20 kDa), both the result of cleavage by a proprotein convertase (Westenberg *et al.*, 2002) and disulfide-linked. The HearNPV F protein proved to be a functional analog of GP64, as the infectivity of an AcMNPV *gp64* deletion mutant was rescued by the introduction of the HearNPV F gene. It was also demonstrated by chemical cross-linking that HearNPV F is present in BVs as an oligomer whereby, unlike GP64, disulfide-bonds are not involved. Deglycosylation assays indicated that both the F_1 and F_2 possess N-linked glycans. However, these glycans did not have a C3 fucose modification when F was made in Hz2E5 cells usually present in insect cells. Since C3 fucose is a major inducer of an allergic response in humans, the present observation makes the HearNPV-Hz2E5 system an attractive alternative for the production of recombinant glycoproteins for therapeutic use in humans.

INTRODUCTION

The *Baculoviridae* are a large family of enveloped DNA viruses that are almost exclusively pathogenic to arthropods, predominantly insects in the order *Lepidoptera* (Theilmann *et al*., 2005). Baculoviruses are divided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GVs). Phylogenetic studies indicate that NPVs can be subdivided into two subgroups: group I and group II (Bulach *et al*., 1999; Hayakawa, *et al*., 2000; Herniou *et al*., 2003). Baculoviruses produce two distinct vision 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 (Horton & Burand, 1993). In contrast, BVs are adapted to propagate infection from cell to cell via receptor-mediated endocytosis and are responsible for the systemic spread of the virus in the infected insect (Volkman & Goldsmith, 1985; Hefferon *et al*., 1999).

Baculovirus, like other envelope viruses from a broad range of different families, overcomes the first barrier of host cells via membrane fusion, which is mediated by specific envelope fusion protein (Weissenhorn *et al*., 1999; Debra *et al*., 2001). The BV phenotype of group I NPVs contains the major envelope glycoprotein GP64, which mediates low-pH-triggered membrane fusion (Blissard *et al*., 1992; Kingsley *et al*., 1999; Plonsky *et al*., 1999) and is necessary for efficient budding from the cell surface of infected cells (Oomens *et al*., 1999). Recent genomic research of group II NPVs and of GVs indicates that they lack a GP64 homolog. A different BV envelope fusion protein has been identified from the group II NPVs *Lymantria dispar* (Ld)MNPV and *Spodoptera exigua* (Se)MNPV with structural similarity to vertebrate virus envelope fusion proteins (IJkel *et al*., 2000; Pearson *et al*., 2000). Like the GP64-like protein, these proteins mediate low-pH-dependent membrane fusion during BV entry and were named F proteins (Westenberg *et al*., 2002; Pearson & Rohrmann, 2002). Cleavage of the SeMNPV F protein into two disulfide-linked subunits by a cellular convertase (furin-like) is necessary for the low-pH-triggered membrane fusion activity and viral infectivity (Westenberg *et al*., 2002). This is in contrast to LdMNPV F, which was found as an uncleaved BV protein (Pearson *et al*., 2000), despite the presence of a furin-like cleavage site. A recent study showed that both LdMNPV F and SeMNPV F can rescue virion production and infectivity of an AcMNPV virus with a deleted *gp64* gene, which suggests that these F proteins of group II NPVs are functionally analogous to GP64 of group I NPVs (Lung *et al*., 2002). F homologs occur ubiquitously in envelopes of vertebrate viruses (Eckert & Kim, 2001).

As in other group II NPVs, no *gp64*-like gene is present in the *Helicoverpa armigera* (Hear)NPV genome (Chen *et al*., 2001), but an *f* homolog has been found as open reading frame (ORF) 133 (*Ha133*). In this chapter the HearNPV F protein is identified on BVs by protein sequence analysis. To show the analogy between this HearNPV F protein and the SeMNPV or LdMNPV F proteins, the conformation of the HearNPV F protein in BVs and its ability to rescue BV production and infectivity of an AcMNPV virus with a deleted *gp64* gene were studied. In addition, deglycosylation experiments revealed the absence of C3 fucosylated N-glycans on the HearNPV F protein when produced in Hz2E5 cells but not in *Trichoplusia ni* cells. The oligomeric structure of the baculovirus F proteins was studied in more detail by chemical cross-linking of BV proteins.

MATERIALS AND METHODS

Cells and virus

Helicoverpa zea cell line Hz2E5 (McIntosh and Ignoffo, 1983) was cultured in plastic tissue culture flasks (Nunc) in CCM3-HyQ medium (Hyclone), supplemented with 5% fetal bovine serum (FBS). *Spodoptera frugiperda* cell line IPLB-SF-21 (Vaughn *et al*., 1977) and *Trichoplusia ni* BTI-Tn-5B1-4 (High Five) (Granados *et al.*, 1994) were cultured at 27^{o}C in plastic tissue culture flasks (Nunc) in Grace's insect medium, pH5.9 to 6.1 (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS). The HearNPV G4 isolate was originally obtained from *H. armigera* (Lepidoptera, Noctuidae) in the People's Republic of China (Sun *et al.,* 1998). The *gp64*-null AcMNPV bacmid was described by Lung *et al.* (2002), and obtained from Dr. G.W. Blissard (USA).

Purification of HearNPV BVs

Hz2E5 cells were infected with a multiplicity of infection (m.o.i.) 5 $TCID_{50}$ units/cell of HearSNPV. After three days the cell culture supernatant was collected and clarified at 2,000 x *g* for 10 min at 4^{o}C. The supernatant was passed through a 0.45 μm pore-size filter. BVs in the filtrate were sedimented through a 25% (w/w) sucrose cushion made up in 0.1 × TE (10mM Tris-HCl pH 7.5, 1.0 mM EDTA) by centrifugation at 100,000 x *g* for 90 min at 4^{o}C. The BVs were resuspended in 0.1 × TE and directly used or stored frozen at -20 ^{o}C.

Protein sequencing

Purified HearNPV BVs were disrupted in Laemmli buffer (125mM Tris-HCl, 2% sodium dodecyl sufate [SDS], 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH6.8) and incubated for 10 min at 95^{o}C. BV proteins were separated in a 12% SDS-PAGE gel and transferred by semi-dry blotting 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 (59kDa) from HearNPV BV was N-terminally sequenced (Protein Research Facility Amsterdam, The Netherlands).

Production of polyclonal antibodies

The HearNPV ORF133 regions coding for amino acids 25(M) to 173(R) of F_2 and amino acids 174(N) to 599(G) of F_1 were amplified by high-fidelity Expand long-template PCR (Roche) with HearNPV G4 genomic DNA as template. Primers used to amplify F_1 were 5'-CAAGGATCCAAACATTGGATTGAACTTCGTTG-3' (underlined nucleotides generate a *Bam*HI restriction site) and 5'-AATAAGC**TTA**TCCCGTACTTAAATTCCAACCGC-3' (underlined and boldface nucleotides generate a *Hin*dIII resitriction site and a translation stop codon, respectively). Primers used for amplifying F_2 were 5'-GGATCCTATGTCGTTGACGCCGAGTCGTCG-3' (underlined nucleotides generate a *Bam*HI restriction site) and 5'-TTTAAGC**TTA**TCGTTTGTTGCGACTCGAGAATGTTG-3' (underlined and boldface nucleotides generate a *Hin*dIII restriction site and a translation stop codon, respectively). The PCR products were cloned into the *Bam*HI and *Hin*dIII cloning sites

of expression vector pET28a (Novagen) to generate pET28-HaF1Δ600-678 and pET28-HaF2Δ1-24.

Proteins F_1 and F_2 were expressed in *Escherichia coli* BL21 cells. The insoluble fractions containing the HaF_1 and HaF_2 protein were purified by continuous-elution electrophoresis using the Model 491 Prep Cell (Bio-Rad) according to manufacturer's protocol. Elution fractions were collected and electrophoresed in 12.5% SDS-PAGE gels and the protein bands visualized by silver staining. Fractions containing F_1 or F_2 protein were pooled and concentrated using Centriprep filter devices (Amicon). Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad).

Two chickens were injected intramuscularly each with 200 μg purified HaF_1 or HaF_2 proteins using a water-in-oil adjuvant. The chickens were boosted after 6 weeks with 100 μg purified protein. Two weeks after the booster, eggs were collected daily for 4 weeks. Antibodies were purified from the egg yolk as described previously (Westenberg *et al.,* 2002).

Western blot analysis

HearNPV BVs were disrupted either under reducing conditions in Laemmli buffer as described above or under nonreducing conditions in 125mM Tris-HCl, 8% SDS, 37.5 mM iodoacetamide, 10% glycerol, 0.001% bromophenol blue, pH6.8 by incubation for 10 min at 95°C. Proteins were separated by SDS-PAGE and subjected to Western analysis as described (Ausubel *et al.*, 1994; Long *et al.,* 2003). The antisera were used in a 1:1000 dilution and the proteins were detected by treatment with horseradish peroxidase conjugated rabbit anti-chicken immunoglobulin (Sigma) diluted 1:10,000 followed by ECL technology as described by the manufacturer (Amersham).

Chemical cross-linking

Purified HearNPV BVs were suspended in cross-linking buffer (10mM HEPES, 100mM NaCl, pH 8.3). Ethylene glyco bis-(succinimidyl succcinate) (EGS), as a cross-linking agent (Sigma), was added to a final concentration of 125μM-500μM. After incubation on ice for 1 h, EGS was quenched with 50mM Tris-HCl (pH 6.8) for 10 min. BV proteins were separated in a 6% non-reducing SDS-PAGE gel and transferred onto Immoblilon-P membranes for Western analysis.

Deglycosylation assay

Deglycosylation experiments were performed on cell culture derived and sucrose-purified HearNPV BVs. BVs were denatured by boiling for 10 min in the presence of 0.5% SDS and 1% of β-mercaptoethanol. Denatured BV proteins were incubated overnight either in Endo H G5 buffer (50mM sodium Citrate, pH 5.5, 5mM PMSF) containing 1 U Endo H (New England Biolabs) or in PNGase F incubation buffer (PBS, pH 7.4, 20mM EDTA, 0.5% NP40, 5mM PMSF) containing 1 U PNGase F (Roche). Deglycosylated BV proteins were separated by SDS-PAGE, immobilized to Immobilon-P membrane and probed with antibodies (IgY) against HaF_1 and HaF_2.

Pseudotyping *gp64*-null AcMNPV with HearNPV *F*

A whole coding region of the *F* gene was amplified by high-fidelity Expand long-template PCR (Roche) with primers Ha133F*Hin*dIII 5'-AAGCTTATGGTTGCGAT AAAAAGTAGTATG-3' and Ha133R*Hin*dIII/*Bam*HI 5'- **GGATCC**AAGCTTCGTAGGGA

TTTGCCGTCGGCATCCCTAAACGGCAGC-3' (underlined and boldface nucleotides generate a *Hin*dIII and *Bam*HI restriction site, respectively) and HearNPV G4 genomic DNA as template. The PCR product was cloned as a *Hin*dIII fragment, of which the protruding ends were filled in by T4 DNA polymerase, into the also blunt-ended *Eco*RI and *Bam*HI sites of the plasmid pΔFBgusAcgp64 (Lung *et al*., 2002) to obtain pΔFBgusHaF.

The expression cassette pΔFBgusHaF was transposed into the attTn7 transposition sites of the *gp64-null* AcMNPV bacmid (Lung *et al.,* 2002) according to the Bac-to-Bac Baculovirus Expression Systems manual (Invitrogen) to obtain the *gp64null*-Ha133-AcMNPV Bacmid. 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').

Sf21 cells (9.0×10^5) were seeded into 35 mm tissue culture plates (Nunc). Cells were transfected with approximately 1 µg of bacmid DNA using 10 µl of Celfectin (Invitrogen). At four days post transfection cells were stained for GUS activity according to the Bac-to-Bac protocol (Invitrogen) to monitor transfection efficiency. The supernatant (passage 1) was clarified at 2,000 x *g* for 10 min at 4^oC and subsequently filter-sterilized (0.45 µm pore size). To investigate the presence of infectious BVs one-fourth (500 µl) of the supernatant was used to infect 9.0×10^5 Sf21 cells. Seventy-two h p.i. the cells were stained for GUS activity. The *gp64*-positive AcMNPV bacmid and the *gp64*-null AcMNPV bacmid served as positive and negative controls for transfection and infection, respectively (Lung *et al*., 2002).

Computational analysis

The HearNPV F protein was analyzed by using the EXPASY sever (Appel *et al*., 1994) to predict the transmembrane domain (TM), signal peptide (SP), heptad repeats (HR) and potential glycosylation sites.

RESULTS

Identification of HearNPV F

To identify the HearNPV envelope fusion protein, BVs were purified from an infected Hz2E5 cell culture. Sucrose gradient purified BVs proteins were separated in a 12.5 % SDS-PAGE gel and the proteins were visualized by Coomassie brilliant blue staining. Four protein major bands were visible with molecular sizes of 59kDa, 45kDa, 33kDa and 18kDa, respectively (Fig. 1, lane 1). An N-terminal sequence NIGLNF was obtained from the purified 59 kDa protein by protein sequencing. This obtained sequence is located in the open reading frame of *Ha133* (Chen *et al.,* 2001), immediately downstream of the predicted furin cleavage site R-N-K-R within the primary translational product. This confirms that *Ha133* encodes the Hear F protein and that it is a homolog of group II NPV F proteins. This analysis also indicates that the HearNPV F protein, similar to the SeMNPV F protein (IJkel *et al*., 2000; Westenberg *et al.,* 2002), is post-translationally cleaved by a furin-like cellular convertase.

Immunodetection of the HearNPV F protein

Post-translational cleavage of the fusion protein of enveloped viruses seems to be a general mechanism used to regulate viral membrane fusion activity (Klenk and Garten, 1994; Eckert & Kim, 2001). The cleavage products could remain associated by covalent or noncovalent linkages. In case of the SeMNPV F protein the cleavage products remain covalently linked by a disulfide-bridge (Westenberg *et al*., 2002). To investigate which situation exists for the F protein HearNPV BV proteins were disrupted, separated in a reducing SDS-PAGE gel and subjected to Western analysis using F_1 (α-HaF_1) or F_2 (α -HaF_2) specific antibodies (Fig 1, lanes 2-3). These results show that both the F_1 (59 kDa) and F_2 (20kDa) cleavage products are present in the BV.

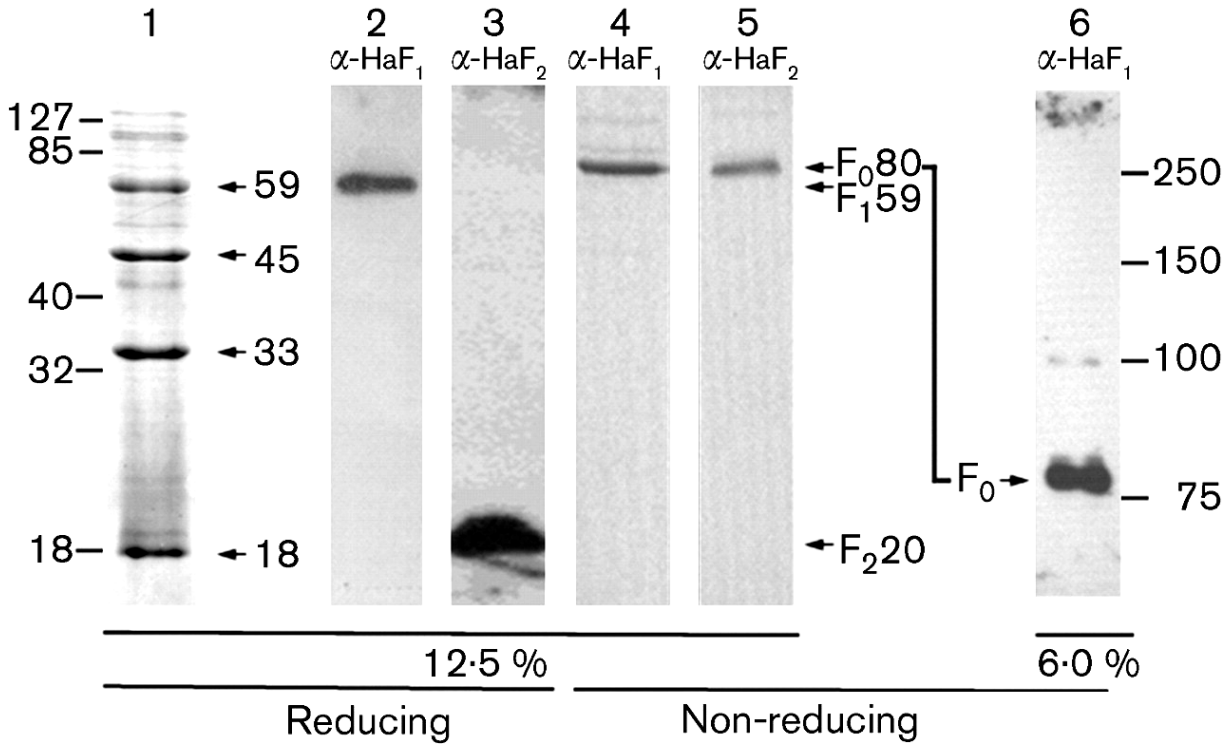

Figure 1. SDS-PAGE and Western blot analysis of HearNPV BV proteins. Cell culture derived BVs were incubated under reducing (lane 1-3) or nonreducing (lane 4-6) conditions and electrophoresed in a 12.5% (lane 1-5) or 6% (lane 6) SDS-polyacrylamide gel. Proteins were Coomassie brilliant blue stained (lane 1) or subjected to Western analysis (lane 2-6), probed with either α -HaF_1 (lane 1, 3 and 5) or α -HaF_2 (lane 2 and 4), and detected with a chemiluminescent substrate. Open arrows indicate the four protein major bands with molecular sizes of 59kDa, 45kDa, 33kDa and 18kDa respectively. F_0 (~ 80 kDa) = F_1 (~59 kDa) and F_2 (~20 kDa) disulfide linked subunits of HearSNPV F protein. Size standards are indicated in kDa.

To examine if the cleavage products remain covalently associated, HearNPV BVs were treated with iodoacetamide (modifies cysteine residues and thereby prevents HearNPV F protein from forming artificial disulfide linkages during further treatment or electrophoresis). The proteins were denatured in the absence of reducing agents and electrophoresed in a 12.5% SDS-polyacrylamide gel. Western analysis with α -HaF_1 and α -HaF_2 detected in both cases a single protein with a molecular weight of 80 kDa (F_0) (Fig. 1, lanes 4-5). The absence of F

protein subunits (59kDa and 20kDa bands) indicates that the F_1 and F_2 are covalently associated by a disulfide-linkage.

Oligomerization of HearNPV F protein

Viral fusion proteins generally form higher order oligomers (Hernadez *et al*., 1996). In case of the envelope fusion protein GP64 of the group I NPVs this oligomer is formed by three stable disulfide-linked GP64 proteins (Oomens *et al*. 1995). To determine whether HearNPV F proteins form disulfide-linked oligomers, non-reduced HearNPV BV proteins were separated in a 6% SDS-PAGE gel and transferred onto Immunobilon-P membranes and probed with α -HaF_1 (Fig. 1, lane 6). A major monomeric band of 80 kDa (F_0) was detected and no band was observed at about 160kDa or 240kDa, indicating that, in contrast to GP64 (Oomens et al., 1995) possible oligomeric forms of the HearNPV F protein are not disulfide-linked.

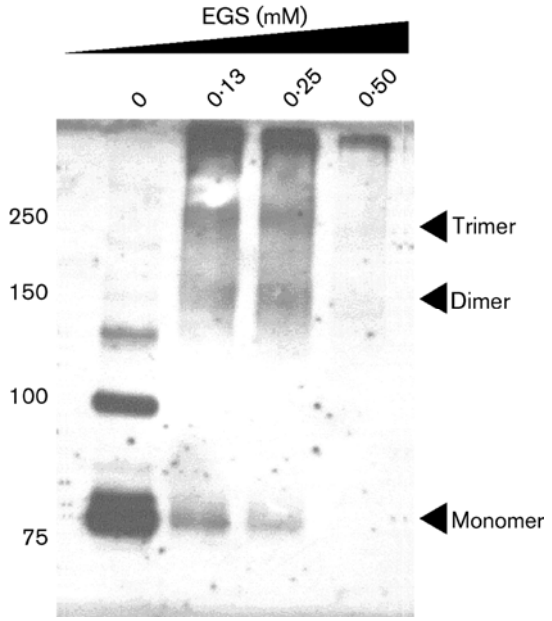

Figure 2

Multimerization of HearNPV F on BVs. BV total proteins were cross-linked with different concentrations (0-0.5 mM) of EGS. Cross-linked proteins were incubated for 10 min at 95°C under non-reducing condition and electrophoresed in a 6% SDS-polyacrylamide gel and subjected to Western analysis using α-HaF_2 and detected with a chemiluminescent substrate. The HearSNPV protein monomer (80 kDa) and multimers (160 and 240 kDa) are indicated by arrows. Size standards are indicated in kDa.

BV proteins were also chemically cross-linked with different concentrations of EGS. Afterwards proteins were separated in a 6% SDS-PAGE gel, transferred onto Immunobilon-P membranes and probed with α-HaF_1 to detect F (Fig. 2). In addition to the monomeric form (~80 KDa), a dimeric (~160 kDa) and a trimeric (~240 kDa) form of the F protein were clearly visible.

Pseudotyping AcMNPV *gp64*-null bacmid

It has been shown that the SeMNPV and LdMNPV F proteins are functional analogs to GP64, as they can rescue the infectivity of an AcMNPV *gp64*-null bacmid (Lung *et al*., 2002). To determine whether the HearSNPV F protein also could rescue the *gp64*-null bacmid, its coding region was placed under the control of the AcMNPV *gp64* promoter and inserted together with a *gus* reporter gene into the *gp64*-null bacmid ($vAc^{gp64\text{-}/HaF}$). The *gp64*-null

bacmid with no envelope fusion protein gene (vAc^{gp64-}) and the repair bacmid ($vAc^{gp64-/gp64}$), in which the *gp64* gene was reinserted were used as negative and positive controls, respectively. The bacmids were transfected into Sf21 cells (Fig. 3A, panel 1-3). Cells transfected with vAc^{gp64-} (panel 1) showed single infected cells with GUS which did not produce infectious virus as expected (Monsma *et al.*, 1996). When cells were transfected with $vAc^{gp64-/HaF}$, a large number of infected cells was observed (panel 3) as was the case in the positive control using the repair bacmid $vAc^{gp64-/gp64}$ (panel 2). Supernatant of both $vAc^{gp64-/gp64}$ and $vAc^{gp64-/HaF}$ transfected cells produced infectious BVs, when tested on uninfected Sf21 cells (panel 5 and 6).

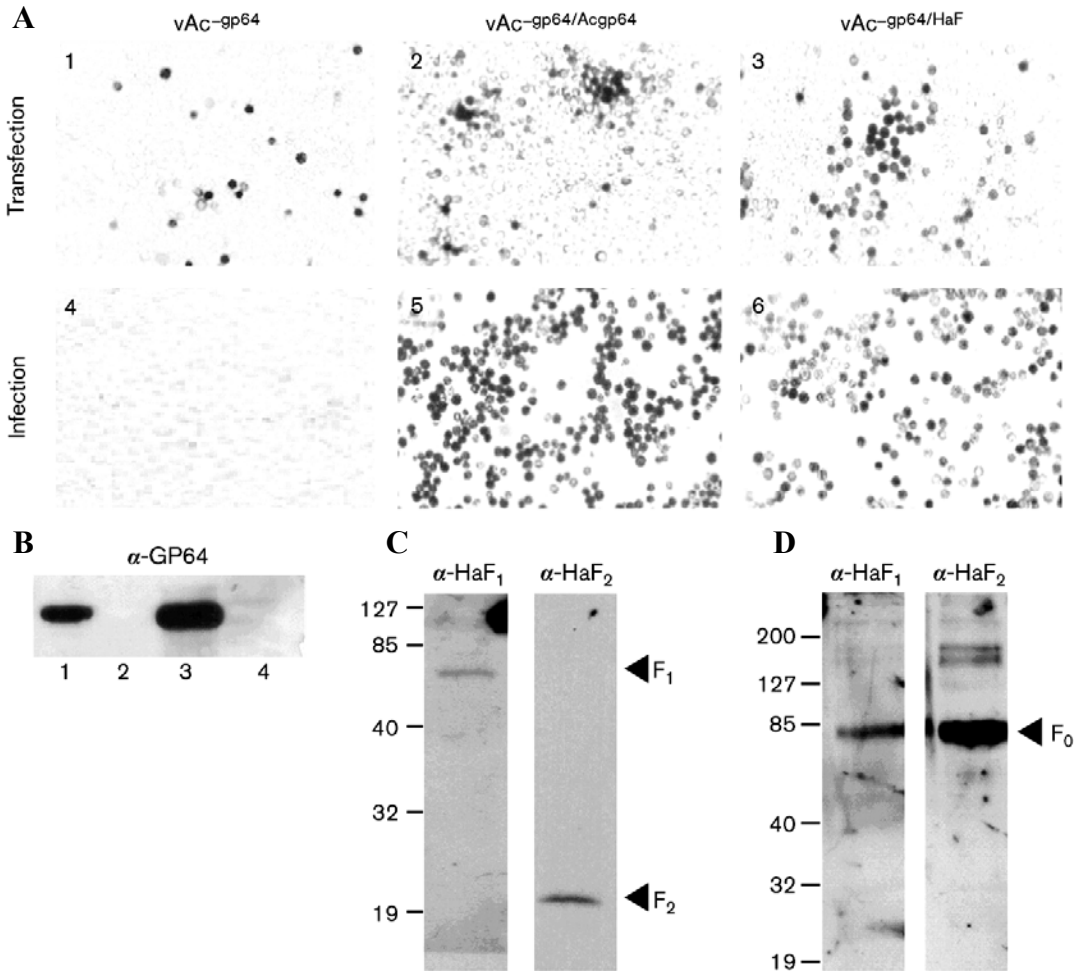

Figure 3. Pseudotyping *gp64*-null AcMNPV with HearNPV F. Sf21 cells were transfected with indicated mutant *gp64*-null AcMNPV bacmids, incubated 96 h, and stained for GUS activity (A panel 1-3). Supernatants from the transfected cells were used to infect another group of Sf21 cells, which were incubated for 72 h and subsequently stained for GUS activity (A panel 4-6). $vAc^{gp64-/gp64}$ (B, lane 1) and $vAc^{gp64-/HaF}$ BVs (B, lane 2; C; D) as well as cells infected with $vAc^{gp64-/gp64}$ (B, lane 3) and $vAc^{gp64-/HaF}$ (B, lane 4; C; D) were harvested, incubated for 10 min at 95°C under reducing (B and C) or nonreducing (D) conditions and electrophoresed in a 12.5% (B and C) or 6% (D) SDS-polyacrylamide gel. Proteins were subjected to Western analysis using antibodies against GP64 (α -GP64) (B), α -HaF_1 or α -HaF_2 (C and D). Size standards are indicated in kDa.

Infectious viruses were produced from cells transfected with $vAc^{gp64-/HaF}$ (Fig. 3A, panel 3 and 6) and from the positive control Ac^{gp64-} (Fig. 3A, panel 2 and 5). Infected cells as well as BVs produced by those cells were harvested and subjected to western analysis antibodies against GP64 (α -GP64) as a probe (Fig. 3B). GP64 was only detected in the $vAc^{gp64-/gp64}$ BVs (Fig. 4B, lane 1) and $vAc^{gp64-/gp64}$ infected cells (Fig. 3B, lane 3), but not in the $vAc^{gp64-/HaF}$ BVs (Fig. 3B, lane 2) and $vAc^{gp64-/HaF}$ infected cells (Fig. 3B, lane 4).

The $vAc^{gp64-/HaF}$ BVs were further analyzed for the presence of F and its post-translational cleavage products F_1 and F_2 in this pseudotyping system. BV proteins were separated in a 12.5% or a 6% SDS-PAGE gel, either under reducing (Fig. 3C) or nonreducing (Fig. 3D) conditions, respectively, and finally detected by Western analysis with α -HaF_1 or α -HaF_2. All detected F protein in the BVs was entirely cleaved into F_1 and F_2 (Fig. 3C), which remains associated by disulfide bonds when analyzed under non-reducing conditions (Fig. 3D). This result indicates that the HearNPV F protein is able to functionally pseudotype the *gp64*-null AcMNPV virus and that the F protein ends up in the BVs in a similar subunit configuration as in the authentic HearNPV BV.

Glycosylation of HearNPV F

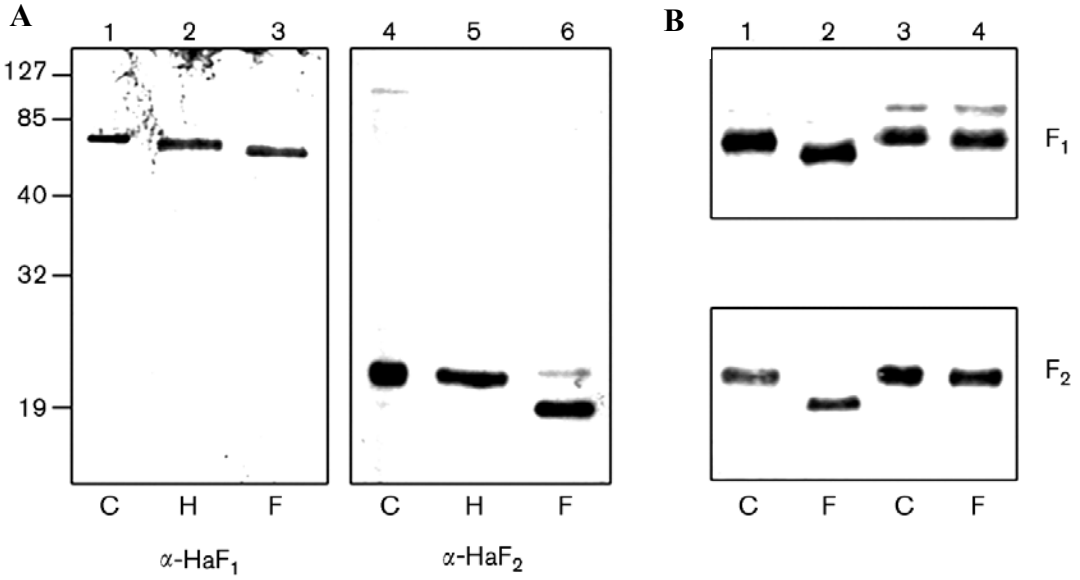

Figure 4. Deglycosylation assays on HearNPV F. BV proteins were incubated under reducing conditions. **(A),** denatured HearNPV BV proteins were incubated overnight with Endo H (H), PNGase F (F) or no deglycosidase (C). **(B),** HearNPV BV (lanes 1 and 2) and *Tni*-High5 cells derived $vAc^{gp64-/HaF}$ BV (lanes 3 and 4) proteins were incubated overnight with PNGase F (F) or no deglycosidase (C). Deglycosylated and non-deglycosylated proteins were electrophoresed in a 12.5% SDS-polyacrylamide gel, subjected to Western analysis and probed with either α -HaF_1 (A, lanes 1- 3 and B, upper) or α -HaF_2 (A, lanes 4-6 and B, lower), and detected with a chemiluminescent substrate. Size standards are indicated in kDa.

The LdMNPV F protein appeared as a N-linked glycoprotein by tunicamycin treatment of infected Ld625Y insect cells (Pearson *et al*., 2000). To obtain more in-depth insight in the nature of the N-linked glycosylation status of the HearNPV F protein, deglycosylation assays were performed. Denatured HearNPV BV proteins were treated with Endo H and PNGase F,

then separated by SDS-PAGE and subjected to Western analysis using either α -HaF$_1$ (Fig. 4A, left panel) or α -HaF$_2$ (Fig. 4A, right panel). Both F$_1$ and F$_2$ appeared to be N-glycosylated. The N-glycans on F$_1$ are EndoH sensitive (Fig. 4A, lane 2), implying that that those N-glycans are of the high mannose type or hybrid type. Furthermore, the N-glycans on both F$_1$ and F$_2$ are PNGase F sensitive (Fig.4A, lane 3 and 6), indicating that none of the glycans contains C3 fucose, which is generally present in insect glycoproteins (Tomiya *et al*., 2004). Subunit F$_2$ is insensitive to EndoH (lane 5), but sensitive to PGNAse F (lane 6), suggesting it contains a paucimannose.

N-linked glycosylation of HearNPV F protein expressed in *T. ni* High5 cells was analysed by PNGase F treatment of vAc$^{gp64\text{-}/HaF}$ BVs collected 5 days p.i. (Fig. 4B). Both F$_1$ and F$_2$ expressed in *T.ni* High5 cells were resistent to PNGase F (Fig. 4B, lanes 3 and 4). This result suggests that the HearNPV F protein expressed in *T. ni* High5 cells isC3 fucosylated and also that the C3 fucosylation on F protein does not affect the infectivity of vAc$^{gp64\text{-}/HaF}$ in these two cell lines.

DISCUSSION

Baculoviridae are a large family of enveloped viruses (Theilman *et al.,* 2005). The BV phenotype of these viruses enters cells by an endocytic mechanism facilitated by the presence of an envelope fusion protein. GP64 is the major BV envelope protein of group I NPVs (e.g. AcMNPV) (Blissard *et al*., 1992; Kinsley *et al*., 1999; Plonsky *et al*., 1999). Recently, a novel type of envelope fusion protein that is unrelated to GP64 was identified in SeMNPV (SE8) and LdMNPV (LD130) BVs (IJkel *et al.,* 2000; Pearson *et al*., 2000). This envelope protein, named F protein (Westenberg *et al*., 2002) was similar to the envelope fusion protein of vertebrate viruses (IJkel *et al*., 2000; Pearson & Rohrmann, 2002). This report demonstrates that the *Ha133* gene in HearNPV G4 genome (Chen *et al*., 2001) being an SNPV encodes a functional homolog of the LdMNPV and SeMNPV F proteins. This result shows that both single (S) and multiple (M) nucleocapsid NPVs of group II NPVs have an F protein in the BV particle.

Four major proteins (59, 45, 33 and 18 kDa) were found in the HearNPV BV (Fig 1, lane 1). The 59 kDa protein appeared to be the transmembrane anchored F$_1$ domain of the F protein. In HearNPV F the furin like cleavage occurrs at the predicted cleavage site (SRNKR↓NIGLNF) resulting an N-terminal sequence NIGLNF of F$_1$. N-terminal protein sequencing of the 45 kDa BV protein showed that this protein is actin, which was confirmed by Western analysis using actin-specific antibodies (data not shown). The origin of the major 18 kDa BV protein is not known, but is is not the F$_2$ subunit of the HearNPV F protein. Using western analysis it appeared that F$_2$ was about 20 kDa (Fig. 1, lane 1). This band is larger than the predicted molecular weight (18 kDa) of F$_2$ with the signal peptide removed. Most likely, this small difference is due to the N-linked glycosylation in F$_2$ (see below).

Western analysis also showed that the HearNPV F protein is post-translationally cleaved and that the two cleavage fragments remain associated by a disulfide bridge (Fig. 1). The F$_2$ fragment of the HearNPV F protein contains only a single cysteine (C108). In contrast, the F$_1$ fragment contains 9 conserved cysteines in its ectodomain. Mass-spectrometric analysis of

peptides should reveal which of the cysteines in the F_1 fragment forms the disulphide-link with the F_2 fragment. A similar situation is found for the SeMNPV F protein, for which it has been demonstrated that this post-translational cleavage is important for its fusiogenic activity (Lung *et al*., 2002; Westenberg *et al*., 2002). In contrast, the LdMNPV F protein has been found as an uncleaved protein on BVs produced in Ld625Y cells (Pearson *et al*., 2000), however when this protein was singly expressed in a different cell line it appeared cleaved (Pearson *et al*., 2002). Probably, Ld625Y cells are unable to properly cleave the LdMNPV F protein, but maybe the protein is activated during viral uptake.

Both SeMNPV and LdMNPV F proteins have been used before to pseudotype a *gp64*-null AcMNPV virus (Lung *et al.,* 2002). In a similar experiment we demonstrated that the HearNPV F protein also has the ability to rescue infectious BV from this *gp64* deletion mutant (Fig. 3). The protein was found on the pseudotyped AcMNPV BVs as two disulfide-linked F protein fragments, similar as on the HearNPV BV, which indicates that the posttranslational modification and assembly of the F proteins in the BV envelope is similar in Hz2E5 and Sf21 cells. Pseudotyping of *gp64*-null AcMNPV virus has not been successful for all baculovirus F proteins. The F protein of the *Plutella xylostella* (Plxy) GV could be incorporated in the AcMNPV BVs but those BVs were not infectious (Lung *et al.,* 2002). Maybe, the Sf21 cells possess the F protein receptor for NPVs but not for GVs. Currently, these pseudotyped viruses are used to investigate if the F protein receptors of HearNPV and SeMNPV on Sf21 cells are one and the same.

