MECHANISM AND STAGES OF PACKAGING OF VP8, THE MAJOR TEGUMENT PROTEIN OF BOVINE HERPESVIRUS-1

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By

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ABSTRACT

VP8 (pU_L47), the major tegument protein of bovine herpesvirus -1 (BoHV-1), is crucial for viral replication and induction of host immune responses. VP8 (pU_L47) translocation from the nucleus to the cytoplasm and subsequently to the Golgi results from its phosphorylation within the nucleus by pU_S3. VP8 (pU_L47) phosphorylation mutant contains a significantly lower amount of VP8 (pU_L47) (~30%) than wild type virus. Outside the context of infection, VP8 (pU_L47) is translocated to the cytoplasm if co-transfected with pU_S3 encoding plasmid, but remains cytoplasmic and is not translocated to the Golgi. Based on these previous studies, we hypothesized that VP8 (pU_L47) is partially packaged in the perinuclear region, and localisation of VP8 at the Golgi for final packaging involves another viral factor, presumably a glycoprotein.

Mass spectrometry studies indicated presence of VP8 (pU_L47), and another tegument protein, VP22 (pU_L49), in the perinuclear and mature virus particles. Co-immunoprecipitation and confocal microscopy confirmed an interaction between VP8 (pU_L47) and VP22 (pU_L49) and their co-localisation in the perinuclear region, respectively. In cells infected with virus lacking the VP22 (pU_L49)-encoding gene, VP8 (pU_L47) was absent from the perinuclear space, and the amount of VP8 (pU_L47) in the purified mature virus was reduced by approximately 33%.

To identify the viral factor(s) responsible for the localisation of cytoplasmic VP8 (pU_L47) at the Golgi, a screening of co-precipitating glycoproteins was performed, and glycoprotein M (gM) was observed to be an interaction partner of VP8 (pU_L47) during infection, as well as outside the context of infection. VP8 (pU_L47) and gM (pU_L10) co-localised at the Golgi in infected cells, and gM (pU_L10) was sufficient for localisation of VP8 (pU_L47) at the Golgi outside the context of infection. In recombinant virus lacking gene encoding gM (ΔgM- BoHV-1), the localisation of VP8 (pU_L47) at the Golgi was impeded, and restored with the restoration of gM (pU_L10). Analysis of purified mature virus from ΔgM- BoHV-1 infected cells indicated a reduction of approximately 65% in the amount of VP8 (pU_L47).

The results of this research add to the knowledge of the stages and proteins involved in the assembly of the tegument layer of BoHV-1 with focus on the major tegument protein, VP8 (pU_L47).

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Finally, my sincere prayers to the God, the Almighty for EVERYTHING.

DEDICATION

This thesis is dedicated to my amazingly loving father,

Prof. K. B Rath

I will forever be indebted to him for being the source of inspiration, values and knowledge and for making me the person I am today!

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LIST OF ABBREVIATIONS

- ATP - Adenosine Tri Phosphate
- BoHV-1 - Bovine Herpesvirus 1
- BoHV-5 - Bovine Herpesvirus 5
- BPIV-3 - Bovine Parainfluenza Virus 3
- BRSV - Bovine Respiratory Syncytial Virus
- BVDV - Bovine Viral Diarrhea Virus
- CK2 - Casein Kinase 2
- CRM-1 - Chromosomal Maintenance 1
- DIVA - Differentiating The Infected From Vaccinated Animals
- DMEM - Dulbecco's Modified Eagle's Medium
- *E.coli* - *Escherichia coli*
- EBTr - Embryonic Bovine Tracheal
- EHV-1 - Equid Herpesvirus -1
- ESCRT - Endosomal Sorting Complexes Required For Transport
- FBS - Fetal Bovine Serum
- FBT - Fetal Bovine Testicular
- HCMV - Human Cytomegalovirus
- HEPES - N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
- HHV-1 - Human Herpesvirus 1
- HHV-3 - Human Herpesvirus 3
- IBR - Infectious Bovine Rhinotracheitis
- ICTV - International Committee on Taxonomy of Viruses
- ILTV - Infectious Laryngotracheitis Virus
- IPTG - Isopropyl -B-D-Thiogalactoside
- IR - Inverted repeat