Viral fusion proteins generally form higher-order oligomers (Hernadez *et al*., 1996). Viral fusion protein from members of the *Orthomyxoviridae, Paramyxoviridae, Coronaviridae* and *Herpesviridaea* forms homo-oligomers and this formation is essential for its fusion activity and for the viral infectivity. The baculovirus fusion protein GP64 of the group I NPVs forms a stable disulfide-linked homotrimer (Oomens *et al*., 1995). Disulfide-linkage seems not to be involved in the oligomerization of the baculovirus F protein (Fig. 1; Westenberg *et al*., 2002) as the F protein is detected by Western analysis under non-reducing conditions predominantly as a monomer. Protein bands with a molecular weight higher than 80 kDa (F_0) were found to react with the antibody against F1 (α -HaF_1), but none of them had a multiple size of the monomer.

Chemical cross-linking has been used to identify for instance the envelope fusion protein interactions of paramyxoviruses (Rusell *et al*., 1994; Sechoy *et al*., 1987). The HearNPV BVs were crosslinked with EGS and the F protein was detected both as non-cross-linked monomers and as cross-linked dimers and trimers (Fig. 2). Most likely, this implies that the baculovirus F protein can be found as trimers on the BV particle. This situation also exists with F proteins of vertebrate enveloped viruses (Dutch *et al*., 2000; Colman & Lawrence, 2003). A heptad repeat (HR) domain on the F_1 fragment with a putative leucine zipper (**L**AKNNNA**L**NEQVKE**L**DDELIR**L**) has been identified, which might be involved in the oligomerization of the F protein (IJkel *et al*., 2000; Eckert & Kim, 2001). In-depth studies involving circular dichroism techniques and mutational analysis are in progress to confirm whether this domain indeed can form a multimeric coiled-coil structure.

In general, viral envelope fusion proteins are heavily N-glycosylated, which is critical for proper folding, trafficking and fusogenicity of the fusion protein (Doms *et al.,* 1993; Helenius *et al.,* 2001). N-glycosylation and protein folding are closely interconnected processes that

take place in the endoplasmic reticulum. The addition of N-linked glycans occurs cotranslationally before or while nascent polypeptide chains are translated (Braakman *et al.,* 2000). Six potential N-glycosylation sites are predicted in HearNPV F protein, one in F_2 and five in F_1. Deglycosylation of HearNPV BV proteins by Endo H and PNGase F showed that both F_1 and F_2 are N-glycosylated (Fig. 4). The fact that F_1 is sensitive to Endo H treatment indicates that some of the N-glycans in F_1 contain high mannose glycans, whereas F_2 has mainly pauci mannose type.

Both F_1 and F_2 obtained from BVs produced in Hz2E5 cells were sensitive to PNGase F treatment (Fig. 4A), which implies that none of the N-glycans contain a C3 fucose residue. In contrast, HearNPV F protein from pseudotyped AcMNPV expressed in *T.ni* cells was C3 fucosylated (Fig. 4B). Glycobiology studies are in progress to elucidate the structure of these N-glycans on the F protein and on cellular glycoproteins of *H. zea* cell lines.

The baculovirus insect cell expression system is often used to express a large amount of proteins for pharmaceutical purposes (Jarvis *et al.,* 2003; Kost *et al.,* 2005). The major drawbacks of using the baculovirus (AcMNPV) expression system to produce glycoproteins as therapeutic is *i*) the inability to produce complex types of N-linked glycans as is the case with vertebrate systems and ii) the C3 fucosylation, which causes allergic reactions in humans (Tomiya *et al*., 2003; Tomiya *et al.,* 2004). The observations that HearNPV F proteins do not contain C3 fucosylated N-linked glycans is thus of considerable interest and promotes the development of the HearNPV-Hz2E5 cell system as alternative for the production of therapeutics for human use.

ACKNOWLEDGMENTS

This research was supported in part by grants from the National Science Foundation of China (NSFC 30300012 to Gang Long), the Royal Academy of Sciences of The Netherlands (KNAW) (project numbers 03CDP012 and 04-PSA-BD-02) and a PhD sandwich grant to GL of Wageningen University.

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Chapter 3

Absence of *N*-linked Glycans from the F_2 Subunit of the Major Baculovirus Envelope Fusion Protein F Enhances Fusogenicity

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This chapter was published as J. Gen. Virol. 88:441-449 (2007).

Chapter 3

SUMMARY

The F protein is the major glycoprotein present in envelopes of budded virus (BV) of members of the *Baculoviridae*. F mediates low-pH activated fusion with insect cell membranes. Baculovirus F proteins are first synthesized as a precursor (F_0) and then cleaved post-translationally into two disulfide bonded subunits F_1 (C-terminal, large subunit) and F_2 (N-terminal, small subunit). Recently we demonstrated for *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) (Long *et al.*, 2006a) that both F_1 and F_2 are *N*-linked glycosylated. Sequence analysis frequently predicts that one or more *N*-linked glycosylation sites are present in F_2 subunits of baculovirus F proteins. *N*-glycans on envelope fusion proteins are usually required for proper conformational integrity and biological function, such as infectivity. In this paper we studied the importance of *N*-linked glycosylation of the F_2 subunit of HearNPV F by site-directed mutagenesis. We eliminated the only putative *N*-linked glycosylation site in F_2 by mutating asparagine (N^{104}) into glutamine (Q^{104}) resulting in mutant HearNPV f^{N104Q}. When inserted into an *f*-null HearNPV and a *gp64*-null AcMNPV bacmid, infectious BV could be retrieved which contained unglycosylated F_2. The virulence of HearNPV f^{N104Q} was enhanced as BVs were produced earlier after infection and yielded larger plaques than *f*-null HearNPV repaired with wild-type *f*. The HearNPV ^{fN104Q} BVs also induced much more efficient low-pH activated syncytium formation. Our study indicates that *N*-linked glycosylation of the HearNPV baculovirus F_2 subunit is not essential for viral infectivity and suggests that it is involved in BV production and fusogenicity.

INTRODUCTION

The *Baculoviridae* are a large family of enveloped DNA viruses that are exclusively pathogenic to arthropods, mainly insects (Theilmann *et al.*, 2005). The family is subdivided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GVs). Phylogenetic studies indicate that NPVs can be further subdivided into two subgroups: group I and group II (Bulach *et al.*, 1999; Herniou *et al.*, 2001). Baculoviruses produce two distinct virion phenotypes: occlusion-derived virus (ODV) and budded virus (BV) (Volkman & Summers, 1977). ODVs are present in occlusion bodies (OB) and are able to infect upon release from OBs midgut epithelial cells by direct membrane fusion. In contrast, BVs are adapted to generate infection from cell to cell via receptor-mediated endocytosis and are responsible for the systemic spread of the virus in the infected insect larvae.

Two distinct envelope fusion proteins were identified in BVs, GP64 (in NPV group I) and F protein (in NPV group II and GV) (Blissard & Wenz, 1992; IJkel *et al.*, 2000; Pearson *et al.*, 2000). The F protein is a functional analogue to GP64 (Lung *et al.*, 2002; Long *et al.*, 2006a). Unlike GP64, baculovirus F proteins show similar structural and functional characteristics to class I viral envelope fusion proteins from retroviruses, paramyxoviruses, coronaviruses and orthomyxoviruses, with respect to the location of the signal peptide, heptad repeats, fusion peptide and transmembrane region (Fig. 1) and their respective functions (Eckert & Kim, 2001; Earp *et al.*, 2005). Like many mammalian viral envelope fusion proteins, the baculovirus F protein is first synthesized as a precursor (F_0), which is subsequently cleaved by a cellular furin-like convertase into two disulfide-linked subunits (F_1 and F_2) (Westenberg *et al.*, 2002; Long *et al.*, 2006a). Cleavage of the F protein is necessary to allow low-pH-triggered membrane fusion activity to promote viral infectivity (Westenberg *et al.*, 2002; Lung *et al.*, 2002). F proteins reside in BV envelopes as non-covalently bound homotrimers and both F_1 and F_2 subunits are *N*-linked glycosylated (Long *et al.*, 2006a).

N-glycosylation is a common cotranslational or posttranslational modification for virus envelope fusion protein (Doms *et al.*, 1993; Helenius & Aebi, 2001). *N*-linked glycans not only determine proper folding and intracellular trafficking of virus envelope fusion proteins (Gallagher *et al.*, 1992; Roberts *et al.*, 1993; Hu *et al.,* 1994; Hu *et al.*, 1995; Braakman *et al.*, 2000; Daniels *et al.*, 2003), but are also critical for fusogenicity (Deng *et al.*, 1994; McGinnes *et al.*, 2001; Von Messling & Cattaneo, 2003; Panda *et al*., 2004) and infectivity (Ohgimoto *et al.*, 1998). Despite the high degree of similarity among baculovirus F proteins with respect to the architecture of functional domains, the number and position of predicted *N*-linked glycosylation sites (N-X-S/T) vary between viruses. However, when comparing baculovirus

F_2 subunit sequences the putative *N*-linked glycosylation sites are frequently found at a conserved location. Recent studies on *Helicoverpa armigera* NPV (HearNPV) suggest that the only predicted *N*-linked glycosylation site (N^{104}) in the HearNPV F_2 subunit is most likely used (Fig. 1)(Long *et al.*, 2006a). There is no information on the importance of *N*-linked glycosylation for the function and activity of F-like baculovirus envelope fusion proteins. In the present study, we eliminated the *N*-linked glycosylation site on the F_2 subunit of HearNPV F by site-directed mutagenesis, resulting in a mutant with conservative asparagine-to-glutamine change (N-to-Q) in F_2 (HearNPV f^{N104Q}). We observed that this mutant is capable to rescue the infectivity of an *f*-null HearNPV and of a *gp64*-null AcMNPV bacmid. Interestingly, the F^{N104Q} mutant produces more BVs at earlier times post infection and is more efficient in low-pH activated syncytium formation than BVs of wild type HearNPV.

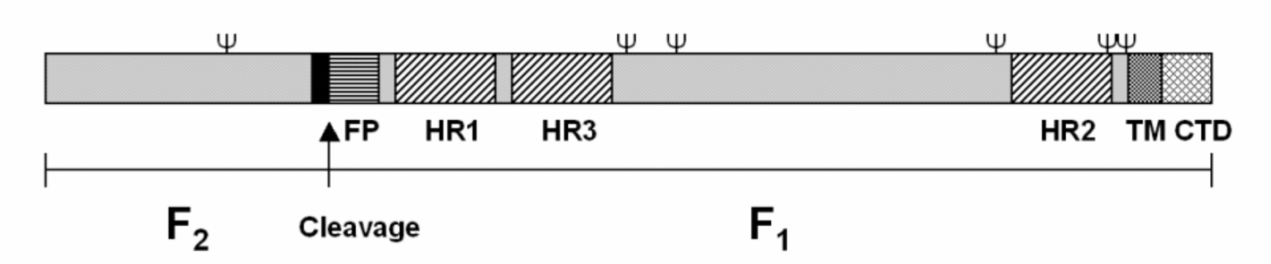

Figure 1. Schematic diagram of the structure and organization of the HearNPV F protein. Location of the cleavage site (arrow), fusion peptide (FP), heptad repeats (HRs), transmembrane domain(TM), cytoplasmic tail domain (CTD) and potential *N*-linked glycosylation sites (Ψ) are showed.

MATERIALS AND METHODS

Cells and Bacmid

The *Heliothis zea* cell line HzAM1 (McIntosh & Ignoffo, 1983) and *Spodoptera frugiperda* cell line IPLB-SF-21 (Vaughn *et al*., 1977) were cultured at 27°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). An *f*-null HearNPV bacmid (Long *et al.*, 2006c) and a *gp64*-null bacmid (Lung *et al.*, 2002) were used for reverse genetics study on the functional role of *N*-linked glycans on the F2 subunit of the HearNPV F protein.

Computational analysis

The amino acid sequence for the F_2 subunits of baculovirus F proteins were obtained from GenBank (accession number: AF271059 for HearNPV; AF081810 for *Lymantria dispar* MNPV; DQ504428 for *Clanis bilineata* NPV; AP006270 for *Adoxophyes honmai* NPV;

DQ123841 for *Agrotis segetum* NPV; AF169823 for *Spodoptera exigua* MNPV; DQ017380 for *Trichoplusia ni* SNPV; AF325155 for *Spodoptera litura* MNPV; AY864330 for *Chrysodeixis chalcites* NPV; AY126275 for *Mamestra configurata* NPV-B; AY394490 for *Leucania separata* NPV.). These sequences were aligned using Megalign software with the Clustal W method. Prediction of potential *N*-linked glycosylation sites was conducted using proteomics tools from the expasy proteomics sever (http://www.expasy.org).

Mutagenesis and bacmids

Site-directed mutagenesis of N^{104} of HearNPV *f* was carried out to replace asparagine (N) of the NXS/T sequon by glutamine (Q). F protein preferred codon for Q (CAG, underlined in the mutagenesis reverse primer) replaces the AAT codon for N^{104} by introducing this codon in the 5' end of mutagenesis reverse primer (5'-CTG TTT ATT CTT GAT CCA TTC AAC TAG A-3'). Inverse PCR (Weiner *et al.,* 1994) was performed using a mutagenesis reverse primer and a forward primer (5'-TTA ACC AGT TGC GAG AAC AGC GA-3'), with prior 5' phosphorylation of the primer pairs, *Pfu*-polymerase (Promega) and the a pFB-F&GFP vector containing f gene cassette (from nt 127811 to nt 130114) and a *p10*-promoter controlled egfp gene (Long *et al.*, 2006c) as a template. Following purification the mutant PCR products were treated with *Dpn*I restriction to eliminate template plasmid DNA. Subsequently, the 5' ends of the purified PCR products were ligated to its own 3'ends generating a new vector containing N104 amino acid residue mutated sequences. Clone containing the desired mutation was sequenced to confirm the mutation. The f^{N104Q} mutant gene cassette was sub-cloned into the pFB-F&GFP vector to replace the wild-type HearNPV *f* gene cassette, by swapping the *Bst*1107I-to-*Hin*dIII fragments, resulting in donor plasmid pFB-f^{N104Q}&GFP carrying the f^{N104Q} mutant gene.

Competent cells containing either an *f*-null HearNPV bacmid (Long *et al.*, 2006c) or a *gp64*-null AcMNPV bacmid was made according to the Bac-to-Bac manual (Invitrogen). Successful transpositions of inserts from donor plasmids, pFB-F&GFP and pFB- f^{N104Q} &GFP, to the *f*-null HearNPV bacmid and the *gp64*-null AcMNPV bacmid produced the recombinant HearNPV and pseudotyped AcMNPV with *f* and f^{N104Q}, respectively. The insertions were confirmed by diagnostic PCR using a gentamycin resistance gene forward primer (5'-AGCCACCTACTCCCAACATC-3') in combination with the M13 forward primer (5'-CCCAGTCACGACGTTGTAAAACG-3') to check for successful transposition. Transfection and infection assays were conducted according to Long *et al.* (2006a)

Western analysis

The expression of the wild type F protein and the F^{N104Q} mutant protein and their

incorporation into BVs were examined by Western analysis using polyclonal antibodies against F_1 and F_2 to probe sucrose-purified BVs or cellular total protein samples throughout infection. Western analysis was performed as described previously (Long *et al.*, 2006a). Briefly, sucrose-purified BVs were disrupted in reducing or non-reducing conditions and were then denatured for 10 min at 95^{o}C. Proteins were separated by SDS-PAGE and subjected to Western analysis. The antisera were used in a 1:1000 dilution and the proteins were detected by treatment with horseradish peroxidase conjugated rabbit anti-chicken immunoglobulin (Sigma) diluted 1:10,000 followed by ECL technology as described by the manufacturer (Amersham).

Plaque assays

To determine the infectivity of HearNPV BVs carrying the F^{N104Q} mutation in the F_2 subunit and the control HearNPV containing wild type F_2, plaque assays were conducted. Briefly, 1x10^{6} HzAm1 cells were transferred to 35mm Petri dishes in Grace's medium (supplemented with 10% FBS). Following 4 days incubation at 27^{o}C formation of plaques on each treatment was examined by UV light microscopy. The relative diameters of ten separated plaques from each infection were measured and statistically evaluated.

One step growth curve

To measure infectious BV productionwas assessed by one-step virus growth curves. HzAM1 cells were infected with HearNPV ^{f} and HearNPV ^{fN104Q}, with a m.o.i. of 5 $TCID_{50}$ units/cell for 1 h. After infection cells were washed with fresh medium once and then incubated in fresh medium. The supernatants were collected 12, 24, 36, 48, 60, 72, 84, 96 and 120 hours post infection (h.p.i.). For each mutant virus and at each time point, triplicate samples were collected. The quantity of infectious BVs from each sample was determined by end point dilution assay on HzAM1 cells (King & Possee, 1992). The results from above were exported to Microsoft Excel software and used for statistical analysis. In addition, infected cells at each time point post infection (p.i.) were collected and subjected to Western analysis to monitor the temporal expression of the F protein upon HearNPV ^{f} and HearNPV ^{fN104Q} infection.

Syncytium formation

Syncytium formation (Sf21-Sf21 fusion or HzAM1-HzAM1 fusion) assays were performed by infection with pseudotyped AcMNPV or recombinant HearNPV BVs (m.o.i. of 5 $TCID_{50}$ units/cell), respectively. Forty eight h.p.i. cells were washed three times with 1 ml Grace's medium (pH 6.1) without FBS and were treated for 5 min in 1 ml acidic Grace's medium at pH 5.0. Subsequently, the acidic medium was removed and replaced with 2 ml

Grace's medium (pH 6.1) supplemented with 10% FBS. Syncytium formation activity was quantified by measuring the number of fused cells relative to the number of total cells (percentage). The obtained results were exported to Microsoft Excel software and used for statistical analysis.

RESULTS

Potential *N*-linked glycosylation sites in baculovirus F_2 subunits

The canonic recognition sequon for *N*-linked glycosylation is NXS/T, where X can be any amino acid residue except proline. Multiple *N*-linked glycosylation sites are predicted in both F_1 and F_2 subunits of baculovirus F proteins. Comparison of the baculovirus F_2 subunits indicates that there are one or two potential *N*-linked glycosylation sites and that at least one is located at a conserved position around Cys^{108} (not shown). A recent study showed that both the F_1 and F_2 subunits of HearNPV F are indeed *N*-glycosylated (Long *et al.*, 2006a). Five putative *N*-glycosylation sites are found in HearNPV F_1 and a single one in F_2 (Fig. 1). The single frequency in HearNPV F_2 and its location at a conserved position suggest that *N*-linked glycosylation of F_2 plays an important role in baculovirus F protein function.

Effects of deletion of *N*-linked glycosylation site from F_2 of HearNPV F

To study the importance of *N*-linked glycosylation of F_2, an asparagine-to-glutamine mutant f^{N104Q} of HearNPV *f* was constructed and the mutation was verified by sequencing. Along with a *p10*-promoter controlled e*gfp* gene, the *f* gene or f^{N104Q} gene was transposed into an *f*-null HearNPV bacmid or into a *gp64*-null AcMNPV bacmid, respectively (Fig. 2A). Bacmid transfection and infection assays were conducted; expression of eGFP signaled successful transfection and virus infection. The transfection and infection experiments demonstrated that infectious BVs of HearNPV ^{f} and HearNPV ^{fN104Q} were produced after transfection (Fig. 2B, left panels). This indicated that HearNPV F lacking a putative *N*-linked glycosylation site on the F_2 subunit, F_2^{N104Q}, was able to rescue infectivity of the *f*-null HearNPV bacmid, as infectious BVs were produced as evidenced by a secondary infection from the transfection supernatant (Fig. 2B, right panels). A similar result was obtained for AcMNPV pseudotyped with the wild type HearNPV *f* gene or the f^{N104Q} gene. The latter gene successfully rescued the infectivity of the AcMNPV *gp64*-null mutant. Together, these results indicate that *N*-linked glycosylation of the HearNPV F_2 subunit is not essential for infectivity of HearNPV (Fig. 2B). *N*-linked glycans on the F_2 subunit are also not required for the production infectious BVs. Most likely, the F^{N104Q} proteins were properly processed and folded and were able to mediate successful envelope fusion processes.

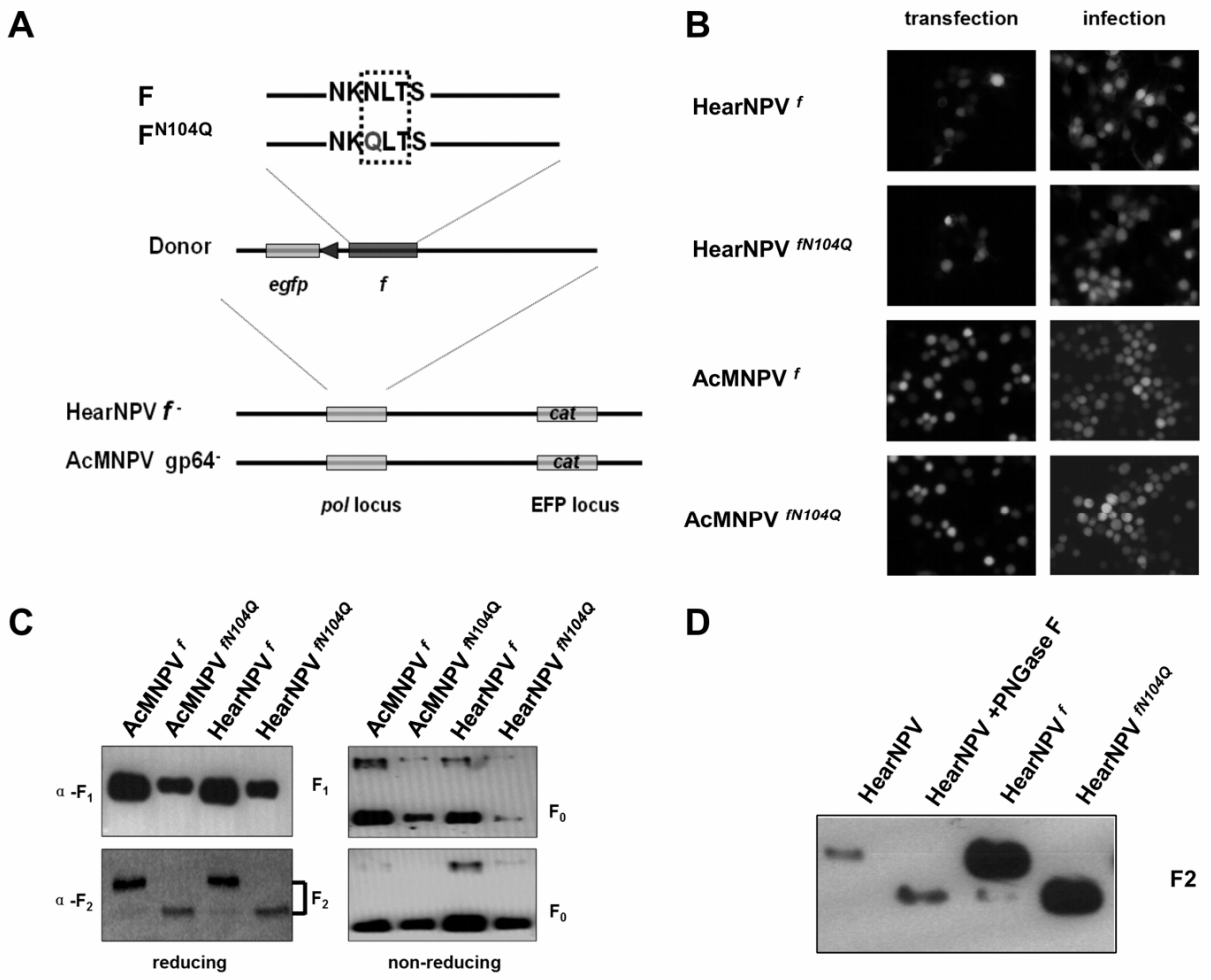

Figure 2. Construction and functional analysis of F_2 *N*-glycosylation knock out mutant (F^{N104Q}). (A) Mutation strategy of f^{N104Q} repaired HearNPV and pseudotyped AcMNPV. (B) HzAM1 (upper 4 panels) or Sf21 cells (lower 4 panels) were transfected with *f*-repaired HearNPV bacmids (HearNPV ^{f} and HearNPV ^{fN104Q}) or pseudotyped AcMNPV bacmids (AcMNPV ^{f} and AcMNPV ^{fN104Q}), respectively (left panels). Infection of HzAM1 cells or Sf21 cells with the supernatants of transfections (right panels) 5 d.p.i. GFP was used to monitored the transfection and infection using epifluorescence microscopy. (C) Incorporation of F_2^{N104Q} and wild type F protein in repaired HearNPV and pseudotyped AcMNPV BVs. BV proteins were separated by SDS-PAGE under reducing (left) and non-reducing (right) conditions followed by Western analysis using antisera against F_1 (α-F_1) and F_2 (α-F_2). The positions of F_0, F_1 and F_2 are indicated (D). Verification of the absence of *N*-linked glycans from the F_2 subunit. Deglycosylation treatment and Western analysis was performed as in C (Long *et al.*, 2006a).

To confirm the correct incorporation of the F^{N104Q} protein into infectious BVs, Western analysis was performed on purified BVs from recombinant HearNPV (HearNPV^{f} and HearNPV ^{fN104Q}) and AcMNPV pseudotyped with *f* (AcMNPV ^{f} and AcMNPV ^{fN104Q}). Under reducing conditions, F_1 (60kDa) and F_2 (20kDa) subunits migrated separately suggesting that

furin-like cleavage had occurred correctly during F protein synthesis in HzAm1 cells and Sf21 cells. Unglycosylated F_2 subunits (19kDa) from HearNPV^{fN104Q} and AcMNPV^{fN104Q} migrated faster than those from HearNPV^{f} and AcMNPV^{f} (Fig. 2C). This reflects the absence of *N*-linked glycosylation of F_2 and the successful construction of an *N*-glycosylation knock out (F^{N104Q}) mutant. Interestingly, unglycosylated F_2 subunits were also present in BVs of both HearNPV^{f} and AcMNPV^{f} (Fig. 2C, left panel). This observation suggests that the only *N*-linked glycosylation site in F_2 subunit is not always glycosylated in the wild type case. To ensure that the F_2^{N104Q} protein is free of *N*-linked glycans, F_2 mobility of deglycosylated F subunit was compared with the F_2^{N104Q} protein by western analysis (Fig. 2D). The mobility of deglycosylated F_2 was the same as that of the F_2^{N104Q} protein.

The *N*-linked glycosylation site of F_2 (N^{104}) is located slightly upstream of the only cystein residue (Cys^{108}) in the F_2 subunit (Chen *et al.*, 2001). This is the only candidate amino acid of the F_2 subunit to form a disulfide bond with the F_1 subunit. To study the effect of the absence of F_2 *N*-linked glycan on disulfide bridging, western analysis was performed after denaturation of BVs under non-reducing conditions (Fig. 2C). F_1 and F_2 subunits co-migrate as F_0 for all the samples with a molecular size of 80 kDa, demonstrating that disulfide bond formation between F_1 and F_2 subunits is not affected by the absence of *N*-linked glycans from F_2.

Infectivity and BV production of wild type and mutant HearNPV

To analyze the effect of *N*-linked glycosylation of the F_2 subunit on BV production, one step growth curves of BVs were compared between HearNPV^{f} and HearNPV^{fN104Q} (Fig. 3). HzAm1 cells were infected with HearNPV^{f} and HearNPV^{fN104Q} at an m.o.i. of 5 $TCID_{50}$ units per cell. BV production at various time points after infection was measured using an end point dilution assay and the whole experiment was carried out in triplo. The results showed HearNPV^{f} and HearNPV^{fN104Q} had comparable BV production kinetics except that HearNPV^{fN104Q} infected cells produced a higher amount of BVs at an early stage of infection while a lower amount of BVs of this mutant was found at a very late stage (Fig. 3A). At the end of the infection the final BV yield was not significantly different between both viruses. Expression of both wild type F protein and F^{N104Q} in cells infected with HearNPV^{f} and HearNPV^{fN104Q} was monitored throughout the infection (Fig. 3B). The cellular expression level of F^{N104Q} is much higher than that of wild type F protein at 24 h p.i.

To further evaluate the effects of the absence of *N*-linked glycans on the F_2 subunit on viral infectivity, plaque size determination was performed on HearNPV^{f} or HearNPV^{fN104Q} infected HzAM1 cells (Fig. 4A, 4B). The results demonstrate that single plaques produced by

HearNPV^{fN104Q} BVs are significantly larger in size than those produced by HearNPV^{f} BVs (Fig. 4C). This suggested that the *N*-linked glycosylation of F_2 was not essential for plaque formation of HearNPV per se, but that the HearNPV^{fN104Q} virus spreads quicker in cell culture than HearNPV containing wild type F.

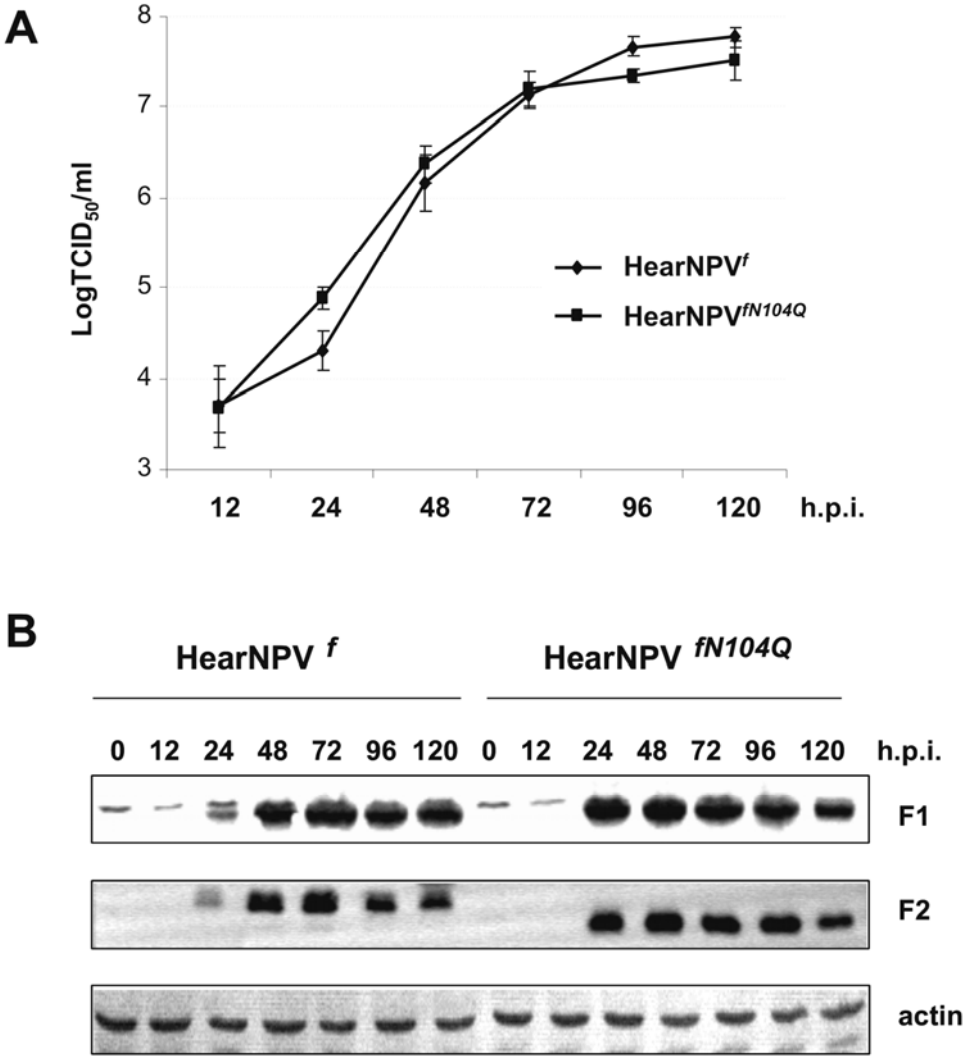

Figure 3. BV production kinetics (A) and temporal expression (B) of wild type *f* gene and *f*^{N104Q} gene repaired HearNPVs. (A) HzAM1 cells were infected at an m.o.i. of 5 $TCID_{50}$ units/cell; supernatant samples were collected at the indicated times p.i. and titered on HzAM1 cells. Each data point represents the average value from three separate experiments; error bars represent the standard deviation. (B) Temporal expression of wild type F and F^{N104Q} in infected HzAM1 cells as in A. Total cellular protein at each time point was analyzed by SDS-PAGE (12% gel). Western analysis was performed as previously described (Long *et al.*, 2006a).

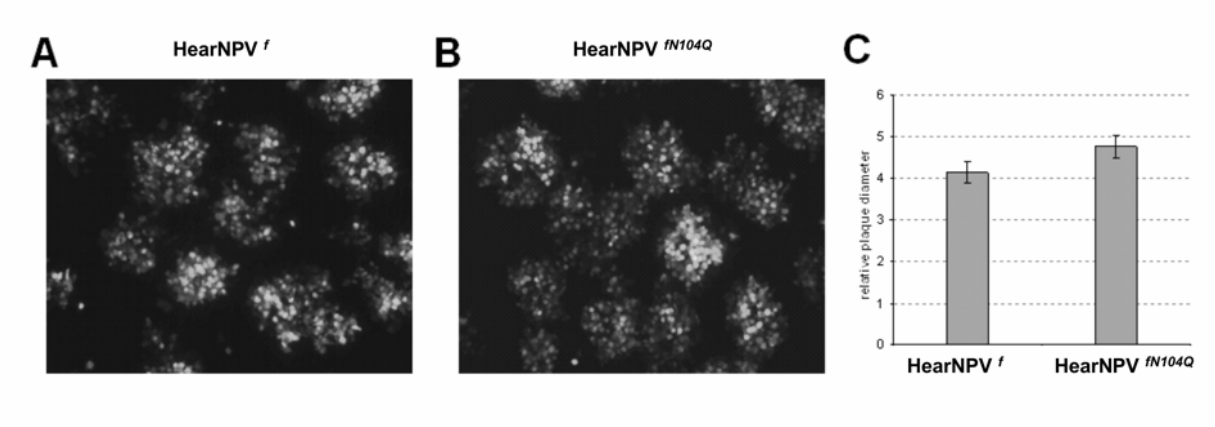

Figure 4. Effect of the F^{N104Q} mutation on plaque formation. (A) 1×10^6 HzAm1 cells were infected with repaired HearNPVs (HearNPV^f and HearNPV^{fN104Q}) at a m.o.i. of 0.01 $TCID_{50}$ units/cell. Four d.p.i. at 27° C the formation of plaques on each treatment was examined by UV light microscopy. (B) Plaque sizes of HearNPV^f and HearNPV^{fN104Q} infected HaAM1 cells. The relative diameters of ten separated plaques from each treatment were measured. The error bars represent the standard deviation of the mean.

Low-pH dependent fusion of wild type and mutant HearNPV

BVs of group II baculoviruses enter insect host cells through a clathrin-mediated and low-pH-dependent endocytic route (Long *et al.*, 2006b). The F protein is responsible for the low-pH-dependent cell fusion (IJkel *et al.*, 2000). As the removal of *N*-linked glycans might change the conformation of viral fusion proteins and thus the fusogenicity of F, we examined syncytium formation at low pH upon virus infection of HzAm1 cells (Fig. 5). Cells were infected at an m.o.i. of 5 $TCID_{50}$ units/cell. Forty-eight h.p.i. infected cells were subjected to low pH (5.0) culture medium and syncytium formation was measured 24 h post acidification. The syncytium formation activity of HearNPV^{fN104Q} -infected HzAM1 cells (Fig. 5A, upper panels, right) was significantly higher than for HearNPV^f infected cells (Fig. 5A, upper panels, left). In the case of syncytium mediated by wild type F protein, the percentage of fused cells was less than 30% in HzAM1 cells, whereas the percentage of fused cells was increased to about 70% percent in the case of syncytium mediated by HearNPV carrying the F^{N104Q} mutation (Fig. 5B). Similar results were obtained with pseudotyped AcMNPV (AcMNPV^f and AcMNPV^{fN104Q}) infected Sf21 cells (Fig. 5A lower panels). This observation indicated that the absence of *N*-linked glycans from F_2 subunits of HearNPV enhanced the low-pH dependent syncytium formation activity of baculovirus F protein.

DISCUSSION

N-linked glycosylation is important for proper protein processing during synthesis, structural integrity and functionality (Imperiali & O'Connor, 1999; Helenius & Aebi, 2001). For viral

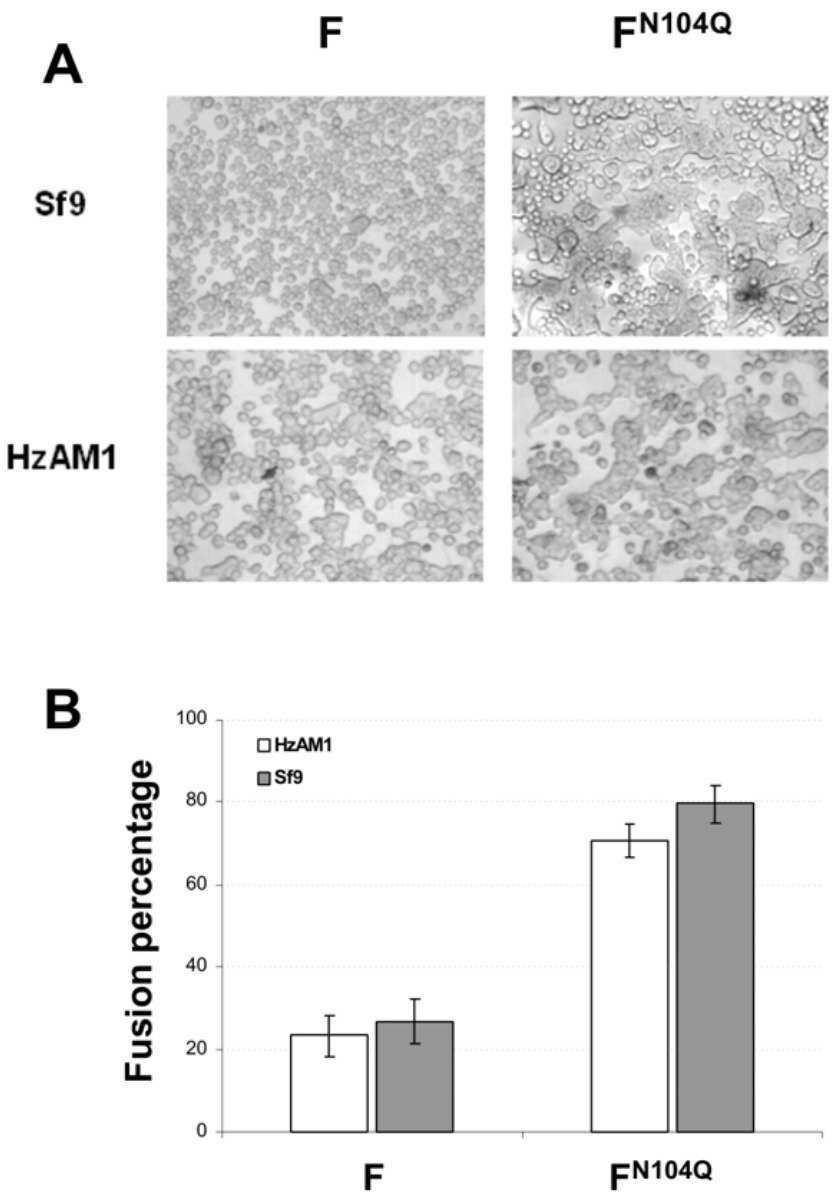

Figure 5. Syncytium formation promoted by HearNPV BV infection. (A) HzAM1 cells (lower panels) and Sf9 cells (upper panels) were infected with repaired HearNPVs (HearNPV ^{f} and HearNPV ^{fN104Q}) and pseudotyped AcMNPVs (AcMNPV ^{f} and AcMNPV ^{fN104Q}), respectively, at an m.o.i. of 5 $TCID_{50}$ units/cell. Fourty-eight h.p.i. infected HzAM1 cells and Sf9 cells were incubated in Grace's insect medium (pH 5.0) for 5 min. After one time wash with fresh Grace's medium, the cells were incubated in Grace's medium plus 10% FBS. Syncytium formation was examined 12 h post low-pH treatment. (B) Comparison of syncytium formation mediated by HearNPV BV with wild type F and with F^{N104Q}. Syncytium formation was measured by the percentage of fused cells relative to the number of total cells. The error bars represent the standard deviation of the mean.

envelope fusion proteins these functions include receptor binding (Ohuchi *et al.*, 1997; Nakayama *et al.*, 1998), envelope fusion (Deng *et al.*, 1994; McGinnes *et al.*, 2001; Von

Messling & Cattaneo, 2003) and virulence (Li *et al.*, 1993; Ohgimoto *et al.*, 1998; Reitter *et al.*, 1998; Wagner *et al.*, 2000; Koch *et al.*, 2003; Panda *et al.*, 2004). In the case of baculovirus F proteins, multiple potential *N*-glycosylation sites are present in the F_1 and F_2 subunits at various positions, but their role in the above functions is unexplored. Both subunits of baculovirus F proteins are *N*-glycosylated (Long *et al.*, 2006a) and the question is which of the potential glycosylation sites indeed contain *N*-glycans and what the consequences of the presence of *N*-glycans are for baculovirus performance for each of these sites.

The F_1 subunit of HearNPV contains five predicted *N*-glycosylation sites, whereas the F_2 subunit has only one such site and contains *N*-glycans (Fig. 1). This suggests that *N*-linked glycosylation of this single site in the F_2 subunit may indeed occur and may be important for F protein function as a whole, perhaps in protein folding, intracellular trafficking, envelope fusion and virus infectivity. In the present study we carried out a mutational analysis of this single putative *N*-glycosylation site of the F_2 subunit. Other group II baculoviruses have F_2 subunits with more putative glycosylation sites, but one is always in a more or less conserved position around Cys^{108}.

Using site-directed mutagenesis and functional rescue of bacmid-derived baculoviruses (Lung *et al.*, 2002) for F proteins, we provide compelling evidence that the only *N*-linked glycosylation site (NLTS) in the F_2 subunit of HearNPV F is indeed occupied with *N*-linked glycans (Fig. 2). An N-to-Q mutation at this sequon aborted the *N*-glycosylation of F_2 but did not inhibit F protein synthesis, BV production and infectivity. However, this putative *N*-linked glycosylation site is not completely glycosylated as unglycosylated F_2 was also found in the wild type HearNPV F protein (Fig. 2C, third lane). The oligosaccharyltransferase recognizes the consensus *N*-linked glycosylation sequon, NXS/T, but it has been shown that the amino acid residue at position 'X' was an important determinant of the glycosylation frequency at an individual site (Shakin-Eshleman *et al.*, 1996). Residues like Trp, Asp, Glu and Leu at position 'X' have been shown to be associated with less efficient *N*-linked glycosylation. This incomplete *N*-linked glycosylation on HearNPV F protein F_2 subunit could be well explained by the presence of a leucine residue at the 'X' position of the NXS/T sequon.

The fact that the f^{N104Q} gene was able to rescue the infectivity of an *f*-null HearNPV and also to rescue a *gp64*-null AcMNPV suggests that morphogenesis and maturation of BVs could occur in the presence or absence of *N*-linked glycans on the F_2 subunit. These processes are apparently independent of the genetic background (HearNPV vs AcMNPV). However, the

infectious BVs production kinetics of the two repaired viruses, HearNPV ^{f} and HearNPV ^{fN104Q}, were significantly different (Fig. 3A). Compared to the wild type repair, HearNPV ^{f}, more infectious HearNPV ^{fN104Q} BVs were produced at the an early stage of infection but less at the later stage. This is in agreement with the larger plaque size for HearNPV ^{fN104Q} (Fig. 5) and may be the consequence of larger BV production and quicker cell-to-cell spread of BVs. We hypothesize that this quicker BV spread may enhance the speed of kill of insects of F_2 *N*-glycan mutants.

The intracellular expression of the F^{N104Q} protein at early times p.i. is much higher than that of wild type F protein (Fig. 3B). This abundance of F most likely triggers larger BV production of the HearNPV ^{fN104Q} mutant at earlier times. The mechanism resulting in higher expression levels of the F^{N104Q} protein and its relation with glycosylation and its pathway remains to be clarified. The absence of glycosylation might result in a slightly different folding of F or enhanced trafficking towards the cell envelope. .

Like the GP64-like proteins baculovirus F proteins were able to mediate low-pH activated membrane fusion (IJkel *et al.*, 2000; Pearson *et al.*, 2000). Syncytium formation activity mediated by the F protein is much less extensive than that mediated by GP64 protein (IJkel *et al.*, 2000). In this study we demonstrated that F^{N104Q} maintained to mediate low-pH activated membrane fusion, suggesting that the removal of *N*-linked glycans from F_2 does not change the overall mechanism of baculovirus F mediated membrane fusion. However, the F^{N104Q} mutant is much more effective in low-pH dependent fusion (Fig. 5). The explanation may be that *N*-linked glycans form a large hydrophilic side on the surface of F proteins. Absence of *N*-glycans forming this hydrophilic face on the F_2 subunit possibly enhances the interaction between F proteins on the virus envelope and the late endosolic cell membrane, thus assists in the low-pH-induced conformational change to generate more effective membrane fusion. It is interesting to note that in HearNPV F_2 the only *N*-glycosylation site (N^{104}) is located just upstream of the only cystein (Cys^{108}). This Cys^{108} might be involved in the disulfide bridging of F_2 with F_1 (IJkel *et al.*, 2000) and it is conceivable that the N-glycans so close to the S-S bridge affect the conformation of the F and hence fusogenicity.

N-linked glycosylation is a prerequisite for proper folding as the glycan addition occurs when the nascent protein folds into its native form. *N*-linked glycans are responsible for the binding of chaperones, which are essential for correct folding in the ER (Hebert *et al.*, 1997; Imperiali & O'Connor, 1999; Parodi *et al.*, 2000; Helenius & Aebi, 2004). The fusogenicity of many viral envelope fusion proteins is dependent on correct folding and *N*-linked glycosylation. Removal of *N*-linked glycans from virus envelope fusion protein often has a

profound and cumulative effect, such as for the VSV G, SV5 F, RSV F and BVDV E2 protein (Machamer *et al.*, 1985; Bagai *et al.*, 1995; Zimmer *et al.*, 2001; Pande *et al*., 2005). In one case, elimination of multiple glycosylation sites in both F_1 and F_2 resulted in viruses with strongly enhanced fusogenicity (Aguilar *et al.*, 2006). Whether this is also the case for baculoviruses remains to be determined. Mutational analysis of the five putative *N*-glycosylation sites of F_1 should clarify this point.

It is tempting to speculate from an evolutionary perspective what would be the benefit for baculoviruses to have *N*-glycans on F_2 and reduced fusogenicity. Since all baculovirus F_2 subunits have this conserved *N*-glycosylation site, it must have a strong evolutionary advantage to have the *N*-glycans maintained. It could be that enhanced fusogenicity and spread kills the insect before it can adequately produce complete polyhedra and that there is an ecological trade-off between the production speed of BV in the insect versus the spatio-temporal spread and survival of the baculovirus via polyhedra in the field or environment. This would imply that the HearNPV F^{N104Q} mutant has shorter lethal time in insects than wild type HearNPV.

In summary, our study demonstrates that *N*-linked glycosylation of the F_2 subunit of HearNPV BVs is not essential for BV formation and fusogenicity with host cells. Mutation of the $N^{104}LT$ sequon and the absence of *N*-linked glycans on the F_2 subunit of HearNPV F resulted in enhanced fusogenicity of BVs. The availability of the HearNPV F^{N104Q} mutant and the potential to generate F_1 *N*-glycosylation mutants will provide important tools to further explore the mechanism of baculovirus F glycosylation, F-mediated fusion and understand the pathobiology of this virus. Mutation of the single putative *N*-linked glycosylation site in baculovirus F_2 might also be a novel strategy to generate baculoviruses with increased speed of action for inundative insect control.

ACKNOWLEDGEMENTS

We thank M. Zwart of the Laboratory of Virology (Wageningen University and Research Center) for technical assistance and helpful discussion. We are grateful to Marcel Westenberg for reading the manuscript. This work was funded by the grants from the Royal Academy of Sciences of The Netherlands (KNAW) (project numbers 04-PSA-BD-02) and a PhD sandwich grant to GL from Wageningen University

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Chapter 4

Functional Entry of Baculovirus into Insect and Mammalian Cells is Dependent on Clathrin-Mediated Endocytosis

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A modified version of this chapter was published as J. Virol. 80:8830-8833 (2006).

SUMMARY

Entry of the budded virus (BV) form of baculoviruses into insect and mammalian cells is generally thought to occur through a low-pH dependent endocytosis pathway possibly through clathrin-coated pits. This insight is primarily based on (immuno) electron microscopy studies, but requires biochemical support to exclude the use of other pathways. Here, we demonstrated chlorpromazine and bafilomycin A strongly inhibited baculovirus infection into insect cells and transduction into mammalian cells, indicating that functional entry of baculoviruses into insect and mammalian cells is primarily dependent on clathrin-mediated and low-pH dependent endocytosis. Surprisingly, inhibition of caveola-mediated endocytosis resulted in an increased baculovirus transduction efficiency. Our results suggest that caveolae are somehow involved in baculovirus entry in mammalian cells. Caveolar endocytosis inhibitor, genistein, enhances baculovirus transduction considerably.

INTRODUCTION

The *Baculoviridae* are a large family of enveloped DNA viruses and exclusively pathogenic to arthropods. Baculoviruses are accommodated taxonomically into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GVs) (Theilmann *et al.*, 2005). Baculoviruses produce two distinct virion phenotypes: occlusion-derived virus (ODV) and budded virus (BV) (Volkman and Summers, 1977), which are responsible for infection of insects and insect cells, respectively. Phylogenetic studies indicate that the NPV genus can be further subdivided into two subgroups: I and II (Bulach *et al.*, 1999; Hayakawa *et al.*, 2000; Herniu *et al.*, 2003). Members of the two NPV subgroups encode two different major BV envelope glycoproteins, GP64 for group I and F for group II, that mediate membrane fusion during viral entry (Pearson *et al.*, 2000).

The initial step for successful virus entry into target cells in general requires virion binding to cell surface specific molecules and followed by internalization for viral infection or virus-based gene delivery to proceed. Viruses from various families utilize different internalization and trafficking pathways to enter target cells, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and phagocytosis (Mellman, 1996; Sieczkarki and Whittaker, 2002; Pelkmans and Helenius, 2003; Dimitrov, 2004; Damm *et al.*, 2005; Marsh and Helenius, 2006). BVs of the baculovirus type species and group I NPV, *Autographa californica* nucleopolyhedrovirus (AcMNPV) (Ayres *et al.*, 1994), are thought to enter insect cells via adsorptive endocytosis (Volkman and Goldsmith, 1985; Hefferon *et al.*, 1999), as evidenced by immunological and (electron)microscopy observations. However, no direct biochemical evidence is available that shows that this is indeed the case for all baculoviruses including group II NPVs, whether alternative routes such as through caveolae (Pelkmans *et al.*, 2002) or macropinocytosis (Sieczkarski and Whittaker, 2002) are used, whether clathrin-coated pitts are involved and whether this holds for the entry of baculovirus into insect as well as mammalian cells.

In order to study and dissect the entry process of baculoviruses, the effects of the inhibitors chlorpromazine (Sigma) (Wang *et al.*, 1993) and genistein (Sigma) (Parton *et al.*, 1994; Dangoria *et al.*, 1996) on baculovirus entry in insect and mammalian cells were studied. Chlorpromazine is a cationic, amphiphilic molecule that acts by shifting clathrin and the AP-2 complex to the late endosomal compartment, thus inhibiting clathrin-mediated endocytosis. Genistein interferes with caveolae-mediated endocytosis by inhibiting viral internalization through caveolae; biochemically it blocks the phosphorylation of tyrosine kinase, which is involved in the formation of caveosomes. Bafilomycin A (Sigma) (Aniento *et al.*, 1996; Brindley and Maury, 2005), a specific inhibitor of endosome proton ATPase, was included to confirm that baculovirus BVs ultimately enter host cells in a low pH-dependent manner. In this chapter, we showed that functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. Additionally, lock up caveolar endocytic pathway enhances baculovirus transduction.

MATERIAL AND METHODS

Cells and drug treatment

The *Heliothis* cell line HzAM1 and *Spodoptera frugiperda* cell line Sf21 were cultured

at 27°C in plastic tissue culture flasks (Nunc) in Grace's insect medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS). BHK21 cells were cultured at 37 °C in Glasgow MEM medium (Invitrogen), supplemented with 10% FBS.

Before infection or transduction, insect and mammalian cells were pretreated with bafilomycine A, chlorpromazine and genistein in serum free medium for 30mins. Following two times of wash with fresh serum free medium, infection was performed at a multiplicity of infection (m.o.i.) of 5 $TCID_{50}$ units per cell and transduction was performed as a MOI of 100. Expression of fluorescent protein was observed 24 hours post infection in insect cells and 24 hours post transduction in BHK21 cells.

Recombinant virus

pFB-GFP (Long *et al.*, 2006b), a donor plasmid with a GFP gene under control of p10 promoter, was used to make recombinant AcMNPV and HearNPV bacmid carrying p10 promoter controlled egfp gene. The pFB-GFP was further modified by inserting a CMV immediate early (IE) promoter controlled monomeric red fluorescent protein (mRFP) gene. Briefly, the CMV IE promoter sequence and mRFP gene were PCR amplified. An upstream Bst1107I restriction site and a downstream BamHI restriction site were introduced into the CMV IE promoter fragment by primers (forward primer: 5'-GGG GTATAC AATAGTAATCAATTACGGGGTCAT-3'and reverse primer: 5'- GCG GGATCC GCTAGCGGATCTGACGGTTCACTA-3'), using vector pEGFP-C2 (GenBank Accession number:U57606) as template. While an upstream BamHI restriction site and a downstream HindIII restriction site were introduced into RFP fragment by primers (forward primer: 5'-GGG GGATCC ATGGCCTCCTCCGAGGACGTC -3'and reverse primer: 5'- GCG AAGCTT TTAGGCGCCGGTGGAGTGGCG -3'), using vector carrying mRFP1 (Dr. Roger Tsien) as template. Three-fragment ligation was performed among the pFB-GFP fragment restricted by Bst1107I and HindIII, CMV IE promoter fragment restricted by Bst1107I and BamHI, and the mRFP gene fragment restricted by BamHI and HindIII. Resulting plasmid named pFB-GR, carrying a p10 promoter controlled gfp and a CMV IE promoter controlled rfp (in opposite orientation), was used to make recombinant AcMNPV bacmid expressing RFP upon transduction.

Transfection

To produce recombinant baculoviruses, bacmid transfection was performed according Bac-to-Bac manual (Invitrogen). BHK21 cells were transfected by either 5 μg of EΔ95/295 or D3Δ2 plasimd using 15μl Lipofectin (Invitrogen). 48 hours after transfection, transfected BHK21 cells were subjected to baculovirus transduction.

RESULTS AND DISCUSSION

After a 30 min treatment with the respective drugs, Sf21 cells were incubated for 1 h with an AcMNPV carrying a GFP gene under control of a p10 promoter (Lopez-Ferber *et al.*, 1995), at a m.o.i. of 5 $TCID_{50}$ units per cell. Infected cells were incubated in drug free Grace's medium supplemented with 10% fetal bovine serum (FBS). GFP expression was examined 24 h post infection (p.i.) by fluorescence microscopy. The infectivity was estimated by percentage of GFP expressing cells for each treatment. Bafilomycin A and chlorpromazine

inhibited AcMNPV infection in Sf21 cells in a dose-dependent manner (Fig. 1A and C). In contrast, AcMNPV infection was not inhibited by genistein, even at a very high concentration, and this supports the view that AcMNPV entry into insect cells is primarily clathrin-mediated. It has been demonstrated that lipid rafts are not involved in AcMNPV entry (Zhang, *et al.*, 2003).

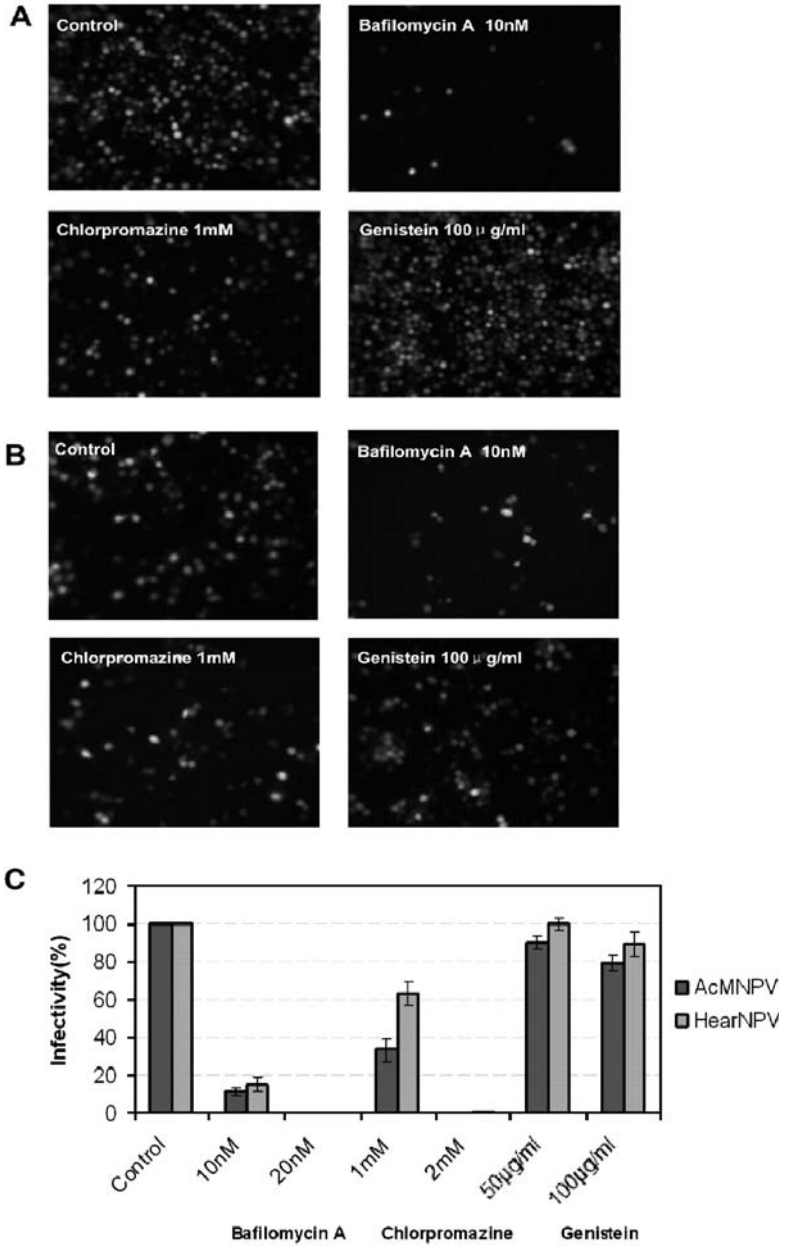

Figure 1. Baculovirus NPVs infect insect cells through a clathrin-mediated and low-pH dependent endocytosis. Insect cells were cultured in Grace's medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO). Sf21(A) and HzAM1(B) cells were pretreated with 10nM Bafilomycin A, 1mM chlorpromazine and 100 μ g genistein and subsequently infected with AcMNPV (A) and HearNPV (B) at a multiplicity of infection of 5 $TCID_{50}$ units per cell for 1h. Cells were rinsed twice with fresh medium and further cultured for 24 h. (C) Virus infectivity was quantified as the percentage of GFP expressing cells relative to total cell number. The data shown are the means and standard deviations from three independent experiments

Recently a novel type of baculovirus BV envelope fusion protein, named F, was identified in NPV group II baculoviruses (IJkel *et al.*, 2000; Pearson *et al.*, 2000; Long *et al.*, 2006a). In order to study the entry pathway of these viruses, the same experiment was performed as in Fig. 1A, but now by using BVs of a group II NPV, *Helicoverpa armigera* NPV (HearNPV), and *H. armigera* Am1 (HzAM1) cells. Similar results were achieved as for AcMNPV (Fig. 1B and C; group I NPV). Chlorpromazine inhibition was almost complete at a concentration of 2mM, for both AcMNPV and HearNPV (Fig. 1C). The results obtained with these inhibitors provide independent, biochemical support for the view that BVs of both group I and group II baculoviruses enter insect cells primarily through clathrin-mediated endocytosis. The caveolae-mediated endocytosis pathway appeared not involved in baculovirus NPV BV infection of insect cells as the virus uptake is unaffected by genistein (Fig. 1C).

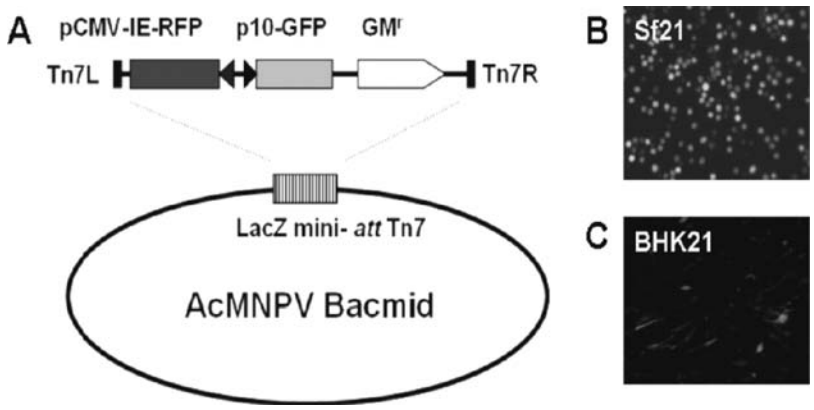

Figure 2. Construction of AcMNPV-GR. (A) A p10 promoter controlled eGFP gene and a CMV immediate early (IE) promoter-controlled RFP gene was introduced into an AcMNPV bacmid resulting in AcMNPV-GR. GFP expression in Sf21 cells (B) and RFP expression in BHK21 (C) cells were examined.

Baculovirus BVs have been reported to effectively deliver genes into mammalian cells and this has provided great impetus for the study and development of more effective baculovirus-based gene therapy vectors (Hofmann *et al.*, 1996; Boyce and Bucher, 1996; Shoji *et al.*, 1997; Pieroni *et al.*, 2001; Kost and Condreay, 2002; Kaikkonen *et al.*, 2006). Understanding of the functional entry pathway of baculovirus into mammalian cells is thus pivotal for a successful entry process. Biochemical evidence suggested that baculovirus transduction into these cells is low-pH dependent and microscopical evidences suggested that baculovirus enters e.g. human hepatoma cells through clathrin-mediated endocytosis and possibly through macropinocytosis (van Loo *et al.*, 2001; Matilainen *et al.*, 2005). But the functional entry pathway of baculovirus into mammalian cells, especially from the cell surface to the early endosome (Marsh and Helenius, 2006), requires further investigation. We therefore studied baculovirus entry into mammalian cells (Fig. 2) using the same set of inhibitor drugs as tested in insect cells (Fig. 1). Using the bacmid system (Luckow and Summers, 1988) a novel recombinant AcMNPV, AcMNPV-GR, carrying a green fluorescent protein (GFP) gene under the control of the AcMNPV p10 (late) promoter to evidence AcMNPV replication and a monomeric red fluorescent protein (mRFP) gene under the control

of the cytomegalovirus (CMV) IE (early) promoter was constructed to evidence entry, uncoating and transport to the nucleus (Fig. 2A).

Sucrose-purified AcMNPV-GR BVs (Long *et al.*, 2006a) were resuspended in PBS and used to transduce BHK21 cells. GFP expression in AcMNPV-GR infected Sf21 cells and RFP expression in AcMNPV-GR transduced BHK21 cells were observed using fluorescence microscopy (Fig. 2B and 2C). Bafilomycin A, chlorpromazine and genistein were applied to dissect the functional entry pathway of AcMNPV into BHK21 cells. After 30 min of drug treatment, BHK21 cells were transduced with AcMNPV-GR at a m.o.i. of 100 $TCID_{50}$ units per cell for 1 h, RFP expression was examined 24 h post transduction. Transduction efficiency was calculated by the percentage of RFP expressing cells. Similar to the results obtained from the baculovirus entry into insect cells (Fig. 1), bafilomycin A and chlorpromazine inhibited AcMNPV transduction into BHK cells (Fig. 3) suggesting that the clathrin-mediated and low-pH dependent endocytic pathway is indeed involved in functional entry of AcMNPV into BHK21 cells.

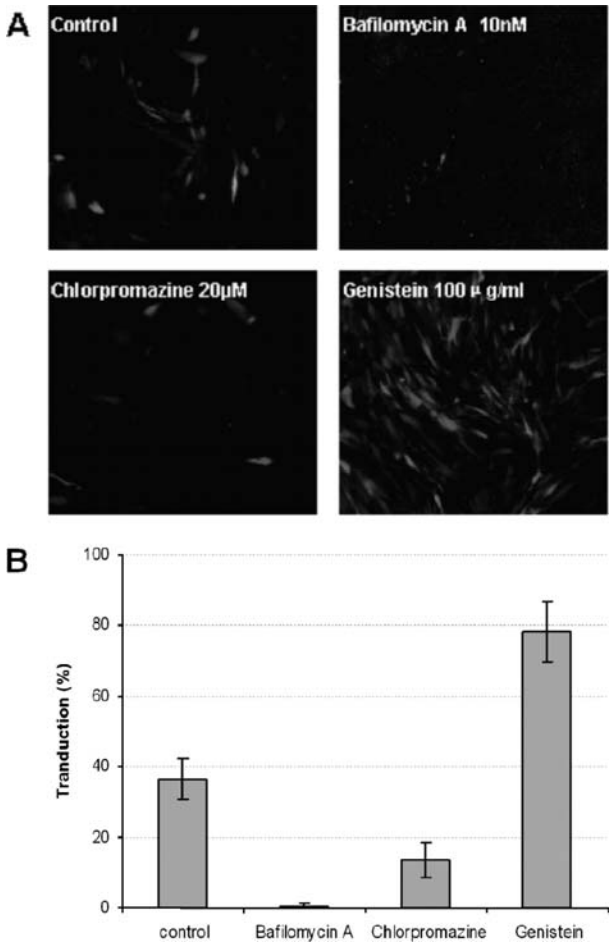

Figure 3. Effects of drugs on AcMNPV-GR transduction into BHK21 cells. BHK21 cells were cultured in DMEM medium (GIBCO) supplemented with 10% FBS.(A) BHK21 cells were pre treated with 10nm bafilomycin A, 20 μ M chlorpromazine and 100ug genistein and subsequently transduced with AcMNPV-GR at a m.o.i. of 100 5 $TCID_{50}$ units per cell for 1h. (B) Transduction efficiency was evaluated as the percentage of RFP expressing cells relative to the total cell number. The data shown are the means and standard deviations from three independent treatments.

Surprisingly, a high concentration of genistein (100 µ g/ml), rather then being without effect on virus entry in insect cells, greatly enhanced AcMNPV transduction from 30% to more than 70% transduced cells (Fig. 3). This is an unexpected result as entry of vertebrate enveloped viruse is either not affected by inhibition of the caveolar pathway, e.g. influenza virus (Sieczkarski and Whittaker, 2002) or is reduced as in the case of murine leukemia virus (Beer *et al.*, 2005). Our result may suggest that caveolae-mediated endocytic pathway is somehow involved in the functional entry of AcMNPV into BHK21 cells or is enhanced as a consequence of increased uptake of cholesterol (Imelli *et al.*, 2004). The enhanced transduction efficiency could also be explained by a lock-up of the caveolae-mediated endocytic pathway by genistein, possibly driving more virus particles to enter mammalian cells through clathrin-mediated endocytosis. Alternatively, the response of insect cells to genistein showing no effect on virus uptake may be different although the primary biochemical response i.e. the inhibition of tyrosine kinase-mediated phosphorylation is the same (Shimabukuro *et al.*, 1996; Arif *et al.*, 2003)

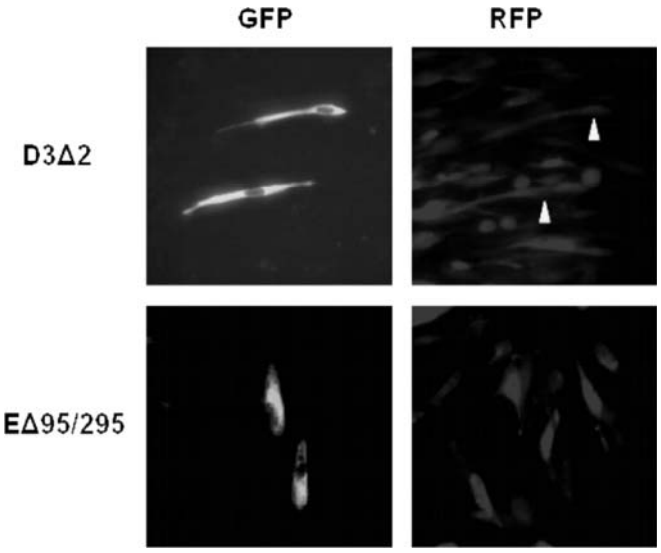

Figure 4. Transduction is inhibited in cells expressing EΔ95/295. BHK21 cells were transfected with plasmids encoding either GFP-D3Δ2 (upper panels) or GFP-EΔ95/295 (lower panels). 48 h after transfection, cells were transduced with AcMNPV-GR BVs. 24 h after transduction, GFP (left panels) and RFP (right panels) expression were examined.

BHK21 cells are sensitive to high concentrations (50 µ g/ml) of chlorpromizine, thus complete inhibition of transduction into BHK21 cells by chlorpromizine does not occur. To confirm that the clathrin-mediated endocytosis is part of the functional entry of AcMNPV into mammalian cells, we used EΔ95/295, a dominant-negative form of Eps15 (EGFR pathway substrate clone 15), which specifically interferes with clathrin-coated vesicle formation at the plasma membrane and thus inhibits virus entry (Benmerah *et al.*, 1999). As a control, we used EGFR mutant D3Δ2, another form of Eps15 with no dominant-negative effect on clathrin-mediated endocytosis. EΔ95/295 or D3Δ2 with an N-terminally fused eGFP gene to allow detection were transiently expressed in BHK21 cells by using Lipofectin (Invitrogen). Fourty-eight hours after transfection BHK21 cells were transduced with AcMNPV-GR at a m.o.i. of 100 $TCID_{50}$ units per cell. Twenty-four hours after transduction, RFP and GFP expression was determined using fluorescence microscopy. As expected, D3Δ2 expression had no effect on AcMNPV transduction, whereas AcMNPV transduction was strongly inhibited in cells transfected with EΔ95/295 (Fig. 4). This result is consistent with the result from chlorpromazine treatment (Fig. 3) and proved that clathrin-mediated endocytosis is the major

functional pathway for AcMNPV entry into BHK21 cells.

In conclusion, our results using various inhibitor drugs and reporter constructs support the view that baculovirus NPVs, regardless whether they belong to group I (GP64) or group II (F) NPVs, primarily enter insect and mammalian cells through a clathrin-mediated, low-pH dependent endocytic pathway. Baculovirus may enter mammalian cells through multiple pathways, but the claveolae-dependent entry is somehow involved as genistein enhances transduction.. These data imply that baculoviruses may be tailored by genetic engineering to enter mammalian cells more efficiently through clathrin-mediated endocytosis and promote that tyrosine kinase inhibitors be used as novel agents to enhance baculovirus-based gene delivery in these cells.

ACKNOWLEDGEMENT

We are grateful to Dr. A. Benmerah (Cochin Institute, Paris) for kindly providing EΔ95/295 and D3Δ2 constructs. This work was funded by the grants from the National Science Foundation of China (NSFC 30300012 to Gang Long), the Royal Academy of Sciences of The Netherlands (KNAW) (project numbers 04-PSA-BD-02) and a PhD sandwich grant to GL from Wageningen University.

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Chapter 5

Functional Role of the Cytoplasmic Tail Domain of the Major Envelope Fusion Protein of Group II Baculoviruses

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This chapter was published as J. Virol.80 :11226-11234, (2006).

Chapter 5

SUMMARY

F proteins from baculovirus nucleopolyhedrovirus (NPV) group II members are the major budded virus (BV) viral envelope fusion proteins. They undergo furin-like proteolysis processing in order to be functional. F proteins from different baculovirus species have a long cytoplasmic tail domain (CTD), ranging from 48 (*Spodoptera litura* MNPV) to 78 amino acid residues (*Adoxophyes honmai* NPV) with a non-assigned function. This CTD is much longer than the CTD of GP64-like envelope fusion proteins (7 aa), which appeared non-essential for BV infectivity. Here we have investigated the functional role of the CTD of *Helicoverpa armigera* single capsid NPV (HearNPV), a Group II NPV. We combined a newly constructed HearNPV *f*-null bacmid knockout-repair system and an AcMNPV *gp64*-null bacmid knockout-pseudotype system with mutation and rescue experiments to study the functional role of baculovirus F protein CTD. We show that except for the 16 C-terminal aa the HearNPV F CTD is essential for virus spread from cell to cell. In addition, the CTD of the HearNPV F is involved in BV production in length dependent manner and is essential for BV infectivity. Tyrosine residue Y658 at 16 aa from the C-terminus seems to be critical. However, HearNPV F without a CTD still rescues infectivity of *gp64*-null AcMNPV BV indicating that the CTD is not involved in processing and fusogenicity. Altogether, our results indicate that F protein is essential for baculovirus BV infectivity and that the CTD is important for F protein incorporation into BV.