- LAP2 - Lamin-Associated Proteins
- MDBK - Madin–Darby Bovine Kidney
- MDV-1 - Marek's Disease Virus 1
- MEM - Eagle's Minimum Essential Medium
- Mg^{2+} - Magnesium Ion
- MOI - Multiplicities of Infection
- NES - Nuclear Export Signal
- NLS - Nuclear Localisation Signal
- NMP - Nuclear Membrane Preparation
- ORFs - Open Reading Frames
- PAGE - Polyacrylamide Gel Electrophoresis
- PBS - Phosphate Buffered Saline
- PMSF - Phenylmethylsulfonyl Fluoride
- SuHV- Suid Herpesvirus
- TIF - Trans Inducing Factor
- TR - Terminal repeat
- U_L - Unique Long
- U_S - Unique Short
- VZV – Varicella Zoster Virus

CHAPTER 1

1. LITERATURE REVIEW

Bovine herpes virus (BoHV-1) causes serious losses to the cattle industry by causing a significant decrease in milk production and fertility of the cattle (Ellis 2009, Jones and Chowdhury 2010). This literature review consists of a comprehensive introduction about BoHV-1, which belongs to the *Alphaherpesviridae* family. There is also a brief explanation about the spread of the disease and the structure, composition and life cycle of BoHV-1. Previous researches showed that the tegument proteins of herpesviruses have a variety of roles including aiding in viral entry (Favoreel, Van Minnebruggen et al. 2005, Jacob, Broeke et al. 2015), DNA encapsidation (Mossman, Sherburne et al. 2000, Cockrell, Huffman et al. 2011, Ohta, Yamauchi et al. 2011, Albright, Kosinski et al. 2015), viral assembly (Bigalke, Heuser et al. 2014, Bigalke and Heldwein 2015, Funk, Ott et al. 2015), viral egress (Mou, Wills et al. 2009, Shindo, Kato et al. 2016) and modulation of host responses (Kelly, Fraefel et al. 2009, Glaunsinger 2015, Afroz, Garg et al. 2018). Despite the versatility of the tegument proteins, the information available about the molecular biology behind their functions is very limited. An introduction about the tegument proteins is also included, which can provide important direction towards future studies of tegument proteins of herpesviruses.

This research aims at analysing the different stages of incorporation of the tegument of BoHV-1 with focus on the major, most abundant tegument protein, VP8 (pU_L47) (Carpenter and Misra 1991). This study also characterises the interactions between VP8 and other proteins (tegument or glycoproteins), which play a role in the incorporation of VP8 (pU_L47) into virions.

1.1 Introduction of BoHV-1

1.1.1 Classification of BoHV-1

Based on the classification by the International Committee on Taxonomy of Viruses (ICTV), BoHV-1 is classified under the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus *Varicellovirus*. The other important pathogens within the genus *Varicellovirus* include human herpesvirus 3 (HHV-3), bovine herpesvirus -5 (BoHV-5) and equid herpesvirus -1 (EHV-1). BoHV-1 shares characteristics with human herpesvirus (HHV-1), another member of the *Alphaherpesvirinae* family. These similarities are present in various aspects such as the viral composition, life cycle and latent infection.

Based on its genetic and antigenic characteristics BoHV-1 has been classified into three subtypes, 1.1, 1.2a and 1.2b (Steukers, Vandekerckhove et al. 2011). Subtype 1.1 is mostly associated with respiratory infections; while subtype 1.2a causes both respiratory and genital symptoms and subtype 1.2b is predominantly associated with genital infection (Wentink, van Oirschot et al. 1993). Viral DNA analysis forms the basis for diagnosis of the respective infections (Muylkens, Thiry et al. 2007, Fulton, d'Offay et al. 2016).