INTRODUCTION

The family *Baculoviridae* are a large group of enveloped, double strand DNA viruses which exclusively infect arthropods, predominantly insects of the order *Lepidoptera* (Blissard *et al.*, 2002). Baculoviruses are divided into two genera, the nucleopolyhedroviruses (NPV) and granuloviruses (GV). The genera of NPV can be phylogenetically subdivided into two subgroups: group I and group II (Zanotto *et al.*, 1993; Herniou *et al*., 2001). During an infection cycle, baculoviruses produce two virion phenotypes that play a distinct role. Virions of the occlusion derived virus (ODV) mediate the transmission of virus from insect to insect, whereas budded virus (BV) mediate the cell-to-cell spread of virus in infected insects and in cell culture.

BVs enter cells via the endocytic pathway including two steps: clathrin-mediated endocytosis and low-pH-dependent membrane fusion (Long *et al.,* 2006b). GP64 is the major envelope fusion protein found in the BV of *Autographa californica* multicapsid NPV (AcMNPV) and other members of the group I NPVs. Previous studies showed that GP64 is involved in BV binding to target cells, necessary for the low-pH-dependent membrane fusion to allow entry and important for efficient virion budding or egress from the cell surface (Blissard and Wenz, 1992; Monsma and Blissard, 1995; Oomens and Blissard, 1999). Recently, a new type of envelope fusion protein named F was identified from *Lymantria dispar* MNPV (Pearson *et al.*, 2000), *Spodoptera exigua* MNPV (IJkel *et al.*, 2000) and *Helicoverpa amigera* NPV (Long *et al.*, 2006) and most likely present in all group II NPVs. The F protein is the major glycoprotein of group II BVs, expressed first as a precursor (F_0) and cleaved into two disulfide bridge linked subunits (F_1 and F_2) by a cellular furin-like convertase (IJkel *et al.*, 2000, Westenberg *et al.*, 2002). The cleavage is essential for activation of F protein into a fusogenic form (Westenberg *et al.*, 2002). In addition, group II NPV F proteins are able to rescue infectious BV production of GP64 knock-out mutant of AcMNPV (Lung *et al.*, 2002). Therefore, these F proteins are functionally analogous to GP64. As such baculovirus F proteins are similar in structure of envelope fusion proteins of vertebrate viruses (Eckert and Kim, 2001).

The cytoplasmic tail domains (CTD) of many viral envelope fusion proteins play an important role in virus assembly and budding through its binding to the nucleocapsid protein (Schmitt and Lamb, 2004). The CTDs of virus glycoproteins are clearly involved in virus assembly, since they bind to the matrix protein of nucleocapsid and collate the viral envelope and nucleocapsid. (Dorfman *et al.*, 1994; Freed and Martin, 1996; Wyma *et al.*, 2000). Another commonly recognized function of the CTD of viral glycoproteins is in glycoprotein trafficking and subcellular localization (Raviprakash *et al.*, 1990; Sauter *et al.*, 1996; Lodge *et al.*, 1997a; Grange *et al.*, 2000; Zhang *et al.*, 2000; Palmarini *et al.*, 2001; Edwards *et al.*, 2002), and in assembly of mature virions (Owens and Rose, 1993; Brody *et al.*, 1994; Cathoman *et al.*, 1998; Murakami and Freed, 2000; Christodoulopoulos and cannon, 2001). In addition, a further role of CTDs in virion morphology, in completion of membrane fusion and in regulation has also been reported (Mulligan *et al.*, 1992; Gage *et al.*, 1993; Ragheb and Anderson, 1994; Rein *et al.*, 1994; Bagai and Lamb, 1996; Januszeski *et al.*, 1997; Melikyan *et al.*, 2000; Aguilar *et al.*, 2003; Waning *et al.*, 2004). Unlike GP64, the BV fusion protein of a group I baculovirus (*Autographa californica* MNPV = AcMNPV) with a short CTD of 7 aa, baculovirus group II NPV F proteins have comparatively long CTDs, ranging from 48 (*Spodoptera litura* MNPV) to 78 aa residues (*Adoxophyes honmai* NPV). The CTD of the

AcMNPV GP64 is not essential for production of infectious BV; removal of the CTD results in a reduction in budding efficiency (Oomens and Blissard, 1999). The role of the CTD of baculovirus F proteins remains to be determined.

In this study, we investigated the functional role of the CTD (49 aa) of HearNPV F protein as a representative of group II baculovirus F proteins. We determined the minimal length of the CTD that still renders infectious BV production by making C-terminal truncations of HearNPV F and found that the CTD can be truncated with 16 aa residues from the C-terminus without loss of the infectivity. However, further F protein CTD truncations maintain to support infectious virus production of AcMNPV *gp64*-null mutants, when pseudotyped. Our results indicate that the CTD of F proteins of group II NPVs is essential for infectious BV production. Deletion of the F protein CTD does not affect F protein synthesis or fusogenicity.

MATERIALS AND METHODS

Cells and virus

The HzAM1 cells(McIntosh and Ignoffo, 1983) and *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 (pH5.9-6.1, Invitrogen), supplemented with 10% fetal bovine serum (FBS). BVs of the HearNPV G4 isolate were used for infections and was propagated in HzAM1 cells (Sun *et al.*, 1998).

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

For generation of a *f* gene deletion mutant, HearNPV bacmid Hz8 (Wang *et al.*, 2003) was used as the parent bacmid. Long primers were designed with on the 5'-end 50 nt corresponding to 50 nt flanking regions of the *f* gene in the HearNPV G4 genome. The forward primer was 5'-GGATTTGCCGTCGTAACAAATTAAACAAAAAAATTTTCATA AAAACAAATAAATTTAAGGGCACAATAACTG-3', containing the viral flanking sequences from nt 127755 to 127804 according to the HearNPV G4 genome sequence (GenBank No AF271059). Reverse primer was 5'-CATACATTTATTAATCTAGTTATT TTTATTAAACCTTTCCGTAATAGTTATTCCTGTGCGCGACGGTTAC-3', with viral flanking sequences from nt 129905 to 129954. The 3' ends (underlined regions) of the primers anneal to the chloramphenicol resistance gene (*cat*) of pBeloBac11 (Shizuya *et al.*, 1992).

To introduce homologous recombination, helper plasmid pBAD-αβγ was eletro-transferred into DH10B cells (InVitrogen) containing HearNPV bacmid HZ8 (Wang *et al.*, 2003). And electrocompetent cells were made according to Muyrers *et al.* (Muyrers *et al.*, 1998). PCR to generate linear fragments was performed by using the high-fidelity 'Expand' long-template PCR system (Roche). Column purified PCR product was digested with *Dpn*I to eliminate template plasmid DNA. After digestion, the linear fragment was recovered from agarose gels and finally collected by ethanol precipitation. One μg of linear fragment was applied for electroporation into DH10B containing the bacmid HZ8 and the resulting *f* gene-deleted bacmid was designated as HearNPV *f*-null. The deletion of the *f* gene in the *f*-null HearNPV bacmid was confirmed by *Eco*RI restriction profiling and PCR.

Donor plasmid and transposition

The pFASTBAC DUAL vector (InVitrogen) is the donor plasmid for the baculovirus insect cell expression system and carries the AcMNPV polyhedrin promoter and p10 promoter in opposite orientations. An eGFP gene was amplified by PCR, using peGFP plasmid (Clontech) as template DNA. An upstream *Nhe*I restriction site and a downstream *Kpn*I restriction site were introduced in the subsequent primers for further cloning. The PCR products were first cloned into a pGEM-Teasy vector (Promega), giving pGFP. After a sequence check, the *Nhe*I-to-*Kpn*I fragment of GFP gene was cloned into the pFASTBAC DUAL vector under control of p10 promoter, resulting in pFB-GFP.

The HearNPV *f* cassette containing the coding sequence of the HearNPV F protein plus 300 nt promoter sequence (from nt 127811 to nt 130144) was amplified by PCR, using *Pfu* ultra DNA polymerase (Stratagene). An upstream *Bst*1107 I restriction site and a downstream *Hin*dIII restriction site (underlined) were introduced in the primers (forward primer: 5'-GGG GTATAC CCRRRRAGAAAACCTTGGTACTGT-3' and reverse primer: 5'-GCG AAGCTT ACAATTTGTGTTCATCATATTGATC-3') for further cloning. After cloning and sequencing verification, the *Bst*1107 I-to-*Hin*dIII fragment of HearNPV F cassette was cloned into pFB-GFP. The resulting vector, carrying the *f* gene cassette and the *p10* promoter controlled eGFP, was named pFB-F&GFP.

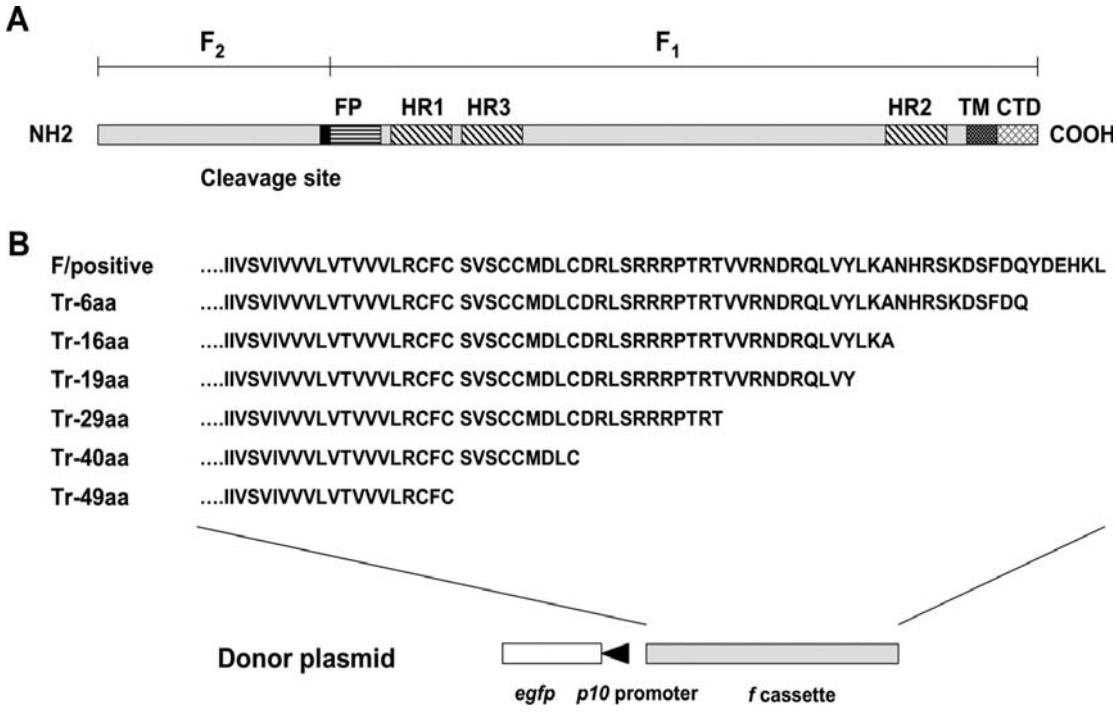

Figure 1. Schematic diagram of the baculovirus Group II *f* gene with proteolytic cleavage site, fusion peptide (FP), heptad repeat (HR) regions, transmembrane region (TM) and cytoplasmic tail (CT) domain of HearNPV F (A) and the C-terminal truncations of F (B). Each donor plasmid carrying HearNPV *f* or truncated *f* genes was used to construct 'repair' HearNPV bacmids and pseudotyped AcMNPV bacmids.

A unique *Sph*I restriction site is located 425 nt upstream of the translational termination codon TAA (Fig. 1). To generate cytoplasmic tail truncation mutations, a forward primer (5'-ATCGCATGCAATTCGAGTTGGAAC -3') covering the *Sph*I restriction site (underlined) and reverse primers (Tr-6aa: 5'-GCG AAGCTT A TTGATCGAAAGAATCTTTAG -3'; Tr-16aa: 5'-GCG AAGCTT A GGCTTTCAAATAGACGAGTTG-3'; Tr-19aa: 5'-GCG AAGCTT A ATAGACGAGTTGACGATCGTTG-3'; Tr-29aa: 5'-GCG AAGCTT A CGTTCGTGTGGGTCTTCGTCT-3'; Tr-40aa: 5'-GCG AAGCTT A GCACAAGTCCATACAACAAGATAC-3' and Tr-49aa: 5'-GCG AAGCTT A ACAAAAACATCGTAAAACTACAACAG-3') located at different positions within the CTD sequence were designed. A translational termination codon TAA and a *Hin*dIII restriction site (underlined) were introduced in each truncation reverse primers to stop translation and to assist further cloning. PCR was performed by using the forward primer covering the *Sph*I site and each reverse primer. All the PCR products were cloned into pGEM-Teasy vector. After sequence verification, the *Sph*I-to-*Hin*dIII fragments were cloning into pFB-F&GFP, resulting in the donor plasmids carrying *f* gene cassettes expressing F protein with various truncations (-6 to -49 aa from the C-terminus) in the CTD. The donor plasmids were used to construct both the repair HearNPV bacmids and to pseudotype AcMNPV bacmids with truncated *f*.

Competent cells containing *f*-null HearNPV bacmid and *gp64*-null AcMNPV bacmid (provided by Dr. G. W. Blissard) were made according to Bac-to-Bac manual (Invitrogen). Transpositions of inserts from donor plasmids to the *f*-null HearNPV and *gp64*-null AcMNPV bacmids were confirmed by diagnostic PCR using a gentamycin resistance gene forward primer (5'-AGCCACCTACTCCCAACATC-3') in combination with the M13 forward primer (5'-CCCAGTCACGACGTTGTAAAACG-3') and sizing the products in 0.6% agarose gels.

Transfection of pseudotyped AcMNPV bacmids and HearNPV *f* repair bacmids into HzAM1 cells

For the transfection of pseudotyping AcMNPV bacmids, Sf21 cells were transfected with approximately 1 µg bacmid DNA according to Bac-to-Bac manual (Invitrogen). The supernatant was harvested 5 days post transfection (p.t.). For the transfection of repaired HearNPV bacmids, a modified transfection method was applied. Briefly, HzAm1 cells were seeded in 6-well plates (Nunc) with $1*10^6$ cells per well. After 24 h incubation in Grace's insect medium supplemented with 10% FBS, cell were washed twice by Grace's insect medium. Then, cells were transfected with approximately 1 µg bacmid DNA using 15 µl Lipofectin (Invitrogen). Supernatants, containing BVs, were harvested 7 days post transfection (p.t.) of HaAM1 cells.

Western-analysis

The expression and incorporation of the complete and truncated F proteins from the HearNPV *f* repair bacmids in BVs was examined by western analysis using polyclonal antibodies against F_1 and F_2 to probe sucrose-purified BVs (Long *et al.*, 2006a). Briefly, sucrose-purified BVs were disrupted in reducing or non-reducing conditions and were then denatured for 10 min at 95°C. Proteins were separated by SDS-PAGE and subjected to Western analysis as previously described (Long *et al.*, 2006a). The antisera raised against F protein in chicken were used in a 1:1000 dilution and the (truncated) F proteins were detected by treatment with horseradish peroxidase conjugated rabbit anti-chicken immunoglobulin

(Sigma) diluted 1:10,000 followed by ECL technology as described by the manufacturer (Amersham).

Budded virus production

To investigate infectious BV production from the F protein CTD truncation mutants relative to the 'wild type' BV and the full *f* repair HearNPV, virus growth curves were generated. HzAM1 cells were infected with a multiplicity of infection (m.o.i.) of 5 $TCID_{50}$ units/cell for 1 h. After infection, cells were washed with fresh medium once and then incubated in new medium. At 12, 24, 36, 48, 60, 72, 84 and 96 h.p.i.), the supernatants were collected. For each mutant virus, and each time point, triplicate samples were collected from triplicate experiments. The quantity of infectious BVs from each sample was determined by an end point dilution assays (EPDA) on HzAM1 cells. To analyze BV production of pseudotyped AcMNPVs compared to wild type AcMNPV, Sf21 cells were infected at an m.o.i. of 5. After 48 h.p.i. for each treatment samples were collected from triplicate supernatant and the quantity of infectious BVs from each sample was determined by EPDA on Sf21 cells. The results from above were exported to Microsoft Excel software and the average of triple log-transferred $TCID_{50}$ values were analyzed and the standard deviations of the mean were determined (Fig. 3). For the infectivity of pseudotyped AcMNPVs (Fig. 6), further one-way ANOVA and post-hoc tests were performed to analyze the significance of the difference in BV production among the pseudotyped viruses.

RNA analysis of HzAM1 cells infected with pseudotyped AcMNPV

HzAM1 cells, susceptible but non-permissive for AcMNPV, were challenged with pseudotyped AcMNPVs at an m.o.i. of 5 $TCID_{50}$ units per cell. Cells from each infection treatment were collected 12 h.p.i. Total RNAs were extracted from each cell sample by Trizol reagent (Invitrogen). Early transcription of *ie-1* was examined by 3' RACE analysis by using a RACE kit (Roche). In brief, first-strand cDNA synthesis was performed by using AMV reverse transcriptase and an oligo (dT) anchor primer, according to manufacturer. The cDNA was amplified with PCR using the anchor primer and an *ie-1* specific primer, *ie-1*-F (5'-'GTACAAATACAGCAGCGTCGCTA-3). The PCR products were analyzed by agarose gel electroporesis. Total RNA samples were served as reverse transcription template control.

RESULTS

Construction of a *f*-null HearNPV bacmid

To study the functionality of the HearNPV *f* gene during virus infection, a *f* null HearNPV bacmid was constructed by deleting the *f* gene from HearNPV bacmid HZ8 according to the method of Muyrers *et al.* (Muyrers *et al.*, 1999). 70-mer primer and a 72-mer oligonucleotides were used to PCR-amplify a linear DNA fragment containing a *cat* gene flanked by 50 bp of sequences homologous to HearNPV genome. After *Dpn*I treatment to eliminate template plasmid, the PCR product was purified and transferred into recombinant competent cells. Bacterial colonies harboring recombinant bacmid were selected on medium containing kanamycin and chloramphenicol. In the resulting *f*-null HearNPV bacmid, the *f* gene was correctly substituted by the *cat* gene (Fig. 2A). The deleted sequence was from 61bp upstream of the translational start codon to 6bp downstream of the stop codon (127803bp to 129904bp according to HearNPV G4 genome sequence). When the *f* gene is present, as in the parental bacmid HZ8 (41), a 10 kbp *Eco*RI band is present (a), whereas two new *Eco*RI bands

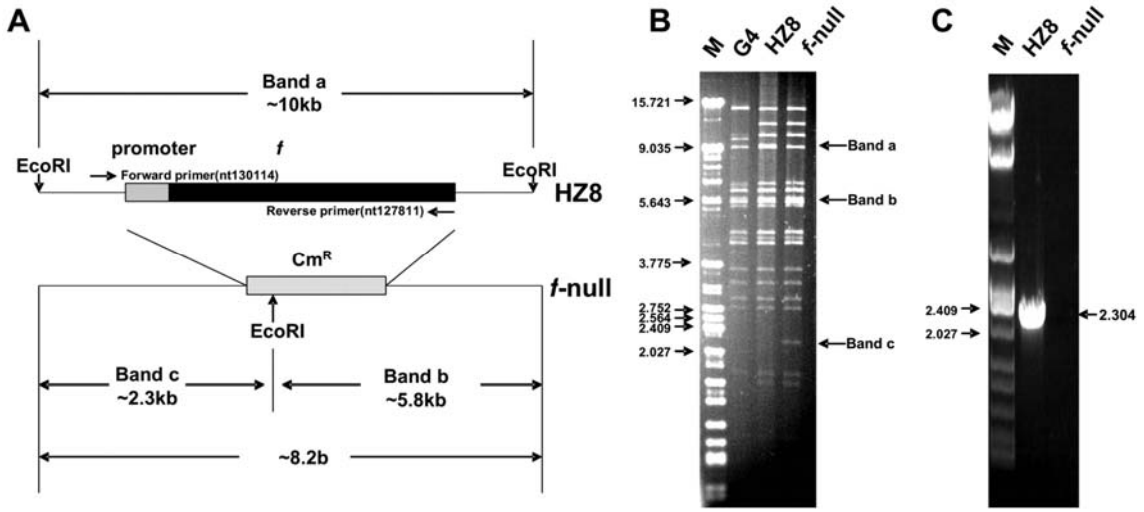

Figure 2. Strategy for the generation of an *f-null* HearNPV bacmid by ET recombination in *E coli* (A) and *Eco*RI restriction profiles (B) and PCR verification (C) of the *f*-null HearNPV bacmid.
(A) At the HearNPV *f* gene (Ha133) locus in the HearNPV bacmid Hz8 (41), a PCR amplified DNA fragment containing the chloramphenicol resistance gene (*cat*) flanked by 50bp HearNPV sequences on both side of *f* gene was used to replace the *f* gene by CM^r gene. (B) *Eco*RI restriction profile of wild type HearNPV (G4 strain) genome, the parental HearNPV bacmid HZ8 and the *f*-null HearNPV bacmid. An around 10kb *Eco*RI restriction fragment containing *f* gene from HZ8 was disrupted by ET recombination resulting in two smaller fragments (with approximately length of 2.3 kb and 5.8 kb, respectively). (C) Diagnostic PCR of the *f*-null HearNPV bacmid. Forward and reverse primers (panel A) were used for the PCR reaction. Bacmid HZ8 is a positive control for the presence of *f*. Sizes of markers (M) are indicated on the left of the gels (in kb).

(b and c), 5.8 and 2.3 kbp in size respectively, are generated when the *f* gene has been replaced by the *cat* gene (Fig. 2b). A PCR product the size of *f* is only generated in bacmid HZ8 and not in the *f*-null HearNPV bacmid (Fig. 2C).

Truncation in the HearNPV F CTD gene and construction of *f* repair HearNPV

The *egfp* gene controlled by the AcMNPV *p10* promoter in association with the HearNPV *f* gene cassette with and without truncation in the CTD was transposed into the Tn7 transposition site of the *f*-null HearNPV bacmid according to the Bac-to-Bac manual (Invitrogen). Bacmid DNA was then isolated from bacterial colonies after diagnostic PCR verification for correct insertion. Approximately 5 µg 'repair' Bacmid DNA from each transposed bacmid was transfected into 1*10E6 HzAM1 cells with transfection of (*f*-null/*egfp*) as negative control. At 7 d.p.t. GFP expression in transfected cells was examined (Fig. 3A). The presence of eGFP in transfected cells signaled effective transfection. Production of infectious BVs from each transfection was determined by infecting new HzAM1 cells with BVs in the transfection supernatant. Five days post infection, eGFP expression in infected cells was examined (Fig. 3B). The 'repair' bacmids with complete *f* (positive control), Tr-6aa and Tr-16aa were able to produce infectious BVs. The other recombinants: Tr-19aa, Tr-29aa, Tr-40aa and Tr-49aa, only showed single cells infected (Fig. 3A) and were not able to produce infectious BV (Fig. 3B). These results indicate that the CTD of F plays an important role in baculovirus BV propagation and that the CTD length is critical

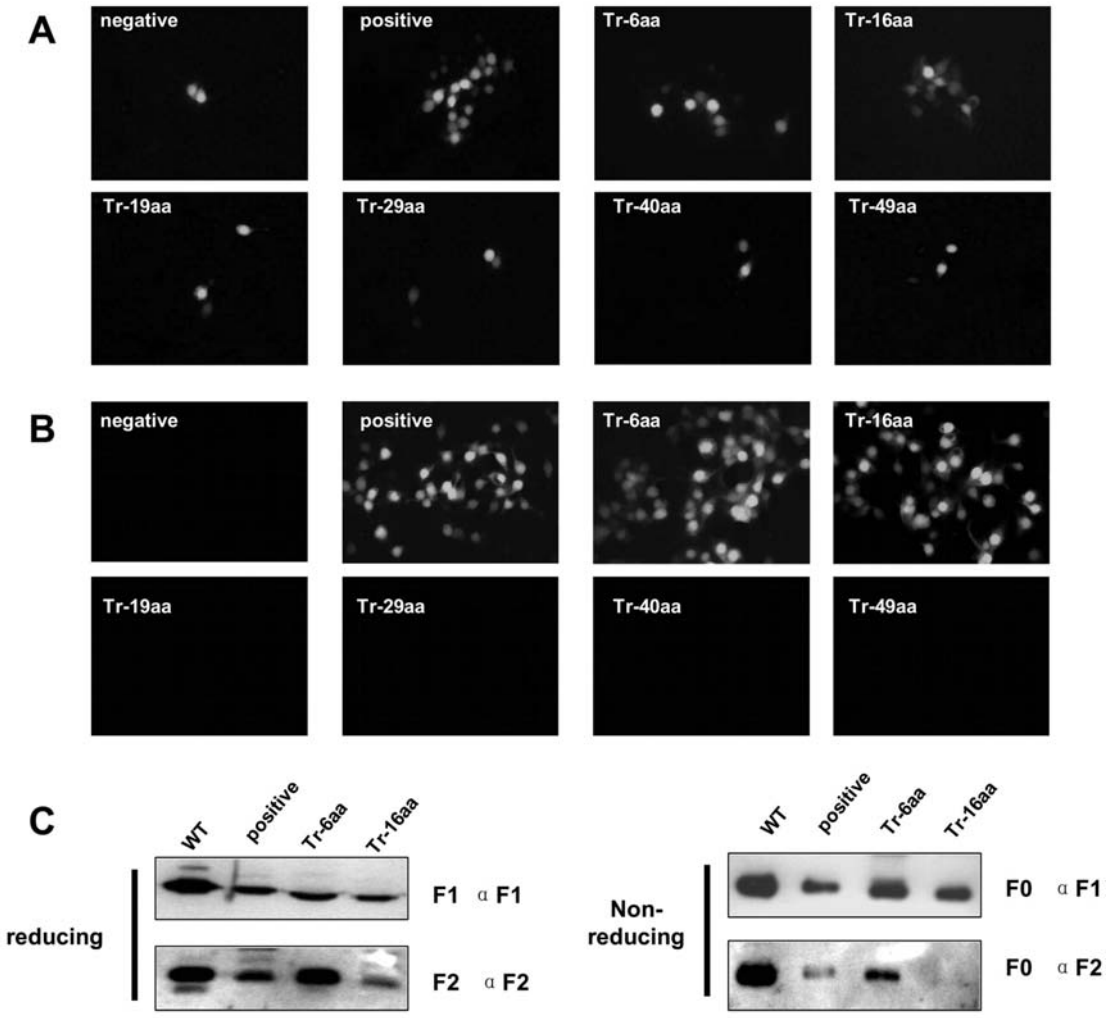

Figure 3. (A) Transfection of 'repair' HearNPV bacmid. (B) Infection assay for viral propagation. (C) Western blot analysis of 'repair' HearNPV BVs. HzAM1 cells were transfected by repaired HearNPV bacmids (positive, Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa and Tr-49aa). GFP expression on each transfection was examined 5 days later (A). Supernatant was collected 7 d.p.t.. After being clarified, this supernatant was used to infect new HzAM1 cell. GFP expression, indicating infectivity, was examined 3 days after infection (B). Western blot analysis was performed on purified wild type HearNPV BVs and f 'repair' HearNPV BVs (positive, Tr-6aa and Tr-16aa), by using anti-F1 and anti-F2 under either reducing or non-reducing conditions.

for infectivity. The F protein CTD can be reduced to 32 aa residues, further truncations of the CTD abort the generation of infectious HearNPV BVs.

To determine the expression of *f* 'repair' as control, and the truncations Tr-6aa and Tr-16aa upon viral infection and incorporation into BVs, we performed western analysis on purified BVs generated from the respective viruses. BVs from wild type HearNPV were used as positive control (Fig. 3C). Under reducing conditions, bands corresponding to a protein with the predicted molecular size of the F_1 subunit (approximately 59 kDa) were detected in wild type HearNPV and *f* 'repair' BVs with an anti-F_1 specific antibody. In the BVs from Tr-

6aa and Tr-16aa 'repair' mutants, the F1 subunits were also detected by anti-F1 antibody, but of smaller size of approximately 58 kDa and 57 kDa, respectively. Bands corresponding to F_2 subunits with a molecular weight of 20 kDa were detected in wild type HearNPV BVs and in the 'repair viruses (*f*-repair, Tr-6aa and Tr-16aa). These results show that F, Tr-6aa and Tr-16aa protein precursors (F_0) were expressed and cleaved by a furin-like convertase in infected HzAM1 cells and that these proteins were incorporated into BVs. To further investigate the processing of F, Tr-6aa and Tr-16aa proteins, western analysis was performed after SDS PAGE under non-reducing conditions (Fig. 3C). Both anti-F_1 and anti-F_2 antibody recognized the F_0 bands. These results indicate that F_1 subunit and F_2 subunit in F, Tr-6aa and Tr-16aa protein in the repaired HearNPV were linked by disulfide bridges as is the case in wild type HearNPV BVs (Long *et al.*, 2006a).

BV production of '*f* 'repair HearNPV

The F, Tr-6aa and Tr-16aa repaired *f*-null HearNPV bacmid produced infectious HearNPV BVs and the further C-terminal truncation mutants do not. This result suggests that the CTD of baculovirus F protein is essential for production of infectious BV and that truncation of the CTD results in inability to rescue infectivity of an *f*-null HearNPV bacmid. To determine if and, if so, to what extent the 6 and 16 aa C-terminal truncations affect infectious BVs production, we compared the *f*-repair (control), and Tr-6aa and Tr-16aa HearNPV F mutants in one-step growth curve assays in HzAM1 cells (Fig. 4). Our results from the one-step growth curve comparisons show that truncation of 6aa and 16 aa from the C terminus of the F CTD reduce the infectious BVs production by about 3-fold and 8-fold, respectively.

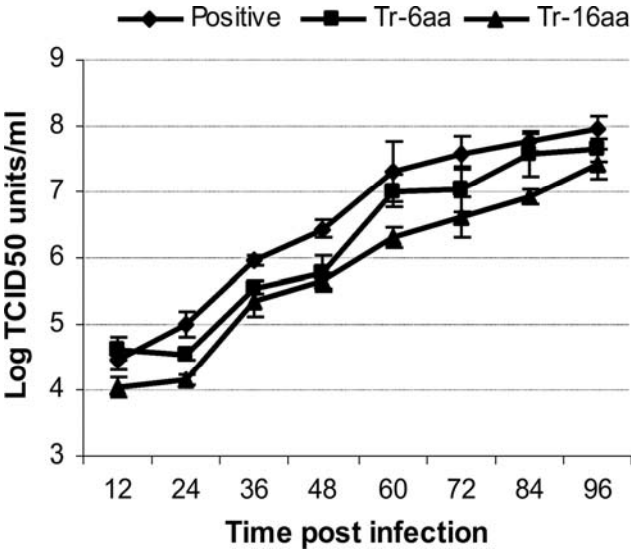

Figure 4. Analysis of BV production by one-step growth curve analysis. Comparison of virus growth curves for the positive (*f*-repair), tr-6aa and tr-16aa repaired HearNPVs. HzAM1 cells were infected in three replicates of HearNPVs with a multiplicity of 5.0 $TCID_{50}$ units per cell. Each data point represents BV production at different time point after infection. Data points represent triplicate infections and titrations, and error bars represent standard deviation from the mean.

CTD truncations of HearNPV F and rescue of AcMNPV *gp64*-null bacmid infectivity

Transfection and infection assays of HearNPV repaired bacmids containing complete *f* and CTD truncations indicate that the CTD of HearNPV F protein plays an important role in infectious virions production. However, it was not clear how the truncation of the CTD would influence infectious BV production. The possible consequences of removal of the CTD from

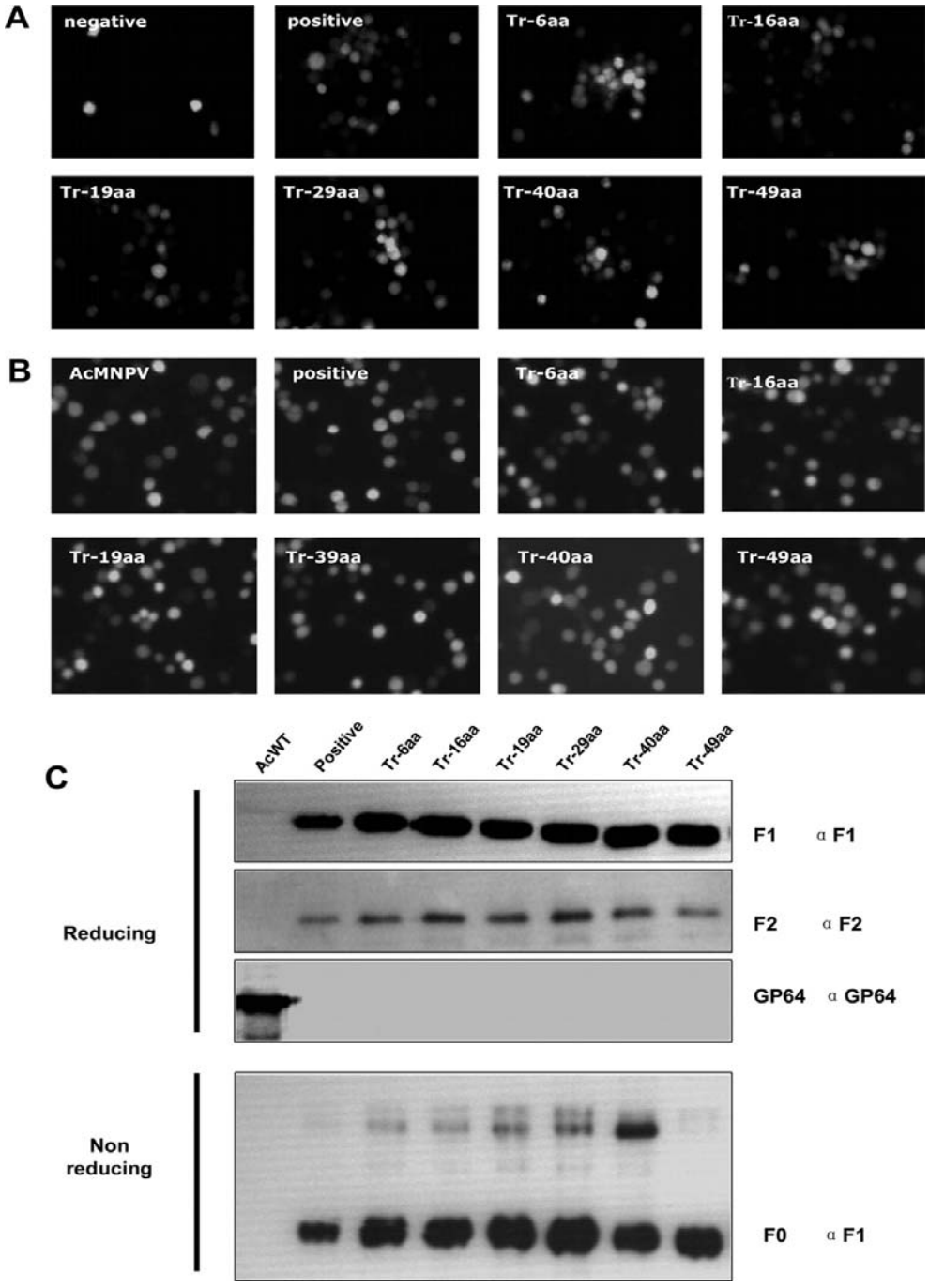

Figure 5. (A) Transfection of *gp64*-null AcMNPV bacmid pseudotyped by the HearNPV *f* gene with or without truncation in the CTD. (B) Infection assay for viral propagation. (C) Western blot analysis of pseudotyped AcMNPV BVs. Sf21 cells were transfected by HearNPV *f* genes pseudotyped *gp64*-null AcMNPV bacmids (f-repair, Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa and Tr-49aa). GFP expression on each transfection was examined 5 days later. A gp64 gene repaired *gp64*-null AcMNPV bacmid served as a positive control (A). Supernatant was collected 7 d.p.i. After clarification, the supernatant was used to infect new Sf21 cell. GFP expression, indicating infectivity, was examined 3 d.p.i. (B). Western blot analysis was performed on purified AcMNPV BVs and pseudotyped AcMNPV BVs (*f* repair, Tr-6aa and Tr-16aa), by using anti-F1, anti-F2 and anti-GP64 under either reducing or non-reducing conditions.

HearNPV F beyond the 16 C-terminal aa would be (i) on F protein processing prior to assembly into BVs, which would result in the impairment of subsequent viral endocytosis and low pH triggered envelope fusion in the next infection; (ii) absence of a linkage between HearNPV F and nucleocapsid proteins leading to inability to proper BV formation. In the first case non-infectious BVs are formed, in the latter case no BVs are formed.