1.1.2 BoHV-1 infection and control strategies

BoHV-1 is the causative agent of infectious bovine rhinotracheitis (IBR) and balanoposthitis (Greig, Bannister et al. 1958). The transmission of the disease occurs through either direct contact or air borne transmission. Transmission also takes place via bodily fluids such as saliva and semen (van Oirschot 1995). BoHV-1 infected cattle may develop respiratory problems, conjunctivitis, genital infections, a reduction in milk production and abortions (Madin, York et al. 1956, van Drunen Littel-van den Hurk 2006). BoHV-1 infection also leads to immune suppression, which makes the animals susceptible to co-infections by bovine viral and bacterial pathogens (Castrucci, Osburn et al. 2000) (Jones and Chowdhury 2007). These co- and secondary infections can be promoted by environmental factors and stress by transport of the cattle, and hence are multifactorial

(Ellis 2001). The viral pathogens that co-infect include bovine viral diarrhea virus (BVDV), bovine parainfluenza virus-3 (BPIV-3) and bovine respiratory syncytial virus (BRSV). The bacterial pathogens involved in co-infections include *Manheimia haemolytica, Mycoplasma bovis, Pastuerella multocida and Histophilus somni* (Taylor, Fulton et al. 2010). Co-infection causes a respiratory disease complex (bovine respiratory disease complex - BRDC) , also termed shipping fever (Jones and Chowdhury 2010, Levings and Roth 2013) as it often occurs after moving calves to the feedlots in North America..

1.1.2.1 Epidemiology of BoHV-1

In 1954, a respiratory infection related to BoHV-1 was first reported in a dairy farm in California (Schroeder and Moys 1954). Later, in 1958, a Canadian research group was the first to isolate the virus, and confirmed that IBR is directly caused by BoHV-1 (Greig, Bannister et al. 1958). Although in some of the European regions like Bolzano of Italy, Bavaria of Germany, Denmark, Finland, Switzerland, Austria, Norway and Sweden, BoHV-1 has been eradicated, BoHV-1 infections are prevalent all over the world with varying severity (Straub 1991, Raaperi, Orro et al. 2014). To define the severity and prevalence of BoHV-1, serum-neutralizing antibodies are measured. In Western Canada serum neutralizing antibodies are present in 10-67% of the cattle (Tessaro, Deregt et al. 2005, Pruvot, Kutz et al. 2014). In different Turkish regions the prevalence ranges from 2.32% to 79.74% (Aslan, Azkur et al. 2015). BoHV-1 infection exists with high incidence in almost every state of India. For example, in the state of Uttar Pradesh the seroprevalance reaches 46.5%, with BoHV-1 subtype 1.1 being the predominantly circulating type (Verma, Kumar et al. 2014, Patil, Prajapati et al. 2016).

A quickly spreading infection by BoHV-1 starts with the introduction of an infected animal into the herd without proper testing and management. Latent infection is established in animals infected with BoHV-1 after the primary infection. In a herd of latently infected cattle, disease outbreak is significant during the months of December to February in the Northern Hemisphere countries. During this time period BoHV-1 related respiratory

symptoms and abortions are very common (O'Neill, Mooney et al. 2014). Calves show lower seropositivity compared to older populations, which might be due to the presence of a prolonged latent infection in the older cattle (Aslan, Azkur et al. 2015). Cattle are the major host of BoHV-1, but BoHV-1 also infects sheep, reindeer, mule, buffalo, pronghorn antelopes and goats (Wentink, van Oirschot et al. 1993, O'Neill, Mooney et al. 2014).

Although BoHV-1 infection is usually not lethal, there is significant loss in the productivity of affected animals (Fulton 2009). A study conducted in the UK has reported a reduction of 2.6 kg in milk production in BoHV-1 positive cows compared to negative cows along with a significant rise in the rate of abortion between 4 and 8 months of gestation (Mahajan, Banga et al. 2013, Statham, Randall et al. 2015). According to a report from India, in the state of Punjab approximately 21.4% of the bovine abortions are caused by BoHV-1 infections (Mahajan, Banga et al. 2013). The rate of abortion is over 50% in the area of Western Turkey (Tuncer-Göktuna, Alpay et al. 2016). In Morocco infections with BoHV-1 and BoHV-4 contribute to 42% of the abortions in cattle (Lucchese, Benkirane et al. 2016). The major impact of BoHV-1 infections includes increase of cost of management and compromised herd health, which also significantly restricts international trade of cattle (Muylkens, Thiry et al. 2007). In North America BoHV-1 is a major component of BRDC (shipping fever), which leads to significant morbidity and mortality.