The CTD of GP64 is not essential for infectious AcMNPV BV production (Oomens and Blissard, 1999), indicating that AcMNPV BV budding is not dependent on an intact CTD. This would suggest that CTD truncations of HearNPV F do not prevent infectious BV formation. Therefore, intact *f*, Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa and Tr-49aa gene cassettes were inserted into the *gp64*-null AcMNPV bacmid system (Long *et al.*, 2006a) to pseudotype *gp64*-null AcMNPV bacmids. The constructs were transposed into the bacmids with an AcMNPV p10 promoter-controlled *egfp* gene. Sf21 cells were transfected with pseudotyped *gp64*-null AcMNPV bacmids, respectively (Fig. 5A). A *gp64*-null AcMNPV bacmid carrying no HearNPV f gene served as a negative control. At 5 d.p.i. the supernatant from each transfection treatment was used to infect new Sf21 cells (Fig. 5B). An AcMNPV virus carrying a *p10* promoter controlled *egfp* gene was used as positive control for infection. Unlike the case of HearNPV 'repair' bacmid, all HearNPV *f* gene cassettes (*f-repair*, Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa and Tr-49aa) rescued the infectivity of the *gp64*-null AcMNPV bacmid mutants and produced infectious BVs. These results confirm that HearNPV *f* gene cassette was correctly expressed and processed in the AcMNPV-Sf21 system, but show in addition that truncation of the CTD of HearNPV F protein does not affect the formation of infectious AcMNPV BVs, indicating that the HearNPV F protein without CTD is fusogenic and able to form infectious BVs.

In addition to the transfection and infection assays, we also examined the incorporation of the HearNPV F protein and F CTD truncation mutants into AcMNPV BVs of (Fig. 5C). When separated in SDS-PAGE under reducing condition and detected by F specific antibodies, F_1 and F_2 were recognized separately in all pseudotyped AcMNPV (*gp64*-null/*f* 'repair' and Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa and Tr-49aa) mutant BVs. The molecular size of F_1 in pseudotyped AcMNPV BVs gradually decreased from 60 kDa (for the full F protein) to 55 kDa (for a CTD truncation of 48aa), whereas the molecular size of F_2 stayed the same at 20 kDa (Fig. 5C). The presence of GP64 in AcMNPV BVs and its absence in pseudotyped AcMNPV BVs confirmed the authenticity of rescue. Under non-reducing condition, bands corresponding to F_0 were detected in all pseudotyped AcMNPV BVs by anti-F_1 antibody (Fig.5C). Anti-F_2 antibody also identified F_0 in pseudotyped AcMNPV BVs (result not shown). These results indicate that HearNPV F proteins with truncations in the CTD are incorporated in AcMNPV BVs as with intact HearNPV F protein. The incorporation of the HearNPV F protein and its truncated forms into AcMNPV suggest that the CTD is not important for proper F protein synthesis, folding, membrane trafficking and fusogenicity.

Infectivity of AcMNPV pseudotyped with HearNPV *f*

To determine the effect of CTD truncations on BV productivity of pseudotyped AcMNPV baculoviruses, the BV production at 48 h.p.i. was evaluated by EPDAs (Fig. 6). AcMNPV produced about 10 times more infectious BVs than the pseudotyped AcMNPVs (*gp64*-null / *f* 'repair', Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa and /Tr-49aa) and that there was no significant difference observed among the pseudotyped AcMNPV viruses. This result

demonstrates that the length of the CTD is not important for infectious BV production in AcMNPV.

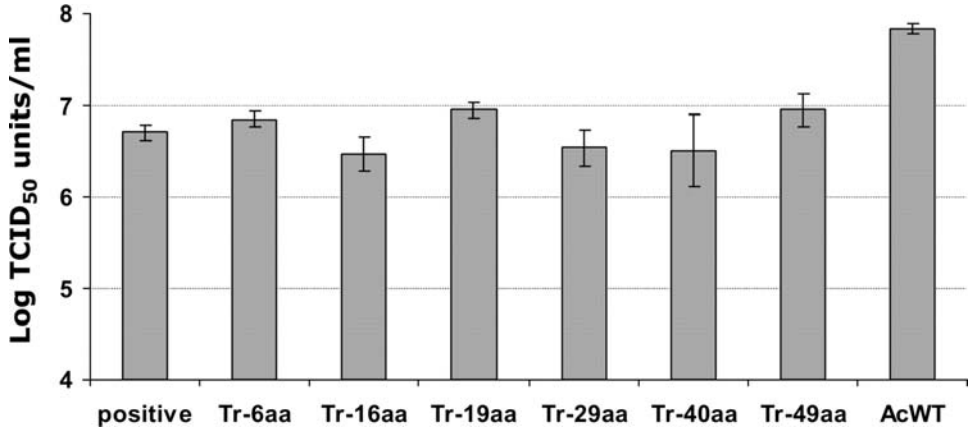

Figure 6. BV production of pseudotyped AcMNPVs. Sf21 cells were infected by F protein pseudotyped AcMNPVs (Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa and Tr-49aa) and wild type AcMNPV. Each data point represents virion production at 48 h.p.i. Data points represent triplicate infections and titrations, and error bars represent standard deviation from the mean.

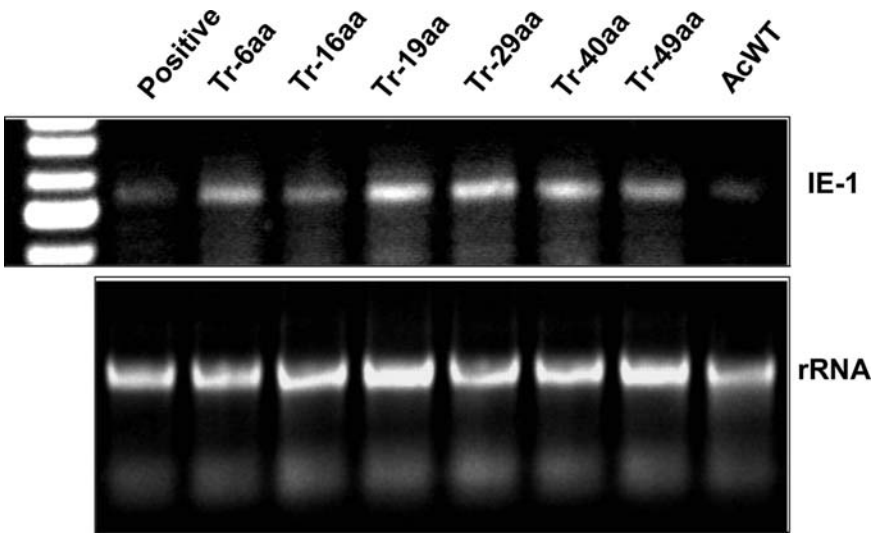

Figure7. Detection of *ie-1* gene early transcripts upon non-productive infection of pseudotyped AcMNPVs to HzAM1 cells. HzAM1 cells were infected by *gp64*-null AcMNPVs, pseudotyped with Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa, Tr-49aa and wild type AcMNPV. Twelve h.p.i., total RNA from infected cells of each infection was isolated. 3' RACE analysis on AcMNPV *ie-1* gene was performed. PCR amplified products were analyzed on 1% argrose gel. Total RNA samples from each treatment were analyzed as template control on reverse transcription.

The previous results show that the CTD of F, except for the 16 C-terminal aa, is essential for HearNPV viral infectivity whereas the CTD is not essential for fusogenicity. It is possible that: (i) the HearNPV F protein without the 16 C-terminal aa of the CTD is incorporated in budded virions which are not infectious, and e.g. entry defective (attaches to cell membrane and envelope fusion) into HzAM1 cells or (ii) the F protein without CTD is not incorporated in virions. To investigate this, a non-productive infection was carried out, in which HzAM1 cells were infected with pseudotyped AcMNPVs(*gp64*-null/*f* repair and the mutants Tr-6aa, Tr-16aa, Tr-19aa, Tr-29aa, Tr-40aa and Tr-49aa). Successful viral entry occurred in all instances as early transcripts of the AcMNPV *ie*-1 gene were detected at 12 h.p.i. (Fig. 7). Thus, viral entry of pseudotyped AcMNPVs into HzAM1 cells was not affected by CTD truncations of the HearNPV F protein.

DISCUSSION

A number of functional roles have been attributed to the CTD of virus glycoproteins. These include regulation of the incorporation of viral glycoproteins into mature virions (Gage *et al.*, 1993; Rein *et al.*, 1994; Christodoulopoulos and Cannon, 2001), determination of the site and efficiency of viral budding (Zhao *et al.*, 1994; Lodge *et al.*, 1997b), governance of the conformation of the glycoprotein extra cellular domain and processing, regulation of cell surface expression of glycoproteins (Sauter *et al.*, 1996; Raviprakash *et al.*, 1990; Grange *et al.*, 2000) and control of virus envelope and cell membrane fusion (Mulligan *et al.*, 1992; Brody *et al.*, 1994; Ragheb and Anderson, 1994; Bagai and Lamb 1996; Cathoman *et al.*, 1998). In the present study, we have investigated the role of the CTD of the major baculovirus group II envelope fusion protein F, more specifically of the HearNPV F protein. Our results show that the CTD of the HearNPV F protein is critical for infectious BV production. In this respect the role of the F CTD differs from that of GP64-like fusion proteins, the major envelope fusion protein from NPV group I members. The minimal length of the CTD to support infectious HearNPV BV production was 32 aa; the C-terminal 16 aa were dispensable. For HearNPV F, however, the CTD is not essential for rescuing BV production and infectivity of a *gp64*-null AcMNPV mutant. This indicates that the CTD of HearNPV F is not involved in fusogenicity. Additionally, HearNPV F protein processing, including synthesis, folding, disulfide bridging, trafficking and susceptibility to cellular convertase (furin-like) cleavage is not affected by removing the CTD from the F protein.

Group II NPV F protein is a newly found baculovirus envelope fusion protein (IJkel *et al.*, 2000; Pearson *et al.*, 2000). Common functional domains, such as furin-like protease cleavage site (Long *et al.*, 2006a; Westenberg *et al.*, 2002), fusion peptide (Westenberg *et al.*, 2004), heptad repeat (G. Long, unpublished data), and cytoplasmic tail, were identified. The arrangement of these domains is similar to the architecture of class I viral envelope fusion proteins from members of viral families of vertebrates. Unlike GP64, baculovirus F proteins have a long CTD, ranging from 52aa to 78aa. The CTD of GP64 (7 amino acid long) was reported to be dispensable for viral infectivity and not essential for efficient virion budding from the cell surface (Oomens and Blissard, 1999). Our experiments demonstrate that a CTD is not essential for infectious BV production of AcMNPV and possibly not for all group I baculoviruses.

However, our results demonstrate that in the case of HearNPV only truncations of up to 16 aa from the C-terminus of the CTD allow the repair of an *f*-null HearNPV bacmids, which

underscores that the CTD is essential for viral infectivity. Other truncations (from Tr-19aa to Tr-49aa) were not able to rescue the *f*-null HearNPV. The tyrosine residue (Y658, the last residue of Tr-19aa) is conserved in all baculovirus F proteins. This may suggest that this internal tyrosine-based signal is important for viral infectivity. There is evidence from other enveloped viruses that an internal tyrosine-based motif is crucial for the functional role of F-like proteins (Beaufils *et al.*, 1993; Zhao *et al.*, 1994; Lodge *et al.*, 1997b). If such motif in baculovirus F protein CTD has a similar function, it may explain that the tr-16aa truncate can repair the *f*-null HearNPV bacmid, whereas the Tr-19aa cannot (Fig.3). Mutational analysis of the tyrosine residue in HearNPV F CTD, which is also present in all other sequenced group II NPVs, should prove the importance of this aa in this process. Additionally, a slight decrease in infectious BV production was observed, associated with truncations Tr-6aa and Tr-16aa. This might suggest that these 16 C-terminal aa, representing the variable region in CTDs of baculovirus F proteins, stabilize the possible interaction between F CTD and nucleocapsid in a length dependent manner, thus guarantee the efficient incorporation of F into baculovirus group II BVs. A slight, but significant decrease in infectious BV production has been noted for AcMNPV, when the 7 C-terminal aa of the CTD were removed (Oomens and Blissard, 1999).

The GP64 protein of AcMNPV is necessary and sufficient for the low-pH-triggered membrane fusion which occurs during BV entry after endocytosis (Blissard and Wenz, 1992; Monsma and Blissard, 1995). Here, a *gp64*-null AcMNPV bacmid pseudotyping system was applied to exclude the possibility that the fusion ability of HearNPV F protein is impaired by eliminating the CTD. HearNPV F proteins with sequential truncations in the cytoplasmic tail (from Tr-6aa to Tr-49aa) are all capable to pseudotype a *gp64*-null AcMNPV bacmid (Fig. 5). This is also in agreement with the observation that the CTD of GP64 is not essential for viral infectivity (Oomens and Blissard, 1999).

Viruses use the host cellular machinery to translate viral proteins. Similar to cellular proteins directed to the secretary pathway, viral glycoproteins are synthesized in a similar fashion. The CTD of transmembrane proteins are exposed at the cytoplasmic side of the ER membrane, thus may interact with cellular proteins that regulate the intracellular processing and trafficking. Deletion in the cytoplasmic portion of viral glycoproteins can have small, but also severe effects on intracellular transport and surface expression (Raviprakash *et al.*, 1990; Sauter *et al.*, 1996; Lodge *et al.*, 1997a; Grange *et al.*, 2000; Waning *et al.*, 2004). As we have shown here, truncation in the CTD of HearNPV F seems to have little or no negative effect on these processes. Truncation of CTD in HearNPV F protein has no influence on furin-like cellular convertase cleavage and subunits disulfide bridging. Successful incorporation into pseudotyped AcMNPV budded virions indicate proper folding of F without CTD and trafficking to viral budding destination. Moreover, the AcMNPV pseudotyping competence of F without CTD confirms our observation that truncations in the F CTD have no effect on the fusogenicity of F protein.

Based on the evidence shown above, the CTD core until the tyrosine residue (Y658) is proposed to be essential for HearNPV viral infectivity but not essential for fusogenicity of F protein. Three possible consequences from CTD deletion may lead to failure to generate infectious HearNPV BVs: (i) HearNPV F without CTD is incorporated in BVs, which are released from the infected cell but are not infectious, because of some defect in viral entry (cell attachment and envelope fusion) into HzAM1 cells; (ii) F without CTD is unable to be

incorporated into BVs as a consequence of impaired protein synthesis and transportation; (iii) F without CTD is properly synthesized and transported to budding sites in infected cells, but not able to be wrapped in mature and infectious BVs. Experiments above showed that HearNPV F without CTD is able to rescue infectivity of a *gp64-null* AcMNPV bacmid. This indicates that F without CTD is properly synthesized and fusion competent. Since AcMNPVs pseudotyped with F without CTD were able to enter HzAM1 cells, as evidenced by the initiation of early gene transcription (Fig. 6), F without CTD can properly fold and be transported. However, F without CTD is apparently unable to generate infectious HearNPV BVs underscoring the importance of CTD in incorporation of F into HearNPV BVs and possibly into BVs of all group II baculoviruses. It is still be possible that lower levels of HearNPV BVs without F protein were produced but they remained below the detection level.

In conclusion, we combined a newly constructed HearNPV *f-null* bacmid knockout-repair system (this paper) and an AcMNPV *gp64-null* bacmid knockout-pseudotype system to study the functional role of the CTD of baculovirus F proteins. We show that the CTD of the HearNPV F protein determines infectious BV production in a length dependent manner and that the CTD is essential for infectious BV production of HearNPV. The HearNPV F protein without a CTD still rescues infectivity of a *gp64-null* AcMNPV, thus the CTD is not involved in F protein processing and fusogenicity. It is possible that the CTD is important for proper incorporation of F into mature HearNPV BVs. Future experiments will determine which CTD domains or aa are involved in this interaction.

ACKNOWLEDGEMENTS

This work was financially supported by the Royal Academy of Sciences of The Netherlands (KNAW) (project 04-PSA-BD-02) and a PhD sandwich grant to GL from Wageningen University. The research was further supported by the 973 project (2003CB114202), PSA project 2004CB720404 from CAS, and by a NSFC grant (30300012) to Gang Long. We thank Professor Hu Zhihong for fruitful discussions and continued interest in this research.

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Chapter 6

N-terminal Heptad Repeat, HR1, of Baculovirus (HearNPV) F is essential for F Function and Intracellular Processing

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Chapter 6

SUMMARY

The heptad repeat (HR), a conserved structural motif of class I viral fusion protein, was accepted to be responsible to form a six-helix bundle structure during envelope fusion process and peptide derived from these HRs can be potent and specific inhibitors of membrane fusion and viral infection(reviews see Dutch *et al.*, 2000; Eckert and Kim 2001). Baculovirus F protein is a newly found envelope fusion protein, which possesses the common feature as class I fusion protein, with respect to the proteolytic cleavage, N-terminal open fusion peptide and multiple heptad repeat domains (HRs) on the transmembrane subunit. Similar to many viral fusion proteins, a conserved leucine zipper motif is predicted in the fusion peptide proximal HR region, HR1, in baculovirus F proteins. To facilitate our understanding of the functional role of this leucine zipper and the HR1 domain in baculovirus F protein processing and viral infectivity, selected leucine residues (Leu209, Leu216 and Leu223) were substituted by alanine(A) and arginine(R) respectively. Pseudotyping *gp64*-null AcMNPV by *f* mutant genes coding single leucine substitution mutant F proteins revealed that the leucine residues were critical for F protein to rescue *gp64*-null AcMNPV and F^{L216R} was not able to rescue. By using AcMNPV as expression system, we further demonstrated that all the mutant F proteins were able to be incorporated in budded virus (BV), indicating substitutions on the leucine had no harm to intercellular trafficking of F protein, although F^{L216R} showed lower viral incorporation rate into AcMNPV BVs. Furin-like protease cleavage was not affected by any of the substitution on leucine residues, however the status of disulfide bridging and N-linked glycosylation was significantly changed. Under non-reducing condition, F proteins carrying F^{L216A}, F^{L216R} and F^{L223A} substitutions was showed to form large amount of malformed oligomers besides F_0 and F^{L216R} was showed to form predominantly malformed oligomers. Glycobiology studies showed that besides the mature N-linked glycosylated form of F_2 subunit carry pausimannose glycans and non-glycosylated F_2 subunit, F^{L216R} was detected as one more heavier N-linked glycosylated form of F_2 in both cells and BVs. Altogether, our results support that leucine zipper motif located in HR1 domain is important to guarantee biological property of baculovirus F protein and HR1 domain is critical for the baculovirus F proper folding and intracellular processing.

INTRODUCTION

Baculoviridae is a large family of The *Baculoviridae* are a large family of enveloped DNA viruses that are almost exclusively pathogenic to arthropods, predominantly insects in the order *Lepidoptera* (Blissard *et al.*, 2002). Baculoviruses are divided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GVs). Phylogenetic studies indicate that NPVs can be subdivided into two subgroups: group I and group II (Zanotto *et al.*, 1993; Herniou *et al.*, 2001). 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. In contrast, BVs are adapted to propagate infection from cell to cell and enter via clathrin-mediated endocytosis. BVs are responsible for the systemic spread of the virus in the infected insect and infected insect cell cultures.

Viral envelope fusion proteins can be sorted into at least two distinct classes, class I and class II, based on their functional characteristics (Earp *et al.*, 2005). Class I fusion proteins are found in many disparate virus families, including retroviruses, othomyxoviruses, paramyxoviruses, arenaviruses, coronaviruses and filoviuruses. Viral fusion proteins of alphaviruses and flaviviruses have been categorized as class II (Earp *et al.*, 2005). In the case of baculoviruses, two distinct envelope fusion proteins were found in BVs, GP64 for goup I NPVs (Blissard and Wenz 1992) and F protein for group II NPVs (IJkel *et al.*, 2000; Pearson *et al.*, 2000; Long *et al.*, 2006a). F proteins from group II NPVs are thus functional analogs to GP64 (Lung *et al.*, 2002; Long *et al.*, 2006a; Long *et al.*, 2006c), as both entire and cytoplasmic tail truncated F genes can rescue the infectivity of a gp64-null AcMNPV. Unlike GP64, baculovirus F proteins have been showed to possess the structural characteristics similar to class I fusion proteins from vertebrate viruses. F proteins are synthesized first as a preprotein which later undergo proteolytic cleavage by a furin like protease (Fig. 1), resulting

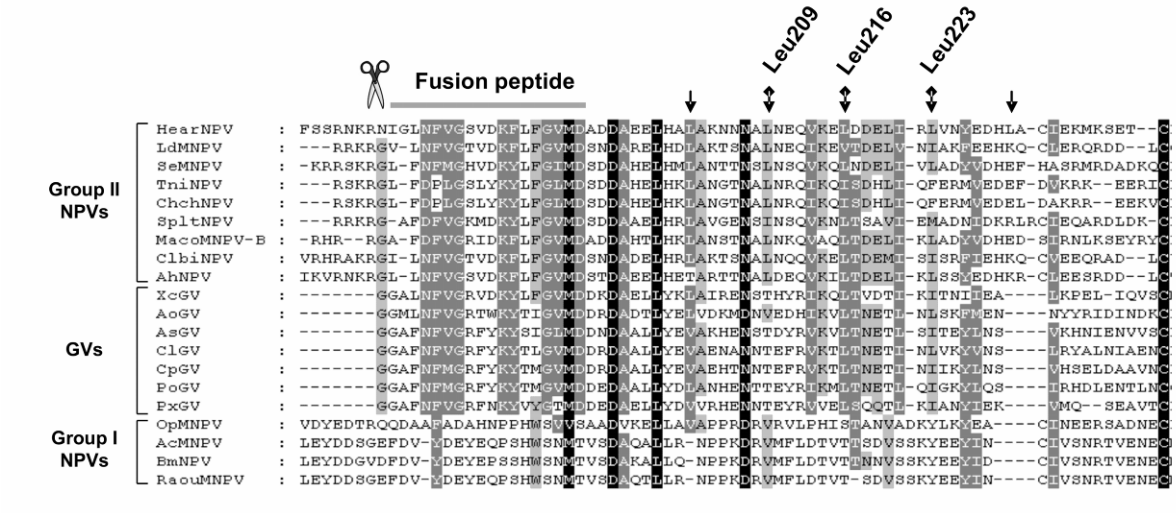

Figure 1. Amino acid sequence alignment of the HearNPV F protein domains near the N-terminal heptad repeat, HR1, with the corresponding domain of F homologous proteins of group II NPVs (A), GVs (B) and group I NPVs(C). Virus abbreviations are shown on the left and the genebank accession numbers of the F proteins are shown on the right.

in two disulfide linked subunits F_1 (C terminal) and F_2 (N terminal) (Westenberg *et al.*, 2002; Long *et al.*, 2006a). Baculovirus F proteins are N-glycosylated and F protein can be found as homo-trimers on the BV particle (Long *et al.*, 2006a). A putative fusion peptide located on the N terminus of the F_1 subunit was reported to be critical to biological property of SeMNPV F (Westenberg *et al.*, 2004). In addition, heptad repeat (HR) regions, HR1 downstream of the fusion peptide region (Fig. 1, Fig. 2) and HR2 upstream of the transmembrane domain, were predicted in baculovirus F proteins.

HR motifs have the capability to form amphipathic helices and have been detected in the transmembrane subunit of the class I viral envelope fusion proteins of RNA viruses. They have been shown to play an essential role in viral fusion and infectivity (Dubay *et al.*, 1992; Cao *et al.*, 1993; Reitter *et al.*, 1995; Chen *et al.*,1998; Weng and Weiss 1998; Luo *et al.*,1999; Sergel *et al.*, 2001; Gianni *et al.*, 2005). After receptor binding or induction by low pH, the trimeric viral fusion proteins undergo a series of conformational change (Bullough *et al.*, 1994; Skehel and Wiley 1998; Baker *et al.*,1999; Colman and Lawrence 2003). X-ray crystallographic studies of various viral fusion protein transmembrane subunits demonstrated that HR regions form coiled-coil trimer or six helix bundle structures, whereby three HR1 helices form a central coiled coil surrounded by three HR2 helices in an anti parallel fashion (Chan *et al.*, 1997; Joshi *et al.*, 1998; Malashkevich *et al.*, 1998; Weissenhorn *et al.*, 1998; Kobe *et al.*, 1999; Zhao *et al.*, 2000; Malashkevich *et al.*, 2001; Xu *et al.*, 2004). Peptides derived from HRs were reported to be a potent and specific inhibitor of membrane fusion and viral infection (Wild *et al.*, 1994; Chen *et al.*, 1999; Ghosh and Shai, 1999; Russel *et al.*, 2001, Bosch *et al.*, 2003; Netter *et al.*, 2004).

Similar to many viral envelope fusion proteins, a HR region with a leucine zipper motif (Alber, 1992; Buckland *et al.*, 1994; Wild *et al.*, 1994; Reitter *et al.*, 1995; Chen *et al.*, 1998; Luo *et al.*, 1999) was located downstream of the fusion peptide of baculovirus F protein (Fig 1). To facilitate understanding of the fusion process mediated by F protein and the functional role of HR1, mutations were introduced into predicted leucine zipper motif within HR1 by replacing selected Leucines (Leu209, Leu 216 and Leu223) by alanine or arginine. A *gp64*-null AcMNPV bacmid pseudotyping system and conventional AcMNPV expression system were combined to test the effects of the substitutions on F protein folding, processing and BV infectivity. This study indicated that the HR1 plays an essential role in baculovirus F protein mediated membrane fusion and viral infectivity and that HR1 is critical for F protein folding and processing.

MATERIALS AND METHODS

Cells and Bacmid

Spodoptera frugiperda cell line IPLB-SF-21 were cultured at 27^{o}C in plastic tissue culture flasks (Nunc) in Grace's insect medium (pH5.9-6.1, Invitrogen), supplemented with 10% fetal bovine serum (FBS). A *gp64*-null bacmid (Lung *et al.*, 2002) was used to study on the functional role of the HR1 region of the HearNPV F protein.

Computational analysis

Sequence for the HearNPV F protein was obtained from GenBank. Prediction of potential coiled coil region and transmembrane domain were conducted by using proteomic tools from the expasy proteomics sever (http://us.expasy.org).

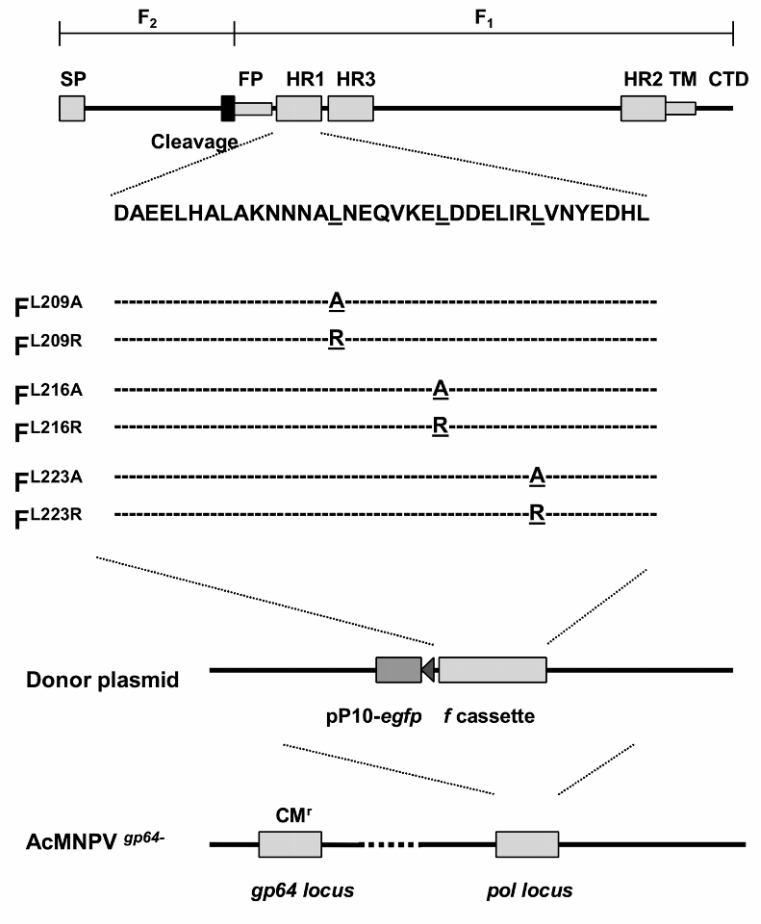

Figure 2. Schematic diagram of pseudotyped AcMNPV bacmid construction. The signal peptide (SP), proteolytic cleavage site, fusion peptide (FP), heptad repeat (HR), transmembrane domain, and cytoplasmic tail domain (CTD) were shown. Selected Leucines (Leu209, Leu216 and Leu223) were substituted by alanine or arginine respecitively. Donor plasmids carrying HearNPV *f* cassette with demonstrated substitutions and a *p10* promoter controlled *egfp* gene were used to construct pseudotyped AcMNPV bacmids.

Mutagenesis and bacmids

Site-directed mutatgenesis of the selected leucines was performed as follows. Preferred codons (underlined) of small uncharged residue alanine (A) and charged residue arginine (R) replaced the codons coding Leu209, Leu216 and Leu223 by introducing in the 5'end of mutagenesis reverse primers (R-L209R, 5'-ACGCGCGTTGTTATTTTTGGCTAAAG-3', R-L209A, 5'-CGCCGCGTTGTTATTTTTGGCTAAAG-3', R-L216R, 5'-ACGTTCTTTCA CTTGTTCGTTGAGC-3', R-L216A, 5'-CGCTTCTTTCACTTGTTCGTTGAGC-3', R-L223R, 5'-ACGACGTATGAGTTCATCGTCGAGTTC-3', R-L223A, 5'-CGCACGTATG AGTTCATCGTCGAGTTC-3'). With prior 5' phosphorylation of the reverse primers and three forward primers (F-L209, 5'-ACCGAACAAGTGAAAGAACTCGAC-3', F-L216, 5'-GACGATGAACTCATACGTTTGGTC-3', F-L223, 5'-GTCAACTATGAAGATCATTTGG CGT-3'), PCRs were performed with the pFB-F&GFP vector (Long *et al.*, 2006c) as template. Phusion-polymerase (Finzyme) was applied in the PCR reaction. After the first purification, the mutant PCR products were treated with DpnI to eliminate template plasmid DNA. Subsequently, the 5' ends of purified PCR products were ligated to their own 3' ends generating new plasmids containing the site-directed mutant sequences in F. After sequence verification, the mutant F gene cassettes were cloned back into pFB-F&GFP vector to replace the f gene cassette, by swapping the *Bst*1107I-to-*Hin*dIII fragments, resulting donor plasmids earch carrying one of the six mutant *f* genes. These donor plasmids were used to transpose six

mutant f genes into a *gp64*-null AcMNPV bacmid and into a wild type AcMNPV bacmid containing the GP64 gene as well.

Competent cells containing either the *gp64*-null AcMNPV bacmid or the wild type AcMNPV bacmid were made according to the Bac-to-Bac manual (Invitrogen). Transpositions of site-directed mutant genes from donor plasmids to either *gp64*-null AcMNPV bacmid or wild type AcMNPV bacmid were confirmed by diagnostic PCR using a gentamycin resistance gene forward primer (5'-AGCCACCTACTCCCAACATC-3') in combination with the M13 forward primer (5'-CCCAGTCACGACGTTGTAAAACG-3'). Transfection and infection assays were conducted according to Long *et al*., 2006a.

Western analysis

The expression of mutant F proteins and the incorporation of the wild type and mutated F proteins in BVs was examined by western analysis using polyclonal antibodies against F_1 and F_2. Sucrose-purified BVs or cellular total protein samples take 48 hs post infection were subjected to Western analysis as previously described (Long *et al.*, 2006a). Briefly, sucrose-purified BVs were disrupted in reducing or non-reducing conditions and were then denatured for 10 min at 95^{o}C. Proteins were separated by SDS-PAGE and subjected to Western analysis. The antibodies were used in a 1:1000 dilution and the proteins were detected by treatment with horseradish peroxidase conjugated rabbit anti-chicken immunoglobulin (Sigma) diluted 1:5,000 followed by ECL technology as described by the manufacturer (Amersham).

Plaque assay

To determine the viral infectivity of pseudotyped AcMNPVs carring wild type and mutant F proteins a plaque assay on Sf21 cells was conducted. Briefly, 1x10^{6} Sf21 cells were transferred to 35mm plastic petri dishes in Grace's medium (supplemented with 10% FBS). After overnight incubation at 27^{o}C cells were infected by pseudotyped AcMNPVs carring wild type and mutant F proteins, respectively, at a multiplicity of infection (m.o.i.) of 0.01 $TCID_{50}$ units/cell. After 90 min of incubation at 27^{o}C, the virus suspension was removed and cells were washed carefully twice with fresh Grace's medium plus 10%FBS. Subsequently cells were overlayed by 2ml of the Grace's medium, containing 10% of FBS and 1.5% of Sea Plaque agarose. After 3 days of incubation at 27^{o}C the formation of plaques for each treatment was examined by UV light microscopy. The plaque sizes of ten separated plaques from each treatment were measured by the numbers of each plaque. The results were exported to Microsoft Excel software and the average of ten plaque sizes was analyzed and the standard deviations of the mean were determined.

Deglycosylation and glycan profiling

Total proteins from sucrose purified BVs of AcMNPV gp64-/f were separated on 12% SDS-PAGE. F2 subunit was recovered from gel with SME method (Scheer and Ryan, 2001). Purified F2 subunit was dissolved in 5 ml 50 mM sodium phosphate buffer, pH 7.3, containing 10 mM EDTA, 10 mM β-mercaptoethanol, and 0.5% SDS (w/v), then denaturated for 5 min at 100°C. After having added Nonidet P40 (Sigma) to a final concentration of 1.5% (v/v), the N-glycans were released PNGase F (Roche). The digestion was carried out with 2 U PNGase F for 24 h at 37°C. The digets was filtered through 4 connected HiTrap columns (4 x 5 ml, 3 ml/min, 50mM NH4HCO3, GE healthcare) UV monitored at 214 nm. The de-N-glycosylated glycoprotein was checked by SDS-PAGE. The released N-glycans, were treated with Calbiosorb Adsorbent (Calbiochem) according to the manufacturer's protocol to remove

detergents. Finally, the N-glycan pool was desalted and purified on a Carbograph-SPE column (Altech) followed by lyophilization.

Purified and lyophilized N-glycans were treated with 0.35 M 2-aminobenzamide (2-AB, Sigma)/1 M sodium cyanoborohydride in dimethyl sulfoxide:acetic acid (7:3, v/v) for 2 h at 65°C. The 2-AB labelled glycans were purified via paper chromatography on acid-pre-treated QMA (Whatman) filter paper strips using acetonitrile (three times) as a mobile phase. Glycans (remaining at the base line) were eluted from the dried paper strips with water, and concentrated (Bigge *et al.*, 1995; Kinoshita and Sugahara, 1999).

Profiling was carried out on a TSK-Gel Amide-80 (GlycoSep-N) column (250 x 4.6 mm, Tosoh Corporation) at 30^{o}C, using a Waters 2690XE Alliance system, equipped with a Waters 474 fluorescence detector ($\lambda_{exc.max}$ = 373 nm, $\lambda_{em.max}$ = 420 nm). A 100-min gradient of 50 mM ammonium formate, pH 4.4, in acetonitrile (25.2-55%, v/v) was used, at a flow rate of 0.8 ml/min, followed by a 3-min gradient to 100% ammonium formate, which was kept for 5 min at 1 ml/min before regeneration started. A 2-AB labelled dextran ladder was used as a standard for Glucose Unit comparison (Guile *et al.*, 1996).

For matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) in the positive-ion mode, N-glycan samples (0.2 µl) were mixed in a 1:1 ratio with a solution of 10 mg 2,5-dihydroxybenzoic acid (DHB) in 1 ml 10 mM aqueous ethanol. N-glycans were analyzed on a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied BioSystems), using a VSL-337ND-N_2 laser (337 nm) with 0.5 ns pulse width. Spectra were recorded in the reflector mode at an accelerating voltage of 20 kV, using an extraction delay of 95 ns.

RESULTS

HR1 region of Baculovirus F proteins

To better understand the architecture with respect to the fusion peptide and the downstream HR region of baculovirus F proteins, we aligned F proteins from members of group II NPV, GV, and group I NPV (Ac23 homologs) (Fig. 1). The comparison of F proteins clearly showed the furin cleavage sites, the conserved fusion peptide and the HR1 region in Fig.1. In agreement to previous report (Westenberg *et al.*, 2004) the areas of furin cleavage and the adjacent fusion peptide are highly conserved among F homologous proteins from group II NPVs and GVs. Also well conserved among is the HR1 region, located immediately downstream of fusion peptide. It is noteworthy that all these conserved functional domains are present in F proteins from group II NPVs and GVs, but non of them is present in the F homologs from group I NPVs. GP64 homologs are the functional envelope fusion proteins of group I NPVs, not the their F homologs. This alignment also showed that F homologs from group I NPVs did not contain the hallmark elements for a functional Class I fusion protein, which explains why the F homologs from group I NPVs are non-functional envelope fusion proteins. A conserved leucine zipper motif (five leucines separated by six other amino acids) is located in the HR1 region at position “d” of the HR1 region. Leucine zippers are frequently identified in the HR1 regions from other class I envelope fusion proteins and proved to be critical for fusion activity and virus infectivity (Wild *et al.*, 1994; Reitter *et al.*, 1995; Chen and Wiley, 1999; Lou *et al.*, 1999).

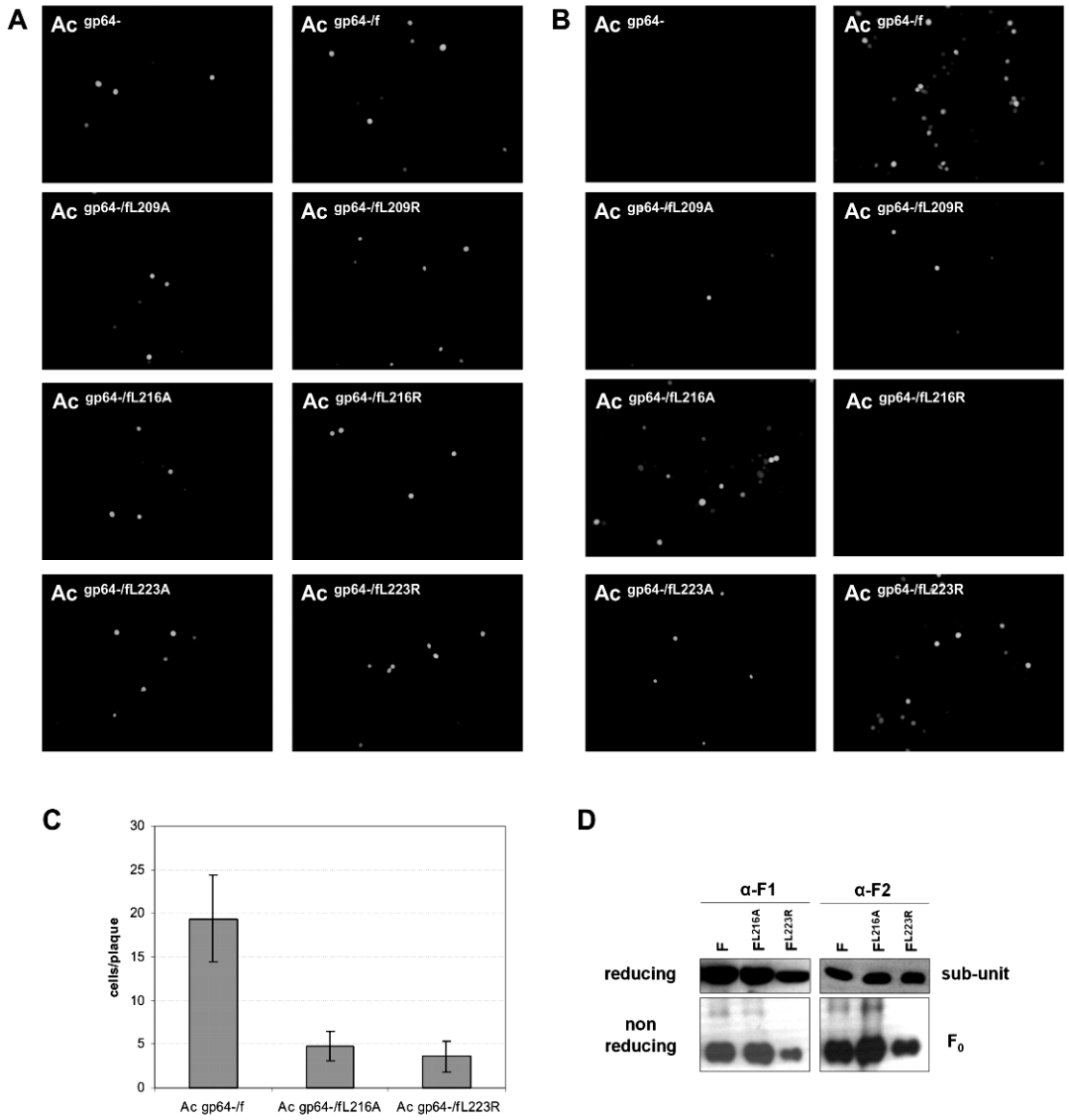

Figure 3. Infectivity of pseudotyped AcMNPVs. (A) Transfection of pseudotyped AcMNPV bacmids. (B) Infection assay for viral propagation. egfp gene expression were used to monitor effective transfection and viral propagation. (C) Plaque sizes of pseudotyped AcMNPVs infected Sf21 cells. The error bars represent the standard deviation of the mean. (D) Western analysis was performed on purified psuedotyped AcMNPVs (AcMNPV gp64-/f, AcMNPV gp64-/fL216A and AcMNPV gp64-/fL223R), by using anti-F1 and anti-F2 antibodies under either reducing or non-reducing conditions.

Leucine zipper is critical for rescuing the infectivity of a gp64-null AcMNPV

To facilitate the understanding of baculovirus F protein mediated membrane fusion and to determine the functional role of the HR1 region immediately downstream of the fusion peptide in F protein, we substituted there leucine residues (Leu209, Leu216 and Leu223) by non-polar small residue alanine (A) and charged residue argine (R) respectively (Fig. 2). The site-directed mutant *f* genes were subsequently transposed into a *gp64*-null AcMNPV bacmid along with a *p10* promoter controlled *egfp* gene, which served as a marker for successful

bacmid transfection and secondary infection assays. Sf21 cells were transfected with the recombinant bacmids, but also with a positive control bacmid, a *gp64*-null AcMNPV bacmid containing HearNPV *f* gene cassette, and a negative control bacmid, *gp64*-null AcMNPV bacmid lacking a fusion protein. Five days post transfection, expression of eGFP in Sf21 cells were examined (Fig. 3A). The supernatant from each transfection was used to infect new Sf21 cells to determine the generation of infectious BVs. Three days post infection, eGFP expression in infected cells was examined (Fig. 3B). The *gp64*-null AcMNPV bacmids carrying mutant *f* genes coding F^{L209A}, F^{L216A}, F^{L223A}, F^{L109R} and F^{L223R} can produce infectious BVs, whereas the *gp64*-null AcMNPV bacmid carrying mutant *f* gene coding F^{L216R} can not.

Effects of mutation on viral infectivity

To study the effect of leucine substitutions on the function of F, we characterized the viral infectivity of the pseudotyped *gp64*-null AcMNPVs carrying mutant *f* genes by plaque assay (Fig. 3C). Sf21 cells were infected by pseudotyped gp64-null AcMNPVs with a m.o.i. of 0.01. Three days p.i. plaques were observed by the expression of eGFP. The sizes of plaques produced by pseudotyped AcMNPVs were quantified by counting the number of infected cells of each plaque. Our result showed that only the AcMNPV $^{gp64\text{-}f}$, AcMNPV $^{gp64\text{-}fL216A}$ and AcMNPV $^{gp64\text{-}fL223R}$ produced plaques, and the size of plaques produced by AcMNPV $^{gp64\text{-}L216A}$ and AcMNPV $^{gp64\text{-}L223R}$ were much smaller than those produced by AcMNPV $^{gp64\text{-}f}$. This result suggested that the leucins (Leu209, Leu216 and Leu223) and the HR1 domain are important for baculovirus F protein function and thus are critical for virus infectivity. Additionally, western analysis was performed on purified BVs of AcMNPV $^{gp64\text{-}f}$, AcMNPV $^{gp64\text{-}fL216A}$ and AcMNPV $^{gp64\text{-}fL223R}$ to confirm that F was made and properly assembled in BVs (Fig. 3D). The result demonstrated that synthesis, processing and viral incorporation were not affected by substitution of Leu216 by alanine and that of Leu223 by arginine.

Expression of F protein mutants in Sf21 cells

To see whether the expression of mutant F proteins was effected by the mutations, which may explain in the defective virus propagation, we transposed all these mutant *f* genes into an AcMNPV bacmid already encoding GP64. Transfection and infection experiment were performed to obtain AcMNPVs carrying mutant f genes as previously described. We infected Sf21 cells with these AcMNPVs at a m.o.i. of 5 $TCID_{50}$ units/cell. Three days p.i. infected cells were collected and the total cellular proteins from each infection treatment were subjected to Western analysis by using antibody against HearNPV F_1 and F_2 separately (Fig. 4A). Expression of GP64 was also tested as loading control. Under reducing condition, F_1 and F_2 subunits were detected in all samples, suggesting all six mutant F proteins are expressed at as similar level in AcMNPV infected Sf21 cells to the parental F protein. The furin-like cleavage of F proteins also properly occurred by any of the substitution of leucine residues, as the subunits F_1 and F_2 were migrated separately in all cases. Surprisingly, the synthesis of F_2 subunits of all the mutant F proteins was influenced. Rather than having two species of F_2 subunits (unglycosylated [ug] F_2 and glycosylated [g] F_2), as in the case of parental F protein, three species of F_2 subunits were found in mutant F proteins, and the third one (higher F_2) was appeared to be around 2kDa heavier than the unglycosylated F_2 (Fig. 4A).. Especially in the case of F^{L216R} protein, a big amount of the higher F_2 was detected, whereas little or no unglycosylated F_2 could be detected. These results suggested that the expression and proteolytic processing of parental and mutant F proteins were similar. However the N-linked glycosylation pattern of the F_2 subunit is changed by the substitutions.

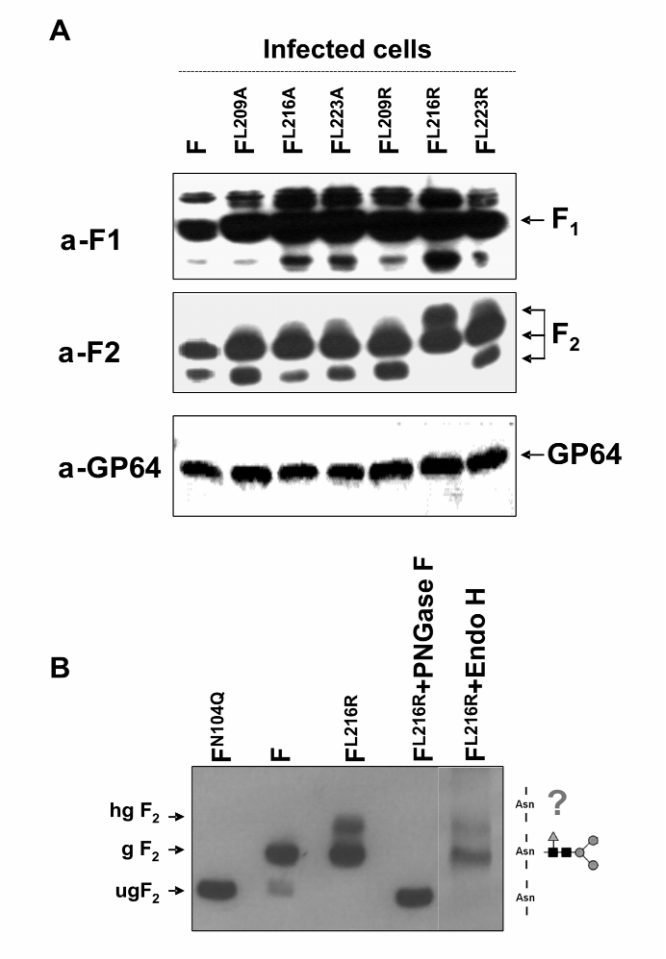

Figure 4. Expression of the parental F and mutant F proteins by baculovirus (AcMNPV bacmid with *gp64* gene) expression system. Sf21 cells were infected by AcMNPVs carrying parental f and mutant f cassettes. 48 hrs post infection, infected Sf21 cells were collected and lysed in SDS-PAGE loading buffer. Total cellular proteins from each infection were probed by anti-F_1, anti-F_2 and anti-GP64 antibodies under reducing condition (A). Deglycosylation assay of F_2 from F^{L216R} protein. (B). Cellular proteins (ranging from 25kDa to 15kDa) from AcMNPV *gp64-fL216R* infected Sf21 cells were recovered from SDS-PAGE by using SME method. Recovered proteins were treated with EndoH and PNGase F respectively. F and F^{N104Q} (a F_2 N-linked glycans removed mutant protein) were used as control.

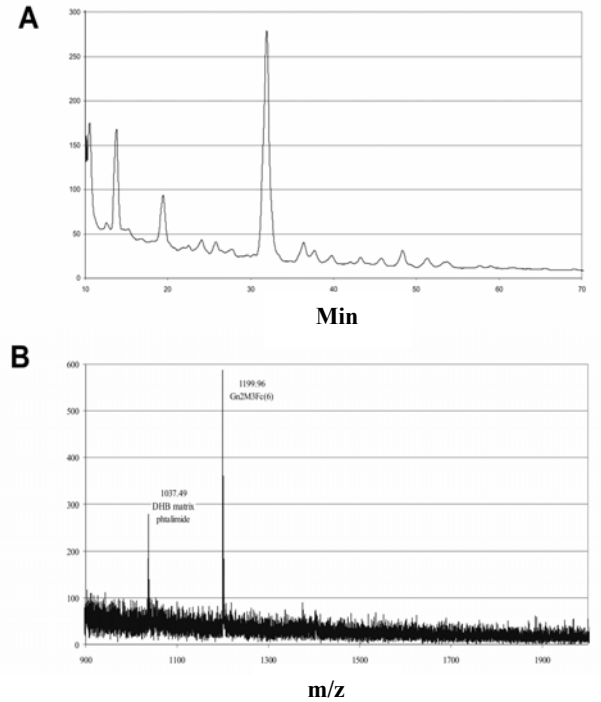

Figure 5. HPLC glycan profiling pattern on GlycoSep-N after PNGase F treatment and purification of the N-glycans of F2 subunit from pseudotyped AcMNPV BVs, which were recovered from SDS-PAGE by using SME method. Only one glycan structure (marked with an *) was found (A) and MALDI-TOF MS spectrum of the 2-AB labelled N-glycan structure found during profiling (B). The peak marked with an b originates from DHB matrix

As the mutations were introduced in HR1 region located in the F_1 subunit, the molecular mass of F_2 subunits should stay theoretically the same. The most likely explanation is that the N-linked glycosylation pattern of F_2 subunit is somehow distorted. Deglycosylation assay on SDS-PAGE recovered F_2 subunit of the F^{L216R} protein showed that the higher (heavier glycan [hg] attached) F_2 subunit is also sensitive to PNGase F treatment (Fig. 4B), with F_2 N-glycosylation absent mutant (F^{N104Q}) as a control (Long *et al.*, 2007). Both the glycosylated F_2 and higher F_2 subunits were gone, producing the unglycosylated F_2. This result confirmed that the third species of F_2 subunit was another N-linked glycosylated form, with large N-linked glycans (around 2kDa) which is 1kDa heavier than the glycans present on glycosylated F_2 subunit. And these large N-linked glycans are not sensitive to Endo H treatment, suggesting they are not of the oligomanose type. In addition, we profiled the N-linked glycans on F_2 subunit of parental F protein incorporated into AcMNPV $^{gp64\text{-}/f}$ BVs. Both NP-HPLC profiling on 2-AB labelled material (Fig. 5A), and MALDI-TOF MS (Fig. 5B) data support that a paucimannose type N-glycan with (α1-6) core-fucosylation (C6), $Fuc_1Man_3GlcNAc_2$ with a *m/z* of 1199, $[M\ 2\text{-}AB + Na]^+$, was present in F_2 subunit of mature F proteins on BVs. These results together suggested that substitution of Leu216 by R affected the glycosylation status of F_2 subunit, giving a heavier N-linked glycan in addition to the mature paucimannose type of N-linked glycans on F_2.

Incorporation of F protein mutants in AcMNPV

To further reveal a mutation effect of mutations of conserved leucine residues in the HR1 region of HearNPV F protein on intracellular trafficking we examined the incorporation of mutated F proteins in AcMNPV BVs. Proteins of sucrose purified AcMNPV BVs were separated in SDS-PAGE under reducing (Fig. 6A) or non-reducing conditions (Fig. 6B), and were subsequently immobilized to membrane for Western analysis. The presence of GP64 protein was detected and served as an internal control for the incorporation in BVs. Under reducing condition (Fig. 6A), F_1 and F_2 subunits were detected separately in all BVs. This result is in agreement with the previous finding that the furin-like cleavage of F protein is not affected by mutating the conserved leucine residues. But incorporation level of mutant F proteins in BVs is different, particularly much lower amount of the F^{L216R} proteins were incorporated in mature BVs. Under non-reducing condition (Fig. 6B), both antibodies (against F_1 and F_2) detected F_0 in the case of parental F protein. F_0s were also detected in the mutant F proteins except for the BV containing F^{L216R} protein. Significant amount of disulfide bond bridged oligomer were detected in the case of F^{L216A}, F^{L223A} and F^{L216R} proteins. The F^{L216R} protein was hardly detected as F_0s but was present mainly as oligomers. These results indicate that the proper disulfide bond formation is dependent on the conserved leucine residues in HR1 region.

DISCUSSION

HR sequences have been widely detected in membrane fusion proteins of paramyxoviruses, influenza virus, coronaviruses and retroviruses and have been shown to play an essential role in viral fusion and infectivity (Dutch *et al.*, 2000; Echert and Kim, 2001). Structural studies of membrane fusion proteins show that HR regions located in the N-terminus and C-terminus of the fusion protein ectodomain can form a coiled coil six-helix bundle structure or the fusion core (Dutch *et al.*, 2000). In the F protein from baculoviruses a group of invertebrate DNA viruses, we found three coiled-coil domains in the F_1 subunit of the HearNPV F protein. The similar architecture of baculovirus F proteins suggests that this F

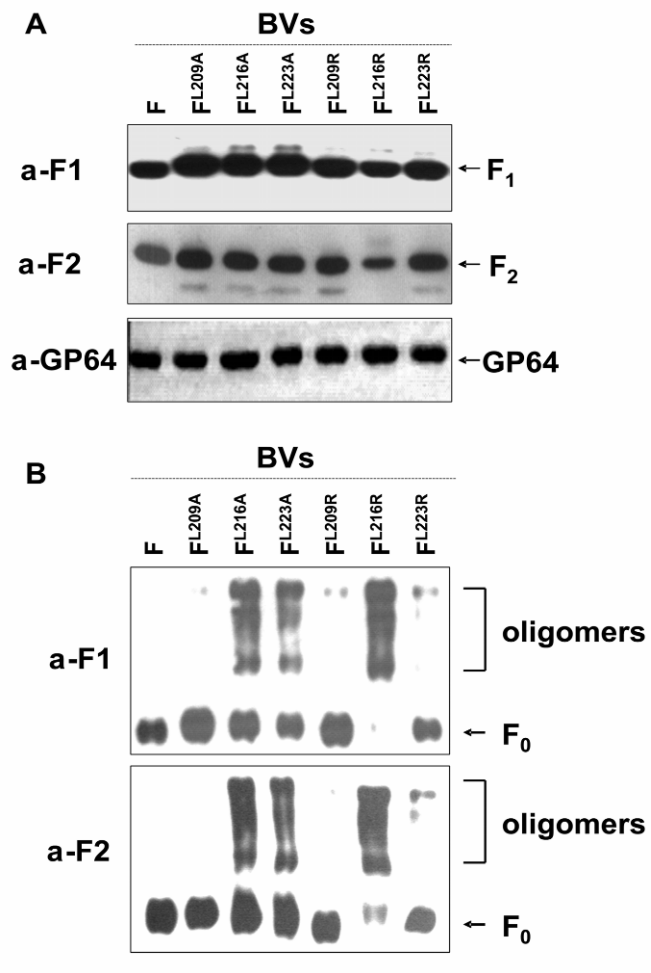

Figure 6. Incorporation of parental and mutant F proteins in AcMNPV. Western analysis was performed on purified AcMNPV BVs carrying both F/F-mutant proteins and GP64, by using anti-F_1, anti-F_2 and anti-GP64 antibodies under either reducing condition (A) or non-reducing condition (B). GP64 was detected in under reducing condition as an incorporation control.

protein is a class I virus fusion protein (Earp *et al.*, 2005), which is usually found in vertebrate enveloped RNA viruses. In order to understand the function role of HR domains in baculovirus F proteins, we studied the functional importance of HR1 by site-directed mutagenesis and reverse genetics.

By utilizing the *gp64*-null AcMNPV pseudotyping system (Lung *et al.*, 2002)we demonstrated HR1 region of HearNPV F is essential for rescuing the infectivity of this AcMNPV bacmid. In addition, we showed a variable but clear effect, when individual leucine residues within the HR1 region were replaced by alanine and arginine (summarized in Table 1). Both the alanine and the arginine substitution of Leu209 resulted in a dramatic decrease of rescued AcMNPV infectivity indicating that the Leu209 is very critical for infectivity. Leu209 is conserved at the "d" position of the second repeat in HR1 in group II baculovirus F proteins, but not in GV F proteins. At the "a" position is a polar asparigine residue. Substitution of the Leu209 with either alanine or arginine probably results in two continuous "non-bulky" hydrophobic residues on the hydrophobic side of the coil. This possibly destabilizes the interaction of HRs during 6-helix bundle formation or fusion and results in marginal infectivity of L209A and L209R when pseudotyped in AcMNPV. This result is also compatible with the observation that the F protein from *Plutella xylostella* GV (PxGV) is not able to rescue the infectivity of *gp64*-null AcMNPV (Lung *et al.*, 2002) as a threonine residue was located at this position.

Table 1. Summary of obtained results

	Coil#	Rescue	Cells per plaque*	Cleavage	N-glycans on F2	Detection of F0	Incorporation
F	++	++	19	+	1.1kDa	++	++
F^{L209A}	+	+	1	+	1.1kDa	++	++
F^{L209R}	+	+	1	+	1.1kDa	++	++
F^{L216A}	++	++	5	+	1.1kDa	++	++
F^{L216R}	-	-	NA	+	1.1/~2.0kDa	+/-	+
F^{L223A}	+	+	1	+	1.1kDa	++	++
F^{L223R}	+	++	4	+	1.1kDa	++	++

NA: Not Applicable.
#: Coil probability was the prediction according to Berger algorithm.
*: Cells per plaque were counted 3days post infection.

Leu216 replaced by alanine resulted in a slight decrease of the F rescuing activity, whereas the substitution of Leu216 by arginine led to a complete failure for HearNPV F to rescue the infectivity of the *gp64*-null AcMNPV. According to the Berger algorithm (Berger *et al.*, 1995) among the six mutant proteins the substitution of Leu216 by arginine is the only one to abolish the coiled coil structure of the HR1 region, which is in good agreement with our results showing no infectivity when F^{L216R} was tested in the AcMNPV pseudotyping system (Table 1). These results further suggest that HR1 is a critical determinant of F function. The substitution of Leu223 by arginine (F^{L223R}) does not affect so much the AcMNPV rescuing activity than that by alanine (F^{L223A}). This might suggest that at the location of Leu223 the size of amino acid residue side chain is more important than its polarity.

In order to study the influence of the six leucine substitutions to F folding, intracellular trafficking and viral incorporation we used the conventional AcMNPV-Sf21 insect-cell expression system (King and Possee, 1992). Mature baculovirus F proteins are located on BV particles as a homotrimer of heterodimers, in which F_1 and F_2 subunits are bridged by disulfide bonds. Three F proteins oligomerize as homotrimer through non-covalent interactions (Long *et al.*, 2006a). Here we demonstrated that on BV particles, substitutions of selected leucines within HR1 did not affect proteolytic processing of F but resulted in multiple disulfide-linked oligomers (Fig. 6.). In particular, the L216R mutant protein hardly produces F_{1+2} as an 80 kDa protein which is the mark of mature and functional F on infectious BV particles. Both antibodies against F_1 and F_2 subunits detected similar malformed disulfide-bridged oligomers (Fig. 6B) suggesting that the disulfide bond between F_1 and F_2 subunits were not affected. Disulfide bonds are involved in the oligomerization of mutant proteins producing malformed oligomers, thus illegitimate cross-linking must have occurred between monomers. Less protein was detected in AcMNPV BVs with F^{L216R} than with the other F mutant and parental proteins. This further suggests that HR1 is not only an important determinant of proper folding and disulfide bond formation of HearNPV F protein, but also important for intracellular HearNPV F protein synthesis.

Like other virus envelope fusion proteins baculovirus F is also modified by N-linked glycosylation of both F_1 and F_2 subunit. It was previously demonstrated that the only potential N-linked glycosylation site in the HearNPV F_2 subunit is genuinely occupied with glycans (paucimannose). N-linked glycosylation is a prerequisite for virus envelope fusion protein

folding and function (Helenius and Aebi, 2004). The sensitivity of N-linked glycans for Endo H treatment was used as a marker of proper glycoprotein folding and intracellular trafficking. Here we showed that replacement of selected leucine residues at "d" positions in HR1 resulted in the attachment of a heavier glycan on the F_2 subunit, with a molecular weight around 2kDa, in addition to the mature Man3GlcNAc2Fuc (Jarvis, 2003). Again in particular for the F^{L216R} protein, a significant amount of F_2 with the heavier glycan was detected (Fig. 4A). Traces of F_2 with heavier glycans were also detected for the other five mutant F proteins (Fig. 4A). As compare to mammalian cells insect cells possess a truncated N-linked glycosylation pathway and a paucimannose type of N-linked glycan was frequently identified in mature glycoprotein expressed in insect cells (Jarvis 2003). The large N-linked glycans from the F^{L216R} F_2 subunit might be from intermediate folding form (oligomannose) on the F^{L216R} protein. It may be equally possible, that the large N-linked glycans are the end product of further extension on the GlcNacMan3GlcNac2Fuc structure. In insect cells only oligomannose type of N-linked glycans can reach a molecular weight of around 2kDa. This view can be supported when assuming the involvement of the calnexin/calreticulin glycoprotein quality control cycle (CNX/CRT cycle) (Helenius and Aebi, 2004). In that case glycoproteins carrying GlcMan9GlcNac2 (with molecular weight around 2kDa) are kept cycling in ER. However, this is an unlikely interpretation for the following reasons: i) F_2 with the heavier glycans were also found to be transported to the cell membrane incorporated in mature AcMNPV BVs (Fig. 6A); this is in contrast to the ER retention of malfolded glycoprotein in the CNX/CRT cycle; ii) these heavier glycans cannot be removed from F_2 by using Endo H treatment (Fig. 4), which is also not in agreement with the assumption that the cycling proein-carried GalMan9GlcNac2 glycan is of the high mannose type which is sensitive to Endo H; iii) the CNX/CRT cycle in the ER seems not present in insect cells, as co-expressed calreticulin and calnexin is needed to promote proper folding of human lipoprotein lipase expressed in Sf21 cells (Zhang *et al.*, 2003). Therefore the heavier glycans are most likely the end product of N-glycan extension. We do not know the detailed structure of this heavier glycan. Further N-linked glycan profiling and lectin binding assays to determine the structure will be necessary and will contribute to the understanding of N-linked glycoprotein quality control mechanism in insect cells.

Baculovirus BVs enter host cells through a clathrin-dependent endocytosis and the nucleocapsids are released upon a low pH activated fusion (Long *et al.*, 2006b), mediated by either GP64 (group I) or F (group II). The HR region in GP64 plays critical role in GP64 mediated membrane fusion (Kingsley *et al.*, 1999). Here, we have shown by reverse genetics that the fusion peptide-proximal HR1 is an important determinant of baculovirus F protein function, virus infectivity and F protein proper folding. This HR1 is well conserved in the F homologous proteins from group II NPVs and GVs, whereas HR1 region can not be found in the truncated F homolog from group I NPVs, which posses the GP64 homologous protein as the functional envelope fusion protein (Fig. 1) (Lung *et al.*, 2003) Therefore our results indicate that the presence of the HR1 region in F homologs in group II NPVs and in GVs is a hallmark for a functional baculovirus F protein.

ACKNOWLEDGEMENT

We are grateful to Prof. Dr. J. F. G. Vliegenthart from the Bijvoet Center for Biomolecular Research of Utrecht University for introducing G. Long to the Research Group Bio-Organic Chemistry to set up N-linked glycan profiling study. This work was funded by

the grants from the Royal Academy of Sciences of The Netherlands (KNAW) (project numbers 04-PSA-BD-02) and a Ph.D sandwich grant to GL from Wageningen University.

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Chapter 7

General Discussion

GENERAL DISCUSSION

Baculoviruses were originally studied in view of their application against pest insects in agriculture and forestry. The prototypic baculovirus AcMNPV, a group I NPV, is well studied and this paved the way to its use in modern biotechnology. In the last two decades significant advances were made in the baculovirus insect cell expression system and the use of baculovirus as gene delivery vector for gene therapy purposes in vertebrates.

Instead of the major envelope fusion protein GP64, found in group I NPVs, a new type of baculovirus envelope fusion protein, designated F, was recently identified in group II NPVs (IJkel *et al*., 2000; Pearson *et al*., 2000). In this thesis we studied the structure-function relationship of the group II baculovirus envelope fusion protein F with HearNPV as an example. Firstly the protein was studied with respect to its general characteristics including post-translational modification and fusion function (Chapter 2). This study was extended in Chapter 3 where the influence of N-linked glycans attached to F_2 on virus infectivity and F fusogenicity is described. In Chapter 4 the functional entry pathway of baculovirus into insect and mammalian cells were compared. Chapters 5 and 6 clarified the functional role of two important domains of F, CTD and HR1, respectively. The implications of the obtained results with respect to the baculovirus entry mechanism, intracellular processing and egress are discussed in Chapter 7.

BACULOVIRUS ENTRY

BV Entry through endocytosis

Two types of enveloped viruses are produced in the baculovirus infection cycle: the ODV, responsible for horizontal or insect-to-insect infection, and the BV, responsible for systematic or cell-to-cell infection. To initiate successful virus infection these two types of virions have to attach selectively to their host cells through specific interactions between certain cell surface receptors and the viral surface proteins. Following the specific interaction, ODVs enter the midgut epithelium cells through direct fusion, whereas the BVs enter the target insect or mammalian cells through an endocytic pathway. This thesis focuses on BVs.

As obligatory intracellular parasites enveloped viruses have to penetrate the cellular membrane barrier to deliver their genetic material into the cytosol or the nucleus. In general, the virus penetration strategy is either direct fusion between virus envelope and plasma membrane at neutral pH or low pH-dependent fusion between virus envelope and membrane of the intracellular organelle (Marsh and Helenius, 2006). The functional entry of baculovirus BVs into insect and mammalian cells is dependent on clathrin-mediated endocytosis. Of the endocytic pathways taken by viruses, the clathrin-mediated endocytic route is most frequently utilized. One of the advantages is that clathrin-mediated endocytosis gives the baculovirus BVs a “free ride” deep into cytoplasm, transporting them with the receptors into early and late endosomes. Another advantage is that clathrin-mediated endocytosis is a continuous process for virus entry in a usually rapid and efficient way (Marsh and Helenius, 1989). It is not surprising that baculovirus BVs select the same route to ensure successful gene delivery into insect and mammalian cells. However, the baculovirus BVs appeared to enter mammalian cells through multiple unusual entry pathways, including macropinocytosis for HepG2 cells (Matilainen *et al.*, 2006) and caveolar/raft endocytosis for BHK21 cells (Chapter 4).

Microvillar-like structures are observed with high occurrence in HepG2 cells suggesting macropinocytosis an entry route of baculovirus into these cells (Matilainen *et al.*, 2006). The final destiny of the virus uptake through this route is not clear. The caveolar/raft pathway is dependent on cholesterol and the activation of tyrosine-kinase signaling cascades. Inhibition of tyrosine-kinase appeared to enhance the transduction efficiency of baculovirus to BHK21 cells (Chapter 4), suggesting that baculovirus BVs are taken up through the caveolar/raft pathway, which did not seem to be functional for transduction. Inhibition has no effect on baculovirus BVs infection to insect cells, suggesting that baculovirus induced caveolar/raft endocytosis occurs only for mammalian cells. This view can be further supported by the significant difference in lipid compositions of insect cell versus mammalian cell plasma membranes. Plasma membranes of Sf9 cells contain 10 times less cholesterol than the ones isolated from mammalian cells (Renkonen *et al.*, 1972; Luukkonen *et al.*, 1973; Gimpl *et al.*, 1995; Marheineke *et al.*, 1998). Thus, the caveolar/raft route might be avoided by this natural difference in lipid composition and the resulted membrane physiology. Also the entry of group I NPVs (AcMNPV) BVs having GP64 as their envelope fusion protein do not seem to depend on lipid raft (Zhang *et al*., 2003b). This bypass of non-functional entry pathway (caveolar/raft route) during infection could be taken as an evolutionary benefit, because a m.o.i. of 5 $TCID_{50}$ units/cell is enough to ensure complete infection. A much higher MOI is required for baculovirus to transduce mammalian cells suggesting a large portion of viruses might not be able to be endocytosed properly. Therefore, genetic engineered baculoviruses specifically enhancing the uptake through the clathrin-mediated pathway would be a good approach to enhance baculovirus gene delivery in future research.

F mediated fusion

Enveloped viruses use specialized proteins to achieve fusion between a viral envelop and a cellular membrane structure. Baculovirus F proteins from group II NPVs mediate low-pH activated fusion in a similar fashion to its counterpart, GP64, from group I NPVs. Their protein sequence however is entirely different. F protein genes from group II NPVs can rescue the infectivity of *gp64*-knock out AcMNPV (Lung *et al.*, 2002 and Chapter 2), suggesting that these F proteins mediate successfully the receptor binding function and the membrane fusion activity. With respect to fusogenicity, the low-pH dependent fusion mediated by GP64 is much more efficient that the one mediated by F (Westenberg, 2004). AcMNPV pseudotyped with F demonstrated less viral infectivity than wild-type AcMNPV (Lung *et al.*, 2002 and Chapter 5) and a HearNPV carrying both F protein and GP64 exhibits more viral infectivity than the wild-type HearNPV (H. Wang, personal communication). Additionally, GP64 can enhance HearNPV mediated gene delivery in mammalian cells possibly through more efficient endosomic escape through membrane fusion (Liang *et al.*, 2005), although at a very low level.

Despite their genetic divergence, functional, structural and biophysical studies have shown that baculovirus F proteins can be categorized as a class I viral fusion protein, occurring in many enveloped RNA viruses of vertebrates. Known examples of class I viral fusion proteins are the retrovirus ENV protein (GP120; *Retroviridae*), the influenza virus hemagglutinin (HA; *Orthomyxoviridae*), the paramyxovirus fusion protein (F; *Paramyxoviridae*), the Ebola virus glycoprotein (GP2, *Filoviridae*) and the coronavirus spike protein (S; *Coronaviridae*). In general the class I virus fusion proteins are synthesized at first as a single-molecule precursor which is subsequently cleaved by a cellular proprotein convertase into a surface subunit and a transmembrane unit. A general fusion mechanism is

utilized by these virus fusion proteins to accomplish a membrane fusion. Upon receptor binding on the cell surface or acidification in the late endosome, proteolytic cleavage exposes the N-terminal open fusion peptide on the transmembrane subunit which in turn is then subsequently inserted into the cellular membrane. Two heptad repeat (HR) regions, HR1 (immediately downstream of the fusion peptide) and HR2 (proximity upstream of the transmembrane region), are located in the membrane-anchored unit. These HR regions are predicted to be alpha-helical and have a high tendency to form a "coiled-coil" structure. Irreversible conformational changes involving the assembly of these HR1 and HR2 into a highly stable six-helix bundle (6HB) structure or fusion core are believed to drive the outer leaflets of the membranes to reach the so-called hemifusion status. This conformational change finally promotes the formation of the fusion pore through which the virus nuclear capsids can be released into the cytosol for further transportation. Three dimensional structures of this fusion core have been determined of the fusion proteins of various viral families (Fig. 1). In general, synthetic peptides corresponding to the HR regions strongly inhibit virus envelope fusion protein mediated fusion and virus infectivity.