1.1.2.2 Pathogenesis of BoHV-1

The productive infection by BoHV-1 starts at the mucosal surfaces. The primary site of BoHV-1 infection are the epithelial cells of the upper respiratory tract and the genital tract mucosa. The entry of the virus into the host cell is mediated by viral glycoprotein and host cell receptor interactions (Ellis 2009). The entry of the virus into the host initiates the viral replication cycle, and subsequent gene expression leads to new progeny virus production. The new progeny virus is then released from the cell and is spread throughout the infected host by local dissemination or viremia (Muylkens, Thiry et al. 2007). The distant organs

and tissues of the host become accessible to the virus which spreads through blood circulation, resulting in clinical symptoms like abortion (Miller, Whetstone et al. 1991). The spread is probably a result of infected monocytes, which then carry the virus to all parts of the body. Along with the primary BoHV-1 infection at mucosal surfaces, neuroinvasion of BoHV-1 occurs by cell-to-cell spread (Jones and Chowdhury 2007). BoHV-1 establishes latent infection in the trigeminal or sacral ganglia in the sensory neurons of the peripheral nervous system. If the animal is under stress, for example during transport, reactivation of BoHV-1 from latency can take place. Stress in cattle increases the corticosteroid levels triggering reactivation from latency (Jones, da Silva et al. 2011). After the reactivation of BoHV-1 from latency, the virus is transported to the primary site of infection in an anterograde manner and the lytic replication cycle starts again, leading to production of infectious virus particles (Preston CM 2007).

1.1.2.3 Prevention of BoHV-1

The major strategy to control BoHV-1 is vaccination. Vaccines primarily used in the USA, Canada and Australia are inactivated or modified live vaccines (Mackenzie-Dyck, Latimer et al. 2015, Hay, Morton et al. 2016, Lung, Furukawa-Stoffer et al. 2017). Marker vaccines, which allow differentiation of infected from vaccinated animals (DIVA) are most commonly used in Europe. Glycoprotein E (pU_S8) was selected as a marker for DIVA vaccines. The reasons for selection of gE (pU_S8) as a marker are as follows. Firstly, there is a significant reduction in the virulence of the gE (pU_S8)-deleted virus (Kaashoek, Moerman et al. 1994, Weiss, Brum et al. 2015), while without gE (pU_S8) the vaccine virus still preserves very high immunogenicity (Strube, Auer et al. 1996). Secondly, when the vaccine virus is devoid of gE (pU_S8), the ability of the attenuated BoHV-1 to reactivate from latency is lost, although is still able to establish a latent infection in the vaccinated animals (Lemaire, Schynts et al. 2001). Thirdly, the detection of serum gE (pU_S8)-specific antibody allows the differentiation of vaccinated and virus-infected animals (Lemaire, Schynts et al. 1999). The cattle vaccinated by the gE-deleted marker vaccine are devoid of

gE and hence can be easily distinguished from cattle infected by wild type BoHV-1. The cattle detected as infected by DIVA are then slaughtered; this is called the test-slaughter strategy and has been very efficient in eradication of BoHV-1 from many areas in many countries (Ackermann, Müller et al. 1990, Beer, König et al. 2003). Despite significant advantages, the use of gE (pU_S8) as a marker for DIVA, has several limitations. The first limitation is the absolute dependence of the diagnostic test on detection of serum gE (pU_S8)-specific antibody. Low gE (pU_S8)-specific immune responses and limited sensitivity of current gE (pU_S8)-specific antibody tests increase the probability of false-negative results. The most important limitation of using gE (pU_S8) as the marker for DIVA is the biosafety concern as the vaccine itself has the potential to cause abortions in pregnant cattle and also has the ability to gain virulence by recombining with wild type (WT) field strains (Muylkens, Thiry et al. 2007, Fulton, d'Offay et al. 2016). gE (pU_S8)-deleted and gE (pU_S8)- plus thymidine kinase-deleted modified live and inactivated vaccines have been used in Europe, and are efficient in reduction of latency and abortion rates (Raaperi, Orro et al. 2014).