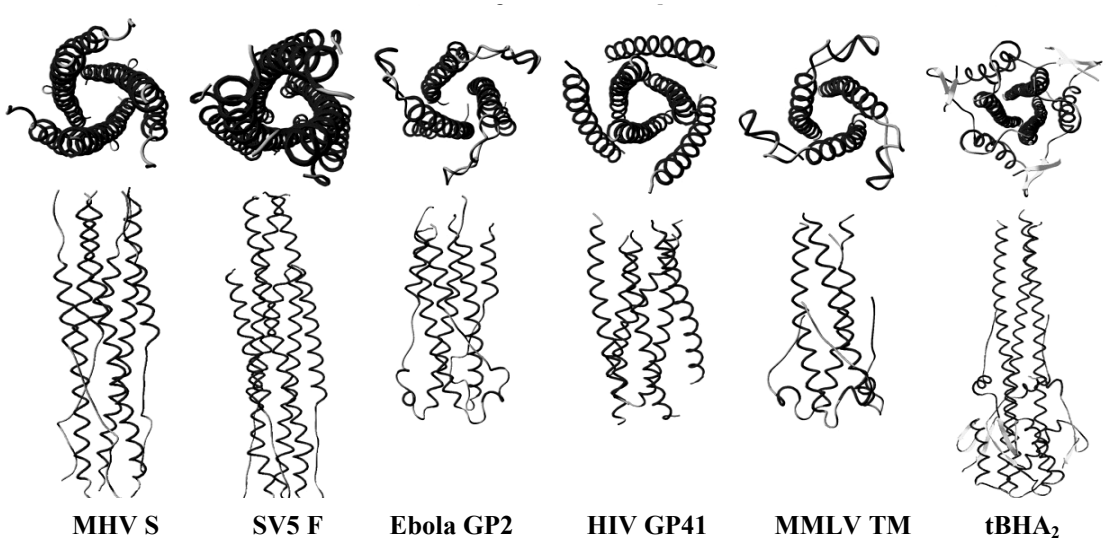

Figure 1. Comparison of MHV fusion core with other viral fusion protein structures. The proteins under comparison include SV5F, Ebola GP2, HIV gp41, MMLV Env-TM, and low pH-induced influenza virus HA, tBHA2. Top and side views are shown for the six fusion core structures (adapted from Xu, *et al.*, 2004, with permission).

Like other class I viral fusion proteins the baculovirus F protein is a type I membrane glycoprotein that is synthesized at first as a precursor in the ER and cleaved subsequently by furin-like cellular convertase in the Golgi, giving the N-terminal subunit F_2 and C-terminal membrane bound subunit F_1. This cleavage is essential for fusogenicity and thus virus infectivity (Westenberg *et al.*, 2002). At the new N-terminal domain of the F_1 subunit a hydrophobic sequence, referred to as the fusion peptide becomes exposed. Similarly, two HR regions (HR1 and HR2) were predicted in the F_1 subunit. And another HR region (HR3) downstream of HR1 was predicted only in HearNPV F. The putative fusion core of HearNPV

F protein consisting of these three HR regions and an inner artificial linker forms a trimer in vitro (Fig. 2). Alteration of the HR1 region by replacing conserved leucine residues abolished F fusogenicity and virus infectivity (Chapter 6). Thus it is reasonable to believe that baculovirus F proteins use the same strategy to accomplish membrane fusion as other class I viral fusion proteins.

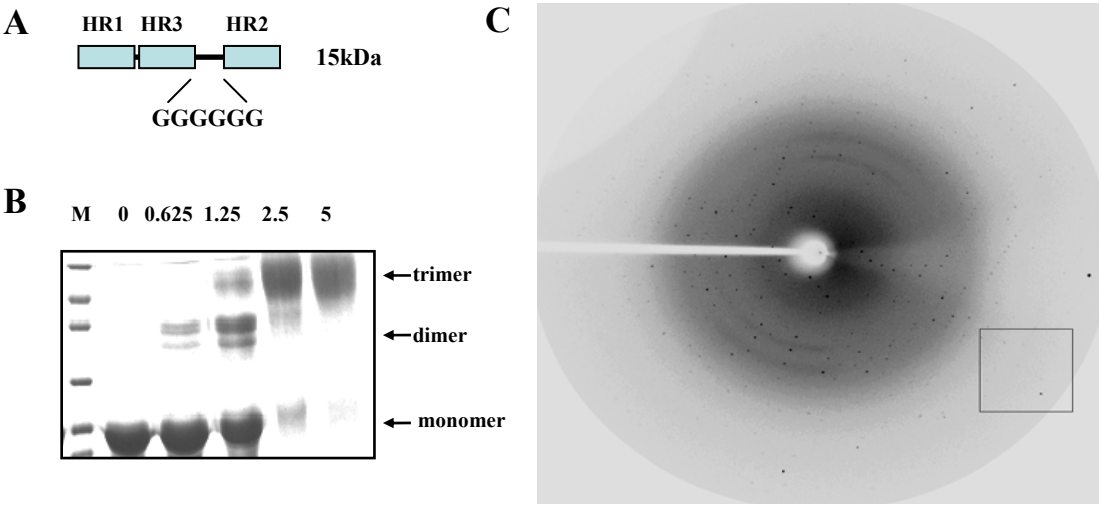

Figure 2. Preliminary characterization of HearNPV F fusion core. (A) Diagram of the putative fusion core of HearNPV F protein. Fragment HR1-HR3 and HR2 was linked by an artificial linker consisting of 6 glycine residues. (B). Chemical cross-linking of the putative fusion core sequence of HearNPV F protein with different concentrations of chemical cross-linker, EGS (Ethylene Glycolbis-succinimidylsuccinate, Sigma). Cross-linked products were separated by 15% SDS–PAGE followed by Coomassie brilliant blue staining. Numbers indicate the concentrations of the EGS used in mM. Bands corresponding to monomer, dimer, and trimer are indicated. (C) Diffraction pattern of obtained crystal.

However, in contrast to what has been found for other vertebrate virus class I fusion proteins, the peptides corresponding to HR regions showed very low inhibitory activity to either membrane fusion or virus infection (Long *et al.*, 2004). Further biophysical and structural research on the baculovirus F protein fusion core will be valuable to understand this observation.

F SYNTHESIS

Disulfide bridging

The formation of disulfide bridges between the correct pairs of cysteine residues is essential for folding and stability of many proteins that are synthesized via the secretory pathway. Especially for virus envelope fusion proteins, correct crosstalk of cysteine residues guarantees correct conformation and functionality (Braakman *et al.*, 2000). Baculovirus envelope fusion proteins, GP64 and F protein, were found to be stabilized by intermolecular and/or intramolecular disulfide bonds. The GP64 fusion protein is located in the BV envelope and on the cell membrane upon egress as a trimer and subunits connected by intra-molecular

disulfide bonds (Oomens *et al.*, 1995; Oomens *et al.*, 1999). The subunits of baculovirus F proteins, F_2 and F_1, are associated with a single disulfide bridge forming a heterodimer F_0. A single and conserved cysteine residue is present in the F_2 subunit and very likely involved in this linkage. Non-convalent interactions mediate the trimerization of baculovirus F protein.

The primary amino acid sequence contains sufficient information to direct correct folding. Changes in the primary amino acid sequence of HR1 of HearNPV F protein by substituting conserved leucine residues resulted in conformationally different F proteins (Chapter 6). Illegitimate and erratic disulfide bonding of F oligomers (Chapter 6) is most likely mediated by the intra-F_1 disulfide bond which is not present in the case of wild-type F protein. This malformation caused a significant effect on F intracellular processing including the trimming process of N-glycans and the trafficking of F to the budding destination in the plasma membrane. As a result, virus infectivity was drastically decreased. In the case of a Cys-HA, a modified HA in which the globular head domains are locked together by engineered intermonomer disulfide bonds, the low-pH dependent membrane fusion was inhibited by preventing HA-mediated membrane fusion (Goldley *et al.*, 1992, Kemble *et al.*, 1992). This information suggests that the proper "crosstalk of cysteines" is a major determinant to guarantee the native tertiary structure of virus membrane fusion proteins. A change in the disulfide bond arrangement by altering the primary sequence puts the function of these proteins at risk. It was assumed that conserved proteins carrying a similar biological function might share a conserved cysteine crosstalk pattern. However, despite the high degree of amino acid sequence homology among the available F proteins from baculovirus group II NPVs (Table 1, Chapter 1), the position and number of cysteine residues vary. This might suggest a variable disulfide-bond pattern among these proteins. Assignment of the F protein intersubunit and the intrasubunit disulfide bonds by mass-spectrometry analysis remains to be necessary to clarify if there is a common disulfide-bond arrangement among baculovirus F proteins.

Besides the common role in stabilizing the functional conformation of virus membrane fusion proteins prior to virus assembly, disulfide-bonds are also found to play an essential role in activating the membrane fusion process during entry. This was first reported in the case of Sindbis virus (Anthony *et al.*, 1991; Abell *et al.*, 1993). Here the authors found that reduction of critical disulfide-bridges stabilized membrane fusion protein during membrane penetration and disrupted the rigid protein-protein associations, thus allowing membrane fusion to occur and allowing the release of the viral genome into the cell. Recently, a more lucid view was drawn in the case of HIV (Fenouillet *et al.*, 2001, Barbouche *et al.*, 2003). Barbouche and colleagues found, changing the redox status through inhibition of the protein-disulfide isomerase (PDI) by anti-PDI antibodies and catalytic function inhibitors, altered ENV-mediated membrane fusion, suggesting an important role of PDI. In 2003 the same group found that after HIV binds to the lymphocyte surface receptors CD4 and CXCR5 and prior to the membrane fusion process, protein-disulfide isomerase-involved reduction of two inter-molecular disulfide bonds of GP120 appeared to be required for fusion. It was also suggested that the PDI mediated thiol/disulfide exchange effectively lowers the activation energy barrier required for ENV conformational change (Markovic *et al.*, 2004). In contrast the membrane fusion, mediated by SARS-coronavirus spike protein, is independent of redox state changes (Lavillette *et al.*, 2006). Thus, disulfide-bond isomerization does not seem to be a general prerequisite for the envelope fusion mediated by all virus fusion proteins. So far it is not clear whether this isomerization scenario is suitable for baculovirus fusion proteins or not.

N-glycosylation

Virus envelope fusion proteins are generally heavily N-glycosylated, as the attached N-linked oligosaccharides are important for their correct folding, stability, trafficking and fusogenicity. Total inhibition of N-glycosylation using tunicamycin and deglycosylation treatment revealed that baculovirus F proteins are also modified by N-glycosylation (Chapter 2 and Fig. 3). In the case of HearNPV F protein, both F_1 and F_2 subunits are N-glycosylated and two major types of N-glycans (the paucimannose and the oligo-mannose structures) were identified. This is in tune with the finding that the glycans of recombinant N-glycoproteins produced in lepidopteran cell lines using the baculovirus expression system were mostly of these two structures. N-glycans produced in HzAM1 and Hz2E5 cells do not contain the C3 fucose group, which is a potential allergen in therapeutic application, as PNGase F can fully and successfully trim the N-glycans off F protein. However, the N-linked oligosaccharides from the same protein expressed in *T. ni* Tn-5B1-4 cells are resistant to PNGase F treatment, suggesting the presence of the C3 fucose group in these cells. Thus, the production of C3 fucose residue is not a general characteristic for insect cells. The molecular percentage of native insect N-glycans with C3 fucose ranges from 2.5% for a *B. mori* cell line (BmN) to 30% for an *M. brassicae* cell line (MB-0503) (Kubelka *et al.*, 1994). Approximately 12-23% of the N-glycans on recombinant glycoproteins produced in Tn-5B1-4 cells carried C3 fucose. There is no detectable C3 fucosylation of latent TGF-β-binding protein-1 expressed in Sf9 cells (Rudd *et al.*, 2000), human interferon gamma expressed in *E. acrea* Ea4 cells (Ogonah *et al.*, 1996), or human transferring expressed in *Lymantria dispar* Ld652Y cells (Choi *et al.*, 2003). The presence of C3 fucose, as judged by blotting with anti-horseradish peroxidase polyclonal antibodies, was restricted to neurons in grasshopper and *D. melanogaster* embryos (Snow *et al.*, 1987; Fabini *et al.*, 2001). A preliminary survey of insect cell cultures was conducted using a similar blotting procedure, which revealed that C3 fucosylation also occurs in Tni368 and in SeUCR cells (data not shown). Thus, selection of cultured insect cell lines from different origins or even from the same insect could be taken to avoid the C3 fucose impediment for recombinant protein production in the baculovirus-insect cell expression system for therapeutic use. Another way to address this problem would be to down-regulate (RNAi) or eliminate the enzymes involved in C3-fucosylation, such as the N-acetylglucosaminidase and C3 fucosyltransferase (Harrison *et al.*, 2006).

N-glycosylation and protein proper folding are closely interconnected processes which occur in the ER. The folding process is assisted by a number of chaperone proteins, at least in mammalian cell systems. Regarding the proper folding of glycoproteins, a well characterized protein quality control system mediated by two chaperones, calnexin and calreticulin, was established and designated as CNX/CRT cycle (Helenius *et al.*, 2001). This unique chaperone system was found in the ER of nearly all eukaryotes (Helenius *et al.*, 1997; Zapun *et al.*, 1999; Parodi *et al.*, 2000). Calnexin or calreticulin binds the N-glycans Glc1Man9GlcNac2, a glycan moiety of glycoproteins trimmed by glucosidase I and II, and forms a complex with ERp57, a thiol oxidoreductase homolog of PDI. However, the expression GFP in secretory pathway using the baculovirus-insect cell expression system dramatically decreased the expression level of two major ER chaperone proteins, bPDI and calnexin (Yun *et al.*, 2005). Baculovirus encoded calreticulin assisted the folding, dimerization and enzymatic activity of lipoprotein lipase (Zhang *et al.*, 2003). These studies might indicate that in baculovirus infected insect cells a stable level of chaperones in the ER is required to guarantee the conformational integrity of heterogeneous proteins. However, in our research a mal-folded HearNPV F protein carrying endo-H resistant glycans was transported to the plasma

membrane for budding, although the incorporation level into BVs was much lower as compared to the wild-type HearNPV F protein (Chapter 6). This finding suggests that glycoproteins expressed in insect cells might not utilize a different system to control protein folding.

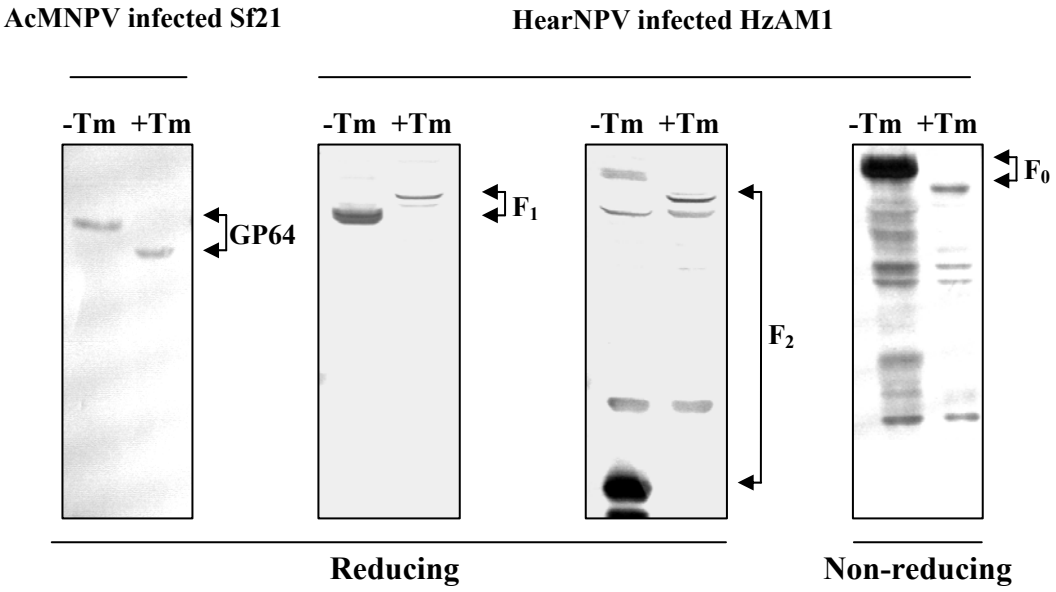

Figure 3. Impact of total inhibition of N-glycosylation in baculovirus envelope fusion protein expression. Sf21 cells and HzAM1 cells were infected by AcMNPV and HearNPV BVs respectively at a m.o.i.of 5 $TCID_{50}$ units per cell. After 48 hours of incubation at 28^{o}C, in the absence or presence of 10ug/ml tunicamycin, total protein of the infected cells was subjected to SDS-PAGE and western analysis under reducing or non-reducing conditions, using antibodies against AcMNPV GP64, HearNPV F_1 and F_2 subunits respectively.

N-glycosylation is a prerequisite for proper glycoprotein folding and intracellular trafficking, and thus is important for protein functionality. For virus membrane fusion proteins these functions include receptor binding, membrane fusion and virulence. In the case of HearNPV F protein, deletion of the N-glycans from the F_2 subunit appeared to not inhibit receptor binding or to block infectivity, but resulted in a hyperfusogen phenotype. The elimination of F_2 glycosylation leads to a more virulent BV. Whether this is also the case *in vivo* remains to be characterized (Chapter 3).

Compared to the tertiary structures of paramyxovirus F proteins (Yin *et al.*, 2005),this N104 glycosylation sequon is situated within the 'neck' region of the baculovirus F protein trimer. Elimination of N-glycans from the N104 site of HearNPV F protein could probably facilitate the conformational changes during the fusion between viral and cellular membranes in the same way as was the case for the N67 site of Nipah Virus (Aguilar *et al.*, 2006). Recently it was found that total inhibition of N-glycosylation by tunicamycin resulted in the production of no infectious BV at all. Most likely this failure to produce infectious BV is a consequence of problems of multiple steps, including virus assembly in the nucleus, intracellular transportation of nucleocapsid and BV budding from plasma membrane (Kelly *et al.,* 1980). With respect to the proper folding and trafficking of F protein, however, tunicamycin treatment significantly inhibited the F_0 cleavage (Fig. 3). This result implies that

the overall N-glycosylation of baculovirus F protein is somehow essential for the proper folding. It has been demonstrated total inhibition of N-glycosylation with tunicamycin does not block the secretory pathway (Wei *et al*., 2005). Thus, the F protein without N-glycosylation in the ER either failed to be transported to the Golgi apparatus where the furin-like convertase mediated proteolysis occurs or made its way to the Golgi but had no accessibility for the proteolysis. Although these possibilities are not yet experimentally approached, the essential role of N-glycosylation in F protein functionality is beyond doubt. Therefore it is postulated here that in case the N-glycans on F_2 are absent, the contribution of N-glycans in baculovirus F protein proper folding and functionality could be covered by the N-glycans in the F_1 subunit.

Proteolysis

Activation of virus membrane fusion proteins is a sophisticated process. The virus membrane fusion proteins are in general synthesized as polypeptide precursors trimerized in the ER of virus infected cells. When the homotrimers are transported to the trans-Golgi complex, the polypeptide precursors are proteolytic cleaved into a receptor-binding surface subunit and a fusogenic transmembrane subunit by cellular furin or furin-like convertase which recognizes an Arg-X-Lys/Arg-Arg motif in the precursors. As described before, baculovirus F proteins were first synthesized as a single-chain precursor in the ER and cleaved in the Golgi by furin-like cellular convertase at a furin cleavage site (Westenberg, *et al.*, 2002 and Chapter 2). This cleavage is the first step in the activation of the viral envelope fusion protein (Westenberg, *et al.*, 2002; Lung *et al.*, 2003.) and is essential for virus fusogenicity and infectivity. This cleavage necessity could be simply explained by the requirement of an N-terminal open fusion peptide according to the consensus fusion mechanism of class I virus fusion proteins (Eckert and Kim, 2001). This cleavage site is not present in the F homologs from group I NPVs (Pearson *et al.*, 2001; Lung *et al.*, 2004), for instance the Ac23 and Op21, which is in agreement with their inability to mediate membrane fusion and to preserve virus infectivity when the GP64 envelope fusion protein gene is knocked out from AcMNPV.

The above described cleavage was initially not found in the LdMNPV F protein (Pearson *et al.*, 2000). This situation can be explained by the lack of proper cellular convertase to execute the cleavage in the host cell of LdMNPV, as the LdMNPV F protein can be successfully cleaved when it is expressed in Sf9 cells (Pearson *et al.*, 2002). Endosomal protease might be involved in the activation of LdMNPV F protein fusogenicity like in the case of hemaglutinin of influenza virus A/WSN/33 (Boycott *et al.*, 1994). Recently two other cases have been reported that endosomal proteolysis of the virus envelope glycoprotein is necessary for Ebola virus and SARS coronavirus infection (Chandran *et al.*, 2005; Simmons *et al.*, 2005; Huang *et al.*, 2006; Schornberg *et al.*, 2006). Mature GP of Ebola virus is a trimer of three disulfide-linked GP1-GP2 heterodimers that are generated by the proteolysis of a GP0 precursor during virus assembly. The endosomal cathepsin B and cathepsin L mediate entry by further proteolysis of the GP1 subunit. For the SARS coronavirus cathepsin L proteolysis of S protein was found to be essential to trigger the conformational changes after receptor-binding, followed by the activation of membrane fusion within endosomes.

Syncytium formation mediated by baculovirus F protein appeared to be much slower than that mediated by the GP64 (Westenberg, 2004). This could probably indicate that F protein mediated fusion needs similar endosomal protease activation. However, this cathepsin L and/or cathepsin B mediated proteolysis within endosome seems not to be required for group

II baculovirus infectivity, as an extensive cysteine protease inhibitor E-64 treatment brought little to no decrease in HearNPV BV infectivity (G. Long, unpublished data). The possibility can not be ruled out that another fusion activation process occurs besides the furin-like proteolysis during F protein synthesis and low-pH activation in the late endosome.

VIRUS BUDDING

Incorporation of actin

The presence of actin in budded enveloped virus or virus-like particles seems to be a general observation (Damsky *et al.,* 1977; Naito *et al.*, 1978; Tyrrell *et al.*, 1978; Ott *et al.*, 1996; Ulloa *et al.*, 1998; Wong *et al.*, 1998; Nermut *et al.*, 1999; Johannsen *et al.*, 2004), indicating that the crossing of the plasma membrane might be an actin-dependent process, possibly related to endogenous multivesicular body formation (Garrus *et al.*, 2001). The further role of actin, however, when incorporated in enveloped virus particles remains to be revealed. In the case of baculoviruses cellular actin is also a structural component of BVs from both group I and II NPVs (Lanier *et al.*, 1998 and Chapter 2). In this thesis research, it was found that cellular actin is a major component of purified HearNPV BVs. The actin makes up about 20% of BVs in terms of protein weight (Fig. 4). Baculovirus nucleocapsids are assembled in the nucleus within the virogenic stroma, where also virus DNA replication occurs. With respect to the BV progeny, pre-assembled nucleocapsids transfer from nucleus to cytoplasm through a poorly-determined mechanism and bud at plasma membrane through exocytosis.

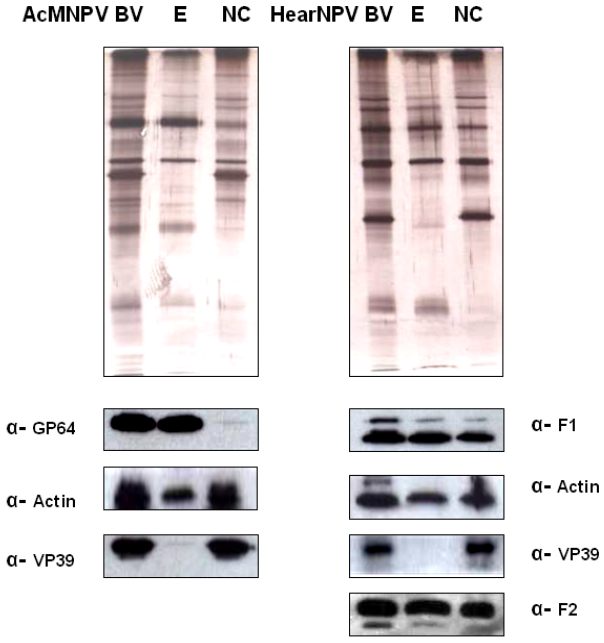

Figure 7. Actin incorporation into AcMNPV and HearNPV BVs. Sucrose purified AcMNPV or HearNPV BVs were fractionated with 1% NP40. Total BV proteins, envelope fraction (E) and nucleocapsid fraction (NC) from each virus were subjected to western analysis using indicated primary antibodies.

It is well documented that cellular actin plays a vital role in baculovirus replication. Immediately after endosome penetration of BV nucleocapsid, F-actin cables can be detected within the cytoplasm (Charlton *et al.*, 1993), which are thought to assist the transportation of the nucleocapsids to the nucleus (Lanier *et al.*, 1998). During late gene expression F-actin appears within the nucleus, both within the central viral stroma (Young *et al.*, 1993) and more significantly within the surrounding "ring zone", adjacent to the inner nuclear membrane

(Volkman *et al.,* 1987; Volkman, 1988; Charlton *et al.*, 1991). This nuclear F-actin is essential for nucleocapsid morphogenesis (Goley et al., 2006). Within the nucleus AcMNPV p78/83 (minor basal capsid protein) and VP39 (major capsid protein) colocalize with the F-actin (Lanier, 1995; Lanier *et al.*, 1998). These two proteins are present in both ODV and BV.

However actin is only found in BVs but not in ODVs (Braunagel *et al.*, 2003; G. Long, unpublished data) indicating that nuclear F-actin detaches from ODV suggesting that nuclear actin is somehow involved in the sorting assembled nucleocapsids depending on their destiny. The nuclear F-actin may act as an essential scaffold protein for nucleocapsid morphogenesis (). A part of the assembled nucleocapsids in the nucleus detaches from nuclear actin and develops into ODV. The remaining nucleocapsids for BV are transported to the plasma membrane for budding either with or without the nuclear actin, depending on whether they obtain actin from the cytoplasm. It is possible that nucleocapsids are transported from the nucleus to the plasma membrane for budding in an actin-assisted manner, as it is difficult to believe that such a macro-structure as an assembled baculovirus nucleocapsid could reach the budding destination simply by diffusion. Using Lantrunculin B inhibiting actin polymerization at various times post infection, Graumann and colleagues proposed that cellular actin polymerization was critical for BV budding and was involved in the terminal stages of BV egress (Graumann *et al.*, 2006). In this model it can be further hypothesized that cellular membrane-associated proteins help actin filaments to attach to the budding membrane structure, which is then incorporated into BVs, like the ezrin, moesin and cofilin which are identified in purified HIV-1 linking the plasma membrane and the actin filaments (Ott *et al.*, 1996; Ott *et al.*, 2000).

Cytoplasmic tail Domain of F

Cytoplasmic tail domains of virus glycoproteins are involved in virus morphogenesis in the cases of HIV-1 and influenza virus, since they bind to the matrix protein of nucleocapsid and collate the viral envelope and nucleocapsid (Ali *et al.*, 2000a; Ali *et al.*, 2000b; Wyma *et al.,* 2000; Wyma *et al.*, 2004). Besides for their role in virus assembly the CTDs of virus glycoproteins are also important for glycoprotein trafficking and fusion (Chapter 2 and 5). F CTD is dispensable for the rescue of the infectivity of gp64-null AcMNPV by HearNPV F. This is compatible with the information that the GP64 CTD is not required for AcMNPV BV production (Oomens *et al.*, 1999). Consequently the F CTD is not required for that either in the AcMNPV context. It is possible that deletion of the cytoplasmic tail of F led to some subtle changes in protein synthesis and F fusogenicity. For instance, the presence of a multimer-like signal was correlated to the length of the cytoplasmic tail, while the F CTD is not critical for F processing, trafficking and fusogenicity. In contrast to the pseudotyping results F CTD appeared to be essential for the generation of infectious HearNPV BVs, suggesting that the F CTD plays an essential role in F protein incorporation into BV after the successful transportation of nucleocapsids to the plasma membrane and before the maturation of HearNPV BVs.

Upon NP40 treatment the GP64 was found almost entirely in the envelope fraction, whereas the F protein was found both in the envelope fraction and the nucleocapsid fraction. This finding is in accordance with the distribution of Op21, the F homologous protein in OpMNPV (Pearson *et al.*, 2001), underscoring the view of the possible interaction between F CTD and nucleocapsids or tegument of the nucleocapsid. However, this interaction does not seem to be a prerequisite for efficient budding of baculovirus BVs, given the fact that the group I and group II NPVs needed a common kinetic force to drive efficient budding. It has

been reported that the F homologous protein Ac23 appeared not to be necessary for infectious BV production (Lung *et al.*, 2003) suggesting that this possible interaction is indeed not essential, at least in the case of AcMNPV. Thus, the group II NPV F CTD would be involved in virus budding in a more complicated way than by direct interactions.

Despite the variation in CTD length and the dissimilarity among the CTDs of different baculovirus F proteins, a cysteine enriched region is located immediately downstream of the transmembrane region in the CTD, suggesting an important functional relevance. A direct inference from this conserved cysteine rich domain would be that some of these cysteines are subjected to palmitoylation, which is a common posttranslational modification that can regulate protein trafficking and protein-membrane interaction. It has been reported that cysteine residues situated at juxtamembrane locations within the CTDs of several viral membrane fusion proteins, including the influenza virus HA (Zurcher *et al.*, 1994; Jin *et al.*, 1996), paramyxovirus F (Sergel *et al.*, 1995), vesicular stomatitis virus G (Whitt *et al.*, 1991), Sindbis virus E1 (Smit *et al.*, 2001), retrovirus ENV (Rosso *et al.*, 2000), baculovirus GP64 (Zhang *et al.*, 2003b) and coronavirus S (Thorp *et al.*, 2006), were palmitoylated. It is generally thought that palmitoylation of viral envelope fusion protein helps to anchor the membranes into lipid rafts, but the functional significance of this modification to virus infectivity varies. For instance, Sindbis, VSV and influenza virus H3 infections are not dependent on their palmitoylation of intra-cytoplasmic cysteins, whereas influenza virus H1, HIV and coronavirus MHV infections clearly are. It is not known yet whether the baculovirus F protein is modified by palmitoylation and whether lipid rafts are involved in group II NPVs budding. It is proposed here that the possibly palmitoylated cysteines are not critical for F function, as HearNPV F without the whole CTD was still properly transported to plasma membrane and fusion competent (Chapter 5). In the case of GP64 it was proposed that the palmitoylation was not capable to guarantee lipid raft binding, as GP64 was not found in a detergent resistant membrane (DRM) fraction (Zhang *et al.*, 2003b). However, this proposition might be open to discussion as the DRM fractions are aggregates of raft domains and thus do not represent the native state of lipid rafts in cell membranes (Munro, 2003). In addition, the lipid composition in insect cell culture, especially the cholesterol content, is different from that in mammalian cells. This disparity in lipid composition could result in distinct physico-chemical properties and lipid raft dynamics. Thus, observation with more appropriate methodology will be important for an authentic view on the possible involvement of lipid rafts in baculovirus budding.

CONCLUDING REMARKS

Based on the observations described in this PhD thesis several aspects on baculovirus F protein were clarified, which might lead to further research on this protein. Baculovirus F protein is first expressed as a precursor which is cleaved by furin-like protease into a heterodimer of two disulfide-linked subunits, F_1 and F_2. F is present in the BV envelope as an oligomer of heterodimers. Future assignment of intermolecular and/or intramolecular disulfide linkages will be helpful to learn the conformation of F. N-glycosylation of F protein is essential for the synthesis, folding and functionality. The functionally significant sites are located in F_1 subunit. Mapping the occupancy of the potential N-glycosylation sites in F_1 and further functional study will be helpful to understand the contribution of N-glycans in F proper folding. Clathrin-mediated endocytosis is the functional entry pathway for baculoviruses to invade insect and mammalian cells and blocking the caveolar pathway of BHK21 cells enhanced baculovirus transduction efficiency. It is worthy to do a survey of other mammalian

cells to determine whether this enhancement is a general scenario or not. A cytoplasmid tail on the baculovirus F protein is required for HearNPV BV budding possibly through an interaction between F CTD and nucleocapsid or tegument proteins. Identification of this interaction, determination of functional domains within the CTD and screening the possible interacting candidate protein will lead to a clear view of group II baculovirus BV budding. Finally, the HR1 region in HearNPV F protein plays an essential role in the folding and function of F. Much more is to be learned about the exact mechanism of baculovirus F-mediated membrane fusion. Future efforts should be invested to determine the 3D structure of baculovirus F protein.

Beyond the research of baculovirus F two other topics are attractive for further attention. In this research actin was identified as a major component of HearNPV BVs. Actin was also found in AcMNPV BVs and it is well documented that cellular actin is involved in AcMNPV infection and morphogenesis. However, no research has been reported on the role of actin in BV budding. Further research on this subject is surely essential to reveal the role of actin in baculovirus BV maturation. The baculovirus-insect cells expression system is increasingly more used to express recombinant glycoproteins for multiple purposes, while how insect cells control glycoprotein folding in the ER is poorly understood. Observations in this thesis study on HearNPV F protein intracellular processing shed some light on a different glycoprotein folding control machinery in insect cells. Further studies on the quality control machinery in insect cells using baculovirus native protein F as a model can be useful in the further tailoring of the baculovirus-insect cell expression system.

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Summary

Baculoviruses are a large group of enveloped, double-stranded DNA viruses exclusively infectious for arthropods, predominantly insects of the order Lepidoptera. Baculovirology has advanced considerably in recent years as a consequence of the successful use of baculoviruses (i) in biological control of insect pests, (ii) as efficient expression vectors for eukaryotic proteins and (iii) as gene delivery vectors into mammalian cells. In the baculovirus infection cycle two phenotypes of virions, occlusion derived virions (ODVs) and budded virions (BVs), are produced. ODVs start the infection in insect larvae by direct fusion with epithelial cells in the midgut, whereas BVs infect cells from other internal tissues and cells in culture through adsorptive endocytosis. Major envelope fusion proteins located in the apical end of BVs play a major role in these processes as they mediate internalization and fusion for successful penetration of cells.

In the family *Baculoviridae* two distinct envelope fusion proteins are identified in BVs. GP64 is the major envelope fusion protein present in the baculovirus *Autographa californica* multi capsid nucleopolyhedrovirus (AcMNPV) and other group I nucleopolyhedrovirus BVs. In group II NPVs, for example *Spodoptera exigua* multi-capsid NPV (SeMNPV), *Lymantria dispar* MNPV (LdMNPV) and *Helicoverpa armigera* single-capsid NPV (HearNPV) (Chapter 1), a different type of envelope fusion proteins, designated as F, was present in BVs. Viral F proteins in general mediate low-pH-dependent fusion during virus entry (endocytosis) and baculovirus F proteins are functional analogs of GP64. In spite of significant divergence in primary sequence, baculovirus F proteins are classified as class I fusion proteins based on striking similarity in the architecture and functional elements (signal peptide, proteolytic cleavage site, fusion peptide, heptad repeats and transmembrane region) with fusion proteins of enveloped RNA viruses. To date baculovirus F proteins are the first class I virus envelope fusion proteins identified in a DNA virus. Therefore, to enhance our knowledge of class I virus fusion proteins in general and to further understand the functional implications of the specific characters of baculovirus F proteins, the HearNPV F protein was chosen as a model for study.

In Chapter 2 the F protein of HearNPV was identified by N-terminal sequencing of the major BV envelope protein (59 kDa). HearNPV F protein was found to be encoded by open reading frame 133 (*Ha133*) of the HearNPV genome. HearNPV F protein was first synthesized as an 80kDa precursor protein (F_0), which was proteolytically cleaved by a furin-like protease, residing in the trans-Golgi network, into two disulfide-linked subunits: F_1 (a subunit of 59 kDa carrying a transmembrane region) and F_2 (a 20 kDa subunit on the extracellular side) both forming a heterodimer. The HearNPV F protein gene successfully rescued the infectivity of an AcMNPV *gp64* deletion mutant, confirming that baculovirus F homologous proteins from group II NPVs are functional analogs of GP64. In addition, by chemical cross-linking it appeared that HearNPV F is present in BVs in the form of multimers whereby, unlike in the case of GP64, disulfide-bonds are not involved. Deglycosylation assays showed that both the F_1 and F_2 subunits of HearNPV possess N-linked glycans. However, these glycans did not have a C3 fucose modification when produced in Hz2E5 cells, but did have such a modification when produced in *Trichoplusia ni* BTI-Tn-5B1-4 (High Five) cells. Since C3 fucose is a major inducer of allergic responses in humans, this observation makes the HearNPV-*Heliothis* cells system an attractive alternative for the production of recombinant glycoproteins for therapeutic applications in animals and humans.

Summary

Virus envelope fusion proteins are generally heavily N-linked glycosylated. N-linked glycosylation is a modification step in protein biosynthesis occurring in the endoplasmatic reticulum. It is important for proper folding and intracellular trafficking, but also for the functionality such as virus attachment, fusogenicity and antigenicity. Baculovirus F proteins are N-linked glycosylated. However the number and location of predicted N-linked glycosylation sites vary from different baculovirus F proteins. One or more N-linked glycosylation sites are present in F_2 subunits of baculovirus F proteins and one site is conserved suggesting a possible role in F functionality. In Chapter 3 the only asparagine (N104) of the only predicted N-linked glycosylation site NXS/T in F_2 of HearNPV F was substituted by a glutamine (Q^{104}) and as a consequence F_2 was not glycosylated. Absence of N-linked glycans on the F_2 subunit of HearNPV F protein did not affect F protein synthesis and incorporation of F into BVs. Plaques produced by recombinant HearNPV expressing a mutant F protein (N104Q) are even slightly larger than those produced by recombinant HearNPV expressing wild-type F protein. The recombinant baculoviruses expressing N104Q also induced much more efficient low-pH activated syncytium formation and produced more BVs at the early stage of infection. These observations suggest that N-linked glycans attached on F_2 of baculovirus F proteins are involved in virus infectivity and F fusogenicity. Total inhibition of N-linked glycosylation resulted in low cellular expression levels of the HearNPV F protein and in poor proteolytic cleavage, indicating that N-linked glycosylation is indeed a prerequisite for proper F protein biosynthesis and F function. It is proposed that the N-linked glycosylation of F_1 is an evolutionary adaptation to modulate the virulence of the virus.

Enveloped viruses enter target cells through envelope fusion mediated by virus envelope fusion proteins. This fusion step occurs either on the surface of the cell membrane at neutral pH or in the late endosome activated by a low pH. Entry of baculovirus BVs into insect and mammalian cells is generally thought to occur through a low-pH-dependent endocytosis pathway, possibly through clathrin-coated pits. This insight, primarily based on (immuno-) electron microscopy studies, lacks biochemical and experimental support. Moreover, other entry mechanisms for virus exist and the relevance of these for baculovirus entry needs to be investigated. In Chapter 4, using specific inhibitors of endocytosis, it was demonstrated that both group I (GP64) and group II (F) NPVs baculoviruses enter insect cells primarily through clathrin-mediated endocytosis. Functional entry of baculoviruses (AcMNPV) into mammalian cells (BKH21) is dependent on the same endocytic pathway. Additionally, using a caveolar endocytosis inhibitor, genistein, the significantly enhanced baculovirus transduction in BHK21 suggests that caveolae are somehow involved in baculovirus entry in mammalian cells. This is not the case in insect cells, where the clathrin pathway seems to be the only one. These observations might promote the engineering of novel baculovirus gene transfer vectors and support a new strategy to enhance baculovirus transduction efficiency in mammalian cells by locking up of the caveolar pathway.

F proteins from different baculovirus species have a cytoplasmic tail domain (CTD), ranging from 48 (*Spodoptera litura* multicapsid NPV [MNPV]) to 78 (*Adoxophyes honmai* NPV) amino acid (aa) residues. This is much longer than the CTD of GP64-like envelope fusion proteins (7 aa) in group I NPV, the latter CTD being non-essential for infectious BV production. In Chapter 5, the functional role of the CTD of HearNPV F was studied using truncation mutagenesis and F swapping approaches. A HearNPV F gene without CTD was not able to rescue an HearNPV virus lacking F, but was able to rescue the infectivity of an AcMNPV lacking GP64. Combining these two lines of evidences, it was demonstrated that

the CTD of HearNPV F is not essential for F biosynthesis, processing, trafficking and fusogenicity, but essential for infectious HearNPV BV production. This finding further suggests that the F CTD is important for F protein incorporation during BV assembly and budding. In group I NPVs such as AcMNPV, F homologs with a CTD are not required for BV production, which might suggest that the assembly of group I and group II NPV BVs occurs differently. It is hypothesized that BV formation of group II NPVs requires a minimal CTD, probably to interact with nucleocapsid proteins.

Heptad repeats (HRs), a conserved structural motif of class I viral fusion proteins, are held responsible for the formation of a six-helix bundle structure of the envelope fusion proteins. Peptides derived from these HRs can be potent and specific inhibitors of membrane fusion and viral infection. Similar to many viral fusion proteins, a conserved leucine zipper motif was predicted downstream of the fusion peptide proximal to the HR region, HR1, in baculovirus F proteins. To study the functional role of the HR1 domain in baculovirus F protein, site-directed mutagenesis was used and mutant F was allowed to rescue the infectivity of an AcMNPV lacking GP64 (pseudotyping) (Chapter 6). The HearNPV F mutant proteins with a single leucine to alanine substitution showed lower rescuing capacity than the wild-type F. Thus the conserved leucine residues were critical for F protein function. The F^{L216R} mutant was not able to rescue the AcMNPV GP64-minus virus for reasons yet unknown. Using AcMNPV as expression vector, it was further shown that all F proteins mutated in HR1 were incorporated in BVs, indicating that substitutions of the leucine residues did not affect intercellular trafficking of F protein, although F^{L216R} showed a lower viral incorporation rate. Proteolysis was not affected by any of the substitutions of the leucine residues. However, folding of F was significantly affected. A large number of malformed multimers of F was detected in F^{L216A}, F^{L216R} and F^{L223A} mutants and F^{L216R} showed predominantly malformed oligomers. In addition to the expected paucimannose N-linked glycans, heavier N-linked glycans were found attached to the F_2 subunits of the F^{L216R} mutant. Collectively these results indicate that the HR1 domain within baculovirus F proteins is important to secure the conformational properties of the F protein and that the HR1 domain is critical more specifically for proper folding and post-translational modification.

The research described in this thesis unraveled some of the important characteristics of baculovirus F proteins in relation to their function in the infection process. These findings further enhanced our knowledge on the role of baculovirus F protein in the uptake in insect cells versus mammalian cells, in the fusion process and in BV maturation and assembly. Finally, in Chapter 7 the data obtained from this study are discussed in the light of the current understanding of the function of baculoviruses and other F proteins.

Summary

Samenvatting

Baculovirussen vormen een aparte groep virussen met een dubbelstrengig DNA als erfelijk materiaal, dat is verpakt in een staafvormig nucleocapside dat op zijn beurt is omgeven door een eiwit-lipidenmantel (envelop). Deze virussen zijn alleen infectieus voor geleedpotigen, vooral voor insecten van de orde Lepidoptera. Ten gevolge van het succesvolle gebruik van baculovirussen als (i) biologisch insectenbestrijdingsmiddel, als (ii) efficiënt vehikel voor de expressie van hoogwaardige eiwitten en als (iii) vervoermiddel voor de afgifte van genen in dierlijke cellen en systemen, is onze kennis over baculovirussen aanzienlijk toegenomen. Bij de vermenigvuldiging van baculovirussen worden twee typen deeltjes of virions gevormd: de door eiwitaggregaten (polyeders) ingesloten virions ('occlusion derived virions' = ODVs) en virions, die via een proces van uitstulping (budding) van de celwand en afgifte aan de lichaamsvloeistof worden verkregen ('butend virions = BV's). ODVs infecteren insectenlarven via directe fusie met de epitheelcelwand in de middendarm, terwijl BVs cellen van inwendige organen en van celculturen infecteren via een opnameproces door adsorptie en interne verwerking door de cellen. Het belangrijkste envelopeiwit van BVs is gelegen aan de apicale zijde van het staafvormige BV deeltje en speelt een belangrijke rol in dit fusieproces, omdat het betrokken is bij de aanhechting aan en het binnendringen in de cel en bij de fusie met het endosoom om het interne nucleocapside af te leveren.

Bij leden van de *Baculoviridae* familie worden twee typen envelopeiwitten met fusieactiviteit in BVs aangetroffen. GP64 is het belangrijkste envelopfusie-eiwit in BVs van het baculovirus *Autographa californica* 'multi capsid nucleopolyhedrovirus' (= AcMNPV) en van andere NPVs, die tot de taxonomische groep I van NPVs (kernpolyedervirussen) behoren. In de kernpolyedervirussen van groep II, bijvoorbeeld *Spodoptera exigua* (floridamot) MNPV, *Lymantria dispar* (plakker) MNPV en *Helicoverpa armigera* (katoenrups) 'single-capsid' NPV (HearNPV) (Hoofdstuk 1), wordt in de BVs een ander envelopfusie-eiwit aangetroffen, aangeduid met F (voor fusie). In het algemeen bemiddelen F eiwitten bij de fusie tussen BVs en het endosoom, die bij een lage zuurgraad plaatsvindt tijdens de endocytose. Baculovirus F eiwitten en GP64 zijn dus functioneel analoog. Ondanks een aanzienlijke verscheidenheid in primaire aminozuurvolgorde worden baculovirus F eiwitten ingedeeld bij de klasse I fusie-eiwitten, omdat zij qua secundaire structuur en aanwezigheid van herkenbare functionele elementen (signaalpeptiden, proteolytische klievingsplaats, fusiepeptide, 'heptad repeats' en een transmembraandomein) lijken op de F eiwitten van RNA virussen met een envelop. Baculovirus F eiwitten zijn uniek omdat klasse I envelopfusie-eiwitten nooit eerder bij DNA virussen zijn aangetoond. Om de kennis van de klasse I envelopfusie-eiwitten in het algemeen te vergroten en de functionele betekenis van de specifieke elementen van het baculovirusfusie-eiwit F meer in het bijzonder beter te begrijpen werd het HearNPV F eiwit als model aan een nader onderzoek onderworpen.

In Hoofdstuk 2 werd het F eiwit van HearNPV geïdentificeerd via N-terminale aminozuurbepaling van het belangrijkste BV envelopeiwit (59 kDa). De genetische code van het F eiwit bevond zich op open leesraam 133 (*Ha133*) van het HearNPV genoom. Het HearNPV F eiwit wordt eerst als een 80kDa voorlopereiwit (F_0) aangemaakt en vervolgens gesplitst door een furine-achtige protease uit het trans-Golgi netwerk in twee eiwitsubeenheden, F_1 (een eiwitsubeenheid van 59 kDa welke een transmembraangebied bevat) en F_2 (een eiwitsubeenheid van 20 kDa met het vermoedelijke domein voor receptorinteractie), die door een zwavelbrug zijn verbonden en dus een heterodimeer vormen. Door het HearNPV

F eiwitgen in te brengen in een AcMNPV *gp64* deletiemutant kon hernieuwde infectiositeit worden verkregen, wat nogmaals bevestigde dat baculovirus F eiwitten van groep II baculovirussen functionele homologen zijn van GP64 van groep I baculovirussen. Via chemische 'crosslinking' bleek dat in BVs HearNPV F aanwezig is in de vorm van multimeren, waarbij, in afwijking van wat wordt gevonden bij GP64, zwavelbruggen niet voor de binding zorgen maar de eiwit-eiwit interactie van F eiwitten zelf. Deglycosyleringsanalyses toonden aan dat zowel F_1 als F_2 subeenheden van HearNPV suikerresiduen bevatten die covalent waren gebonden aan F. Echter, deze zogeheten glycanen hebben geen C3-fucose als onderdeel van de suiker wanneer BVs worden geproduceerd in Hz2E5 cellen, maar wel wanneer ze gemaakt worden in *Trichoplusia ni* BTI-Tn-5B1-4 (High Five) cellen. Aangezien C3-fucose een allergiereactie oproept bij de mens, maakt deze waarneming het HearNPV-*Heliothis* celsystem een aantrekkelijk alternatief voor de productie van recombinante glycoproteinen voor therapeutische toepassingen bij mens en dier.

Virusenvelopfusie-eiwitten zijn over het algemeen sterk geglycosyleerd. N(= asparagine)-gebonden glycosylering is een modificatiestap tijdens de eiwitsynthese, die plaatsvindt in het endoplasmatisch reticulum. Deze glycosylering is van belang voor de juiste vouwing en het juiste intracellulaire tramsport, maar ook voor de functie van het eiwit (zoals bij virusaanhechting aan cellen), voor fusievermogen en antigeniciteit. Baculovirus F eiwitten zijn vaak aan asparagines (N) geglycosyleerd. Echter, het aantal en de plaats van de voorspelde N-gebonden glycosylering variëren bij baculovirus F eiwitten. Een of meer van deze plaatsen is theoretisch aanwezig in F_2 subeenheden. Eén glycosyleringsplaats komt op dezelfde positie in alle F_2 eiwitten voor en speelt wellicht een rol bij in het functioneren van F. Hoofdstuk 3 beschrijft de vervanging van de asparagine (N^{104}) van de enige voorspelde N-gebonden glycosyleringsplaats NXT (waarbij X elk aminozuur kan zijn behalve proline en T threonine of serine is) in F_2 van HearNPV F. De asparagine werd vervangen door een glutamine (Q^{104}) residu. Het gevolg was dat F_2 niet werd geglycosyleerd. De afwezigheid van een suikerresidu op de F_2 subeenheid had geen effect op de synthese van F als zodanig en de opname van F in BVs. Infectiehaarden, gevormd door een HearNPV recombinant met een mutant F eiwit ($N^{104}Q$), zijn zelfs iets groter in omvang dan diegene, die worden gemaakt door HearNPV met een wild-type F eiwit. De recombinante baculovirussen met een mutant F (N104Q) veroorzaakten ook efficiëntere fusie bij lage zuurgraad door de vorming van grote syncytia en de productie van meer BVs in een vroeg stadium van de infectie. Deze waarnemingen suggereren dat N-gebonden suikerresiduen bij F_2 betrokken zijn bij virusinfectiositeit en fusievermogen van het F eiwit. Remming van de N-gebonden glycosylering resulteerde in lagere expressieniveaus van het HearNPV F eiwit en in beperkte slechte klieving van F in twee eiwitsubeenheden. Dit geeft aan dat N-gebonden glycosylering een voorwaarde is voor juiste F eiwitsynthese en het correct functioneren van F. De hypothese is nu dat de N-gebonden glycosylering van F1 een evolutionaire aanpassing is om de virulentie van het virus te moduleren.

Virussen met een glycoproteine-mantel dringen cellen binnen via fusie, waarbij een envelopfusie-eiwit is betrokken. Dit fusieproces vindt plaats ofwel aan het oppervlak van cellen bij een neutrale zuurgraad ofwel in het endosoom, in welk geval het wordt geholpen door een lage zuurgraad. Algemeen wordt aangenomen dat het binnendringen van baculovirus BVs in insectencellen en zoogdiercellen geschiedt via het laatstgenoemde mechanisme, waarbij plekken op het celmembraan zijn betrokken die rijk zijn aan clathrines. Dit inzicht is vooral gebaseerd op (immuno-) elektronenmicrosopische studies, maar biochemische en experimentele ondersteuning voor dit inzicht ontbreken. Er bestaan echter nog meer routes,

waarlangs een virus cellen kan binnenkomen en de betekenis van deze alternatieve routes voor de entree van baculovirus werd verder onderzocht. In Hoofdstuk 4 wordt onderzoek met gebruikmaking van specifieke remmers van endocytose beschreven en vastgesteld, dat zowel groep I (met GP64) als groep II (met F) baculovirussen insectencellen in elk geval via de 'clathrine' route binnentreden. Het binnentreden van baculovirussen (AcMNPV) in zoogdiercellen (BKH21) gaat volgens dezelfde route. Door gebruik te maken van een specifieke remmer, genistein, om het binnentreden van baculovirus via 'caveoli' te blokkeren, werd de opname van baculovirussen (in dit geval spreken we van transductie omdat er geen virusvermingvuldiging, maar alleen transport plaatsvindt), aanzienlijk verhoogd. Dit doet vermoeden dat 'caveoli' op enigerlei wijze betrokken zijn bij het intreden van baculovirussen in zoogdiercellen. Dit is niet het geval bij insectencellen, waar de 'clathrine'-route de enige lijkt te zijn. Deze waarnemingen kunnen leiden tot de constructie van nieuwe baculovirussen voor het efficiënter overbrengen van genen naar zoogdieren (-cellen). In elk geval kan de transductie van baculovirussen naar zoogdiercellen worden verhoogd door de 'caveoli' route te blokkeren.

F eiwitten van diverse baculovirussen hebben aan het C-terminale uiteinde van F_1 een aminozuurstaart, (CTD), die uitsteekt in het cytoplasma en een lengte kan hebben variërend van 48 (*Spodoptera litura* MNPV) tot 78 (*Adoxophyes honmai* NPV) aminozuurresiduen. Dit is veel langer dan de CTD van GP64-achtige envelopfusie-eiwitten (7 aminozuren) in groep I NPVs. Deze CTDs zijn bovendien niet essentieel voor de infectiositeit van BVs. In Hoofdstuk 5 wordt een studie beschreven naar de specifieke rol van de CTD in het HearNPV F eiwit via inkorting van de CTD vanaf het C-terminale uiteinde en het terugzetten van deze truncaten in HearNPV via uitwisseling met het wild-type F eiwit. Het HearNPV F eiwit zonder CTD was niet in staat infectieus HearNPV BVs te maken, maar kon dat wel in AcMNPV dat GP64 miste. Het HearNPV F CTD lijkt dus niet essentieel te zijn voor de biosynthese, de processing, het transport en het fusievermogen van F, maar wel voor het verkrijgen van infectieuze HearNPV BVs. Dit suggereert dat de CTD van het F eiwit van belang is voor de assemblage van BVs of het uittreden van BVs uit de cel. Voor groep I NPVs is de CTD van het F eiwit niet van belang voor BV productie, wat aangeeft dat de assemblage van BVs van groep I en groep II NPVs op een verschillende wijze verloopt. De huidige hypothese is dat de vorming van BVs van groep II NPVs een minimale CTD vereist, die een interactie aangaat met nucleocapside-eiwitten om tot BV vorming te komen.

'Heptad repeats' (HRs), herhalingen van stukken van 6 aminozuren afgewisseld door een leucine, in het baculovirus F eiwit vertegenwoordigen een geconserveerd motief in klasse I virale envelopfusie-eiwitten. Deze HRs zijn verantwoordelijk voor de vorming (aggregatie) van envelopfusie-eiwitten. Peptiden, afgeleid van deze HRs, kunnen krachtige en specifieke remmers van deze structuren zijn en dus van membraanfusie en virusinfectie. Zoals voorkomt in veel andere envelopfusie-eiwitten werd een HR-gebied met regelmatige peptidenherhalingen voorspeld (HR1) op basis van de aminozuurvolgorde van diverse F eiwitten, stroomafwaarts gelegen van het fusiepeptide in F. Om de rol van de HR1 in HearNPV F nader te bestuderen via aminozuurmutagenese werd mutant F geïntroduceerd in een AcMNPV zonder GP64 (pseudotyping) (Hoofdstuk 6). De HearNPV F mutanten, bij welke enkele leucines in de HR1 elk afzonderlijk zijn vervangen door alanines, waren aanzienlijk minder infectieus dan wild-type F. De geconserveerde leucines zijn dus kritisch voor de functie van HearNPV F. De F^{L216R} mutant leverde om onbekende reden geen infectieus AcMNPV virus op. Door de mutante F eiwitten tot expressie te brengen via AcMNPV, maar nu in aanwezigheid van GP64, kon aangetoond worden dat de HR1 mutante

F eiwitten wel gemaakt worden en in BVs terecht kunnen komen. Dit betekent dat de vervangers van de leucines de vorming en het intracellulaire transport niet beïnvloedden, ofschoon de mutant F^{L216R} minder in BVs werd ingebouwd. Proteolytische klieving van F door furine werd niet gehinderd door deze vervanging van leucine. Echter, de vouwing van F werd aanzienlijk beïnvloed. Een groot aantal onjuist gevouwen F multimeren als gevolg van de mutaties F^{L216A}, F^{L216R}, F^{L223A} en F^{L216R} vertoonde overwegend slechtgevouwen oligomeren. In de F^{L216R} mutant werden, naast de verwachte paucimannose als N-gebonden glycaan, ook meer omvangrijke suikergroepen aangetroffen in de F_2 subeenheid. Al met al lijken deze resultaten er op te wijzen, dat het HR1 domein binnen het baculovirus F eiwit zeer belangrijk is om de conformationele eigenschappen van het F eiwit veilig te stellen en dat het HR1 domein zelfs noodzakelijk is voor de juiste vouwing en post-translationele aanpassingen (glycosylering) van F.

Het onderzoek, zoals beschreven in dit proefschrift, heeft enkele verbanden gelegd tussen enkele belangrijke structurele eigenschappen van het baculovirus F eiwit en de functie in het infectieproces. De hier beschreven bevindingen hebben vooral inzicht verschaft in het functioneren van het F eiwit bij de opname door insecten- en zoogdiercellen, bij de fusie met membranen om het nucleocapside vrij te maken voor transport naar de celkern en bij de rijping en assemblage tot BVs. Ten slotte zijn in Hoofdstuk 7 de resultaten van het onderzoek besproken tegen de achtergrond van de huidige inzichten ten aanzien van de functie van envelopfusie-eiwitten in het algemeen.

摘要

杆状病毒是一类有囊膜的双链 DNA 病毒,它们特异性地感染节支动物。由于在昆虫病害的生物防治,真核蛋白的高效表达和基因递呈载体方面的成功应用,杆状病毒领域在近几年取得了长足的发展。在杆状病毒的感染循环中产生两种不同的病毒型,包涵体病毒(ODV)和出芽病毒(BV)。ODV 通过病毒囊膜与昆虫中肠上皮细胞直接的膜融合引发的病毒感染, 而 BV 则通过吸附型内吞侵入其它组织或培养细胞。位于 BV 顶端的主要膜融合蛋白介导了病毒内吞和膜融合等入侵过程。

在杆状病毒属中鉴定有两类截然不同的膜融合蛋白。 GP64 是 AcMNPV 和其它组 I 核多角题病毒(NPV)BV 中的主要膜融合蛋白。而在组 II NPV 中, 如 SeMNPV, LdMNPV 和 HearNPV, 病毒编码另一种命名为 F 的膜融合蛋白。这两类蛋白都能介导病毒内吞后的低 pH 值依赖型膜融合而且 F 是 GP64 的功能类似物 (第一章)。基于病毒膜融合蛋白的功能组件及其排布, 杆状病毒 F 类蛋白与众多 RNA 病毒膜融合蛋白被归类为一型病毒膜融合蛋白。迄今为止,杆状病毒 F 类蛋白是第一个在 DNA 病毒中发现的一型膜融合蛋白。为了增加人们对一型病毒膜融合蛋白的认知并且深入了解杆状病毒 F 类蛋白的功能特性, 在此 HearNPV F 被选为研究对象进行了多角度的研究。

通过对主要BV膜蛋白(59 kDa)的N端序列测定, HearNPV F由 133 号开放阅读框编码(第二章)。HearNPV F蛋白首先合成约为 80 kDa的前体蛋白, 之后在反式高尔基体中被furin类蛋白酶切割成为有二硫键相连的两个亚基:F_1和F_2。HearNPV F能成功地拯救gp64缺失的AcMNPV, 再次证明杆状病毒F类蛋白是GP64 的功能类似物. 另外化学交联实验显示HearNPV F以多聚体的形式存于BV表面, 与GP64 不同的是二硫键并不参与F的多聚化。去糖基化实验表明F_1和F_2均被N糖基化修饰。在BTI-Tn-5B1-4 细胞中表达的HearNPV F的N糖基C3 甘露糖而在Hz2E5 细胞中表达HearNPV F的N糖基却不含C3 甘露糖。由于C3 甘露糖能在人体中诱发过敏反应, 因而HearNPV-Heliothis细胞系统则更适于医用糖蛋白的表达。

通常病毒膜融合蛋白为高度N糖基化蛋白。N糖基化发生在内质网中, 对糖蛋白的正确折叠, 细胞内转运和蛋白功能都至关重要。杆状病毒F为N糖基化蛋白, 然而杆状病毒F类蛋白中预测的N糖基化位点的数量和定位却存在很大的差异。在第三章中, 通过用谷氨酰胺替换天冬酰胺我们对HearNPV F的F_2亚基中唯一的N糖基化位点(N104)进行了功能研究。F_2亚基N糖基化缺陷 (N104Q) 并不影响HearNPV F 的合成和包装。相对于表达野生型F的HearNPV, 表达N104Q的HearNPV产生稍大的病毒感染空斑。另外相当于野生型HearNPV F, N104Q介导更强的低pH依赖型膜融合。这些结果暗示F_2亚基上的N糖基于病毒感染性和F的融合功能相关。细胞N糖基化的完全抑制会导致HearNPV F 的修饰与胞内转运, 这也说明了N糖基化对HearNPV F的必要性。

囊膜病毒对宿主细胞的入侵依赖于病毒膜融合蛋白介导的膜融合。这个过程发生在细胞表面与细胞膜的融合(中性 pH 环境)或者发生在细胞内部与内吞体膜融合(低 pH 环境)。人们普遍认为杆状病毒 BV 的入侵是通过低 pH 依赖的内吞完成,可能是 clathrin 介导的内吞。然而这种认知只是基于免疫或电子显微观察, 缺乏有力的生物化学证据。 而且人们对其它的病毒入侵途径与杆状病毒侵入靶细胞的相关性知之甚少。在第四章中,

胞吞抑制和病毒感染实验表明 NPV BV 通过 Clathrin 介导的胞吞侵入昆虫宿主细胞。AcMNPV BV 对哺乳动物细胞(BHK21)的成功入侵也依赖于 Clathrin 介导的胞吞。抑制 Caveolar 胞吞途径能显著地增强 AcMNPV 对 BHK21 细胞的转导,这暗示 Caveolar 胞吞途径同样是杆状病毒侵入 BHK21 细胞的途径(无效转导途径)。这些观察可能促进杆状病毒基因递呈载体的改造并且提出一个新的策略：通过关闭 caveolar 途径增强杆状病毒基因注意载体的转导效率。

杆状病毒 F 类蛋白都具有较长的胞内区（CTD），48 到 78 氨基酸不等。而 GP64 CTD 只有 7 氨基酸，并且此 CTD 为病毒感染性非必须序列。在第五章中，通过运用截短型突变和反式遗传学方法，我们对 HearNPV F CTD 的功能进行了探讨。研究发现 CTD 截断的 HearNPV F 不能拯救缺失 F 基因的 HearNPV，但能成功拯救缺失 GP64 的 AcMNPV。结合这两组结果，我们推断 HearNPV F CTD 的截断并不显著影响 F 的合成，修饰，转运和融合功能，但是对 HearNPV 感染性 BV 的产生至关重要。这些发现进一步暗示 HearNPV F CTD 在出芽过程中特别是 F 组装入 BV 的过程中发挥重要作用。在 AcMNPV 中同样含有较长 CTD 的 F 同源蛋白，而此蛋白的基因是病毒复制的非必须基因。这也可能暗示组 I 和组 II NPV 运用不同的方式完成感染性 BV 的组装和出芽。

七元重复序列（HRs）是一型病毒膜融合蛋白中的一个保守结构组件，它们在病毒入侵过程中形成一个六螺旋束型结构从而介导病毒囊膜与宿主细胞膜结构的融合。HR 所对应的短肽能高效地抑制病毒的感染和膜融合过程。类似其他的一型膜融合蛋白，在杆状病毒F类蛋白融合肽的下游，同样发现有含有亮氨酸拉链的HR区（HR1）。 为了深入了解杆状病毒HR1 区的功能，我们对HearNPV F HR1 区进行了定点突变和反式遗传学研究（第六章）。研究结果显示用丙氨酸或精氨酸替换HR1 区中的亮氨酸残基显著降低HearNPV F 的功能。F^{L216R}丧失了拯救GP64 基因缺失AcMNPV的能力。利用普通AcMNPV表达系统，我们跟踪了突变型F的表达与转运。结果显示亮氨酸突变并不影响F的剪切和转运但是F的折叠和N糖基化修饰却发生了显著的变化。这些结果表明 HR1 区对于杆状病毒F类蛋白的翻译后修饰和正确折叠至关重要。

本研究就揭示了杆状病毒 F 类蛋白在感染过程中所扮演的角色，增加了人们对杆状病毒入侵， F 类蛋白介导膜融合以及 BV 的成熟与组装过程的认识。最后于第七章, 结合新近病毒膜融合蛋白的研究结果对杆状病毒 F 类蛋白的功能进行了讨论。

Acknowledgements

The first time I heard of Wageningen University and my current promotor Professor Just M. Vlak in the summer of 1999, the last period of my undergraduate study, I was prepared and eager to join Professor Zhihong Hu's lab as a MSc student in Wuhan Institute of Virology, Chinese Academy of Sciences. During my MSc study, I got the first chance to visit Wageningen in the beginning of 2001, joining the cooperative venture between Professor Zhihong Hu and Professor Just M. Vlak on baculoviruse functional genomics study. Immediately after I finished my MSc study in the middle of 2002, I was recruited as a "sandwich PhD student", sponsored by Wageningen University and Wuhan Institute of virology. Since then, I started an extraordinary journey for this thesis describing the functional study on baculovirus envelope fusion protein. Here I take the opportunity to appreciate all the people I met in these years.

First of all, I acknowledge my promotor Prof. Just M. Vlak for offering me the opportunity to work in your lab and pursue this PhD research under your coaching. Thank you for sending me to Coventry in the first half of my PhD study and making me find out a way to perform original and achievable research. Thank you for calling me back again in the second half and backing me up to fight for our scientific stories. I will memorize this in my natural life. And you know that I love my PhD study, in both halves. Thank you for take me to various kinds of concerts and showing me the western culture and your singing talent. I give my special thanks to your wife Ellen for hosting us the international folks in the department during Christmas. The hospitality and friendship of your family made my enjoyable and luxury life in the Netherlands.

I am sincerely grateful to Prof. Zhihong Hu for the extensive supervision during my MSc study and continuous concern during my PhD study. I was deeply moved by your devotion in science. The days under your supervision inspired my desire for science and gave me strength to carry out my research. I have been not only benefited in scientific research from you but also shaped inside by you since the first time we met in the old building of the institute in 1998.

This thesis would not have been completed without the help of many international scientists. My thanks first go to Prof. Johannes. F. G. Vliegenthart, Prof. Johannis. P. Kamerling and Susanne. P. Jongen (Utrecht University, Utrecht, Netherlands) for your generous support to carry out the N-glycan profiling work in your lab and your scientific contributions in my research. I thank Prof. Zihe Rao(Tsinghua University, Beijing, China), for giving me a lab bench to work on the crystallization of baculovirus F protein fusion core. And I thank Dr Yanhui Xu (Princeton University, New Jersey, US) for the kindness and experimental help in Prof. Zihe Rao's lab. I am grateful to Dr Alexandre Benmerah (Institut Cochin, Paris, France) and Dr Nathalie Sauvonnet (Institut Pasteur, Paris, France) for the generous offer of the Eps15 constructs. I also want to thank Dr Gary W. Blissard (Cornell University, New York. US) for providing the *gp64*-null AcMNPV bacmid.

I am grateful to my colleagues in Wageningen and Wuhan. Marcel (Westenberg), you showed me where and how to find chemicals and enzymes, demonstrated basically every thing running in the lab. I learned a lot from you. My study would never happen without your

accomplishment in baculovirus envelope fusion protein. Hangzhong, I loved the bacmid Hz8 which was the fundamental piece for all my reverse genetics work. You squeezed space from your office to accommodate me and incorporated me in your research framework. Monique, you gave me good suggestions in my research. When Just was not around you granted me to order many weird drugs to poison insect and mammalian cells. The entry story would never be produced without your help. Jan, working in your territory was exciting. You taught me the techniques to handle specimens and those microscopes, joking, dancing and whistling. I was benefited a lot from a tiny part of your expertise. I thank Prof. Rob Goldbach for giving me an opportunity to be a tutor for the molecular virology course and for your encouragement which supported me to carry out the function of a tutor. I thank Richard for teaching me to handle mammalian cells and for the scientific input to the research. I also want to thank Magda, Stephen, Els, Hanke, Gorben, Mark, Hans, Marcel (Prins) Liljana, Juliette, Margaret, Remziye, Agata, Xushi, Changyong and Fang, I appreciate your company in Wageningen and the ways you entertained me. I wish to acknowledge all the scientific staff in Wuhan Institure of Virology, Xinwen Chen, Zhengli Shi, Xiulian Sun, Hualin Wang, Fei Deng and Dong Wu, involved in the cooperation for the contributions to the joint research between Wageningen and Wuhan, and thank you all for the assistances to my study. I give my special thanks to Wout for helping with materials and logistics, Thea for the help for my visa application and residence permission, Li Liang, Bichun Zhu, Jianhong Zhang and Li Yuan for the administrative work for my life in Wuhan. I want thank Jun Xu, Huiyuan Wang and Fangliang Zheng for your kind reception in the hospitable dining group.

Here I want to thank the my brothers and their “leaders” in my hometown officially, the family of Xun Liao, the family of Jun Kan, the family of Gang Liu, the family of Hua Yang and the family of Tao He. Every time I went back hometown, you did warm me from the bottom of my heart and amuse me as best as you can. You guys make the hometown always a cherished place.

Above all, I want to thank my family for their constant care and support. Xiaoyu, you bring color to my life when I was gray and you put me together when I was down. Without you I would never have achieved all of this. A few weeks ago, Rob asked me “what was the aim of your scientific research”. I could not answer as I have no clear idea yet. But I surely know at the moment that your smile is the aim of my life. Thank you for all your love and understanding. I thank my parents in law for the sacrifice and support done for Xiaoyu and me. I am deeply indebted to my parents and my sister for their financial and moral support during my studies in China and in Netherlands.

Gang Long

Wageningen, December 2006

List of publications

Pan X., **G. Long**, H. Wang, Y. Zheng, X. Sun, M. Westenberg, F. Deng, H. Wang, J. M. Vlak, and Z. Hu. Deletion of *Helicoverpa armigera* nucleopolyhedrovirus orf107, a gene encoding a virion structural protein, enhances virus infectivity *in vitro* and *in vivo*. Conditionally accepted by J. Gen. Virol.

Long G., S. P. Jongen, X. Pan, J. P. Kamerling, and J. M. Vlak, 2006. N-terminal heptad repeat, HR1, of baculovirus (HearNPV) F is essential for F function and intracellular processing. In manuscript.

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Dong C, D. Li, **G. Long**, F. Deng, H. Wang, and Z. Hu. 2005. Identification of functional domains required for HearNPV P10 filament formation. Virology **338:** 112-120.

Long G, X. Chen, D. Peters, J. M. Vlak, and Z. Hu. 2003. Open reading frame 122 of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus encodes a novel structural protein of occlusion-derived virions. J. Gen. Virol., **84:** 115-121.

PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 22 credits (= 32 ECTS = 22 weeks of activities)

Review of Literature (3 credits)
Enveloped virus entry and fusion protein (2003)

Writing of Project Proposal (4 credits)
Function of Baculovirus F-protein: on fusion and processing (2004)

Post-Graduate Courses (2 credits)
Biomolecular mass spectrometry course (Utrecht University 2004)
Glycobiology and protein quality control (self education 2004-2005)

Deficiency, Refresh, Brush-up and General Courses (6.3 credits)
Techniques for writing and presenting a scientific paper (Mansholt graduate school 2002)
Career perspective (Wageningen graduate school 2005)
Ethical dilemmas for life scientists (Wageningen graduate school 2005)
Professional communication strategies (Wageningen graduate school 2006)

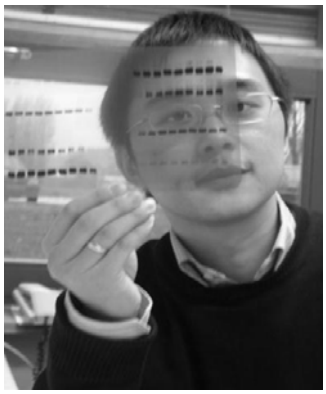
PhD Discussion Groups (3 credits)
Virology discussion and supervision group (Wageningen 2003-2006)
Zoonotic virology discussion group (China 2006)

PE&RC Annual Meetings, Seminars and Introduction Days (0.75 credit)
PE&RC anniversary (2005)
PE&RC day (2005)
PE&RC day (2006)

International Symposia, Workshops and Conferences (4.5 credits)
Dutch annual virology symposia (Utrecht University 2003, 2005)
Annual meeting of society for invertebrate pathology (SIP 2004)
Annual meeting of society for invertebrate pathology (SIP 2006)

Laboratory Training and Working Visits (3 credits)
Characteristics of Baculovirus putative fusion core (Lab of Structure Biology Tsinghua University 2003, 2004)
Profiling of glycans from Baculovirus F (Bijvoet Centre for Biomolecular Research Utrecht University 2004, 2005)

Curriculum Vitae



Gang Long was born on March 27, 1978 in Pingxiang City, Jiangxi Province, P. R. China. He finished his BSc study at the Department of Life Science, Nanchang University, Nanchang, China, in 1999, majoring in bioengineering. In the same year, he started his MSc study on baculovirus unique genes in the lab of Prof. Zhihong Hu, Wuhan Institute of Virology, Chinese Academy of Sciences (WIV, CAS). From the beginning of 2001, he joined the collaborative venture between WIV and Wageningen University and research center (WUR), working in Prof. Just M. Vlak's lab.

In 2002, immediately after his MSc study, he was employed by WIV as a research assistant in the same lab. In the meantime, he was recruited by Prof. Just M. Vlak as a "sandwich PhD student" sponsored by WUR and WIV, carrying functional analysis of baculovirus envelope fusion protein. The results of his PhD research are described in this thesis.

This research was supported in part by grants from the National Science Foundation of China (NSFC 30300012 to Gang Long), from the “973 project” (project number 2003CB114202) to Zhihong Hu, from the Royal Academy of Sciences of the Netherlands (KNAW) (project number 03CDP012), a joint KNAW-CAS grant to Zhihong Hu and Just M. Vlak (Program Strategic Alliances projects 2004CB720404 and 04-PSA-BD-02) and a PhD sandwich grant to Gang Long from Wageningen University.

The HearNPV BV pictures on the cover were taken by Gang Long and Jan van Lent. Student dormitory buildings of Wageningen University were sketched by Xiaoyu Pan.