Certain commercially available vaccines are also available for use in central and South America. These vaccines contain several modified live viruses or inactivated viruses in combination with adjuvants (Walz, Givens et al. 2017); a few examples include inactivated strains of BVDV-1, BVDV-2 and BoHV-1 with aluminum hydroxide as adjuvant; inactivated vaccine strains of BVDV-1, BVDV-2, BoHV-1 and BoHV-5 in an oily emulsion; BVDV-1, inactivated BVDV-2 and thermosensitive modified-live BoHV-1 in Quil A, amphigen and cholesterol. These vaccines are highly immunogenic in vaccinated cattle (Baccili, Martin et al. 2019). Despite being extensively used, modified live vaccines have certain limitations. The modified live virus can become latent and then be reactivated due to stress or immunosuppression by another infection (Petrini, Iscaro et al. 2019). Despite protecting cattle, the modified live virus in the vaccine still has the ability to cause abortions in pregnant cows (Newcomer, Cofield et al. 2017). Conventional modified live vaccines don’t allow differentiation of vaccinated from infected animals. Commercial

BoHV-1 vaccines are also available in North America in combination with bacterial strains like *Campylobacter fetus, Histophilus somni, Leptospira spp, Clostridium chauvoei, Clostridium septicum, Clostridium haemolyticum, Clostridium novyi, Clostridium sordellii* and *Clostridium perfringens*. These multivalent vaccines expand the immune responses in the vaccinated cattle (De Brun, Leites et al. 2021).

1.2 Structure and Composition of BoHV-1 and other alpha herpesviruses

The family *Herpesviridae* consists of viruses with a common virion structure. The most detailed data on the virion structure are provided by cryoelectron tomography. In general, the diameter of the spherical virus particle is approximately 186 nm (Laine, Albecka et al. 2015). The virus particle consists of a core with double stranded DNA encapsulated by an icosahedral capsid made up of 162 capsomeres. The icosahedral capsid is surrounded by a protein matrix called the tegument. The tegument is an amorphous layer of structured regions and consists of approximately 23 proteins (Lee, Vittone et al. 2008). The tegument in turn is surrounded by a lipid envelope in which protruding glycoproteins are present. The protrusion of the glycoproteins make the full diameter of the spherical virus particle to be around 225 nm. The nucleocapsid is closer to the lipid envelope at the proximal pole and further away at the distal pole (Gibson 1996, Owen, Crump et al. 2015). Figure 1.1 illustrates a schematic representation of the virus structure.

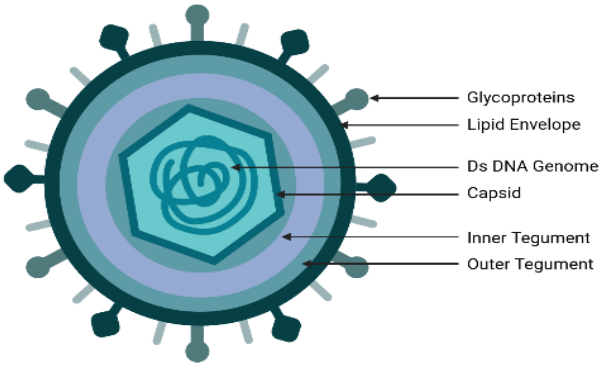

Figure 1.1 Schematic representation of the herpesvirus structure.

1.2.1 The genome of BoHV-1

The genome of BoHV-1 is double-stranded DNA, which is approximately 135 kilo base pairs (kbp) in size (Engels, Loepfe et al. 1987). The genomes of herpesviruses in general are divided into class A-F depending on the orientation and positions of inverted or directly repeated sequences as shown in Fig.1.2. The BoHV-1 genome belongs to class D, and other herpesviruses with a class D genome include varicella-zoster virus (VZV), suid herpesvirus (SuHV) and equine herpesvirus (EHV-1) (Schynts, McVoy et al. 2003). The BoHV-1 genome is divided into a 103 kbp unique long (U_L) region and a 32 kbp unique short (U_S) region. The *U_L* region is in the prototype orientation and is fixed. The *U_S* region is flanked by large inverted repeats, which are either internal repeats (IR) or terminal repeats (TR) (Roizman 2013). The BoHV-1 genome consists of 95 open reading frames (ORFs); 33 of these ORFs are critical for viral replication in tissue culture (Robinson, Meers et al. 2008). The *U_L47* gene, encoding VP8, is a part of the *U_L* region of the genome and is essential for replication of BoHV-1 in cattle, but not in cell culture. *U_L47* has a high-GC content and is flanked by the *U_L46* and *U_L49* genes, which encode VP11/12 (pU_L46) and VP22 (pU_L49), respectively (Lobanov, Maher-Sturgess et al. 2010).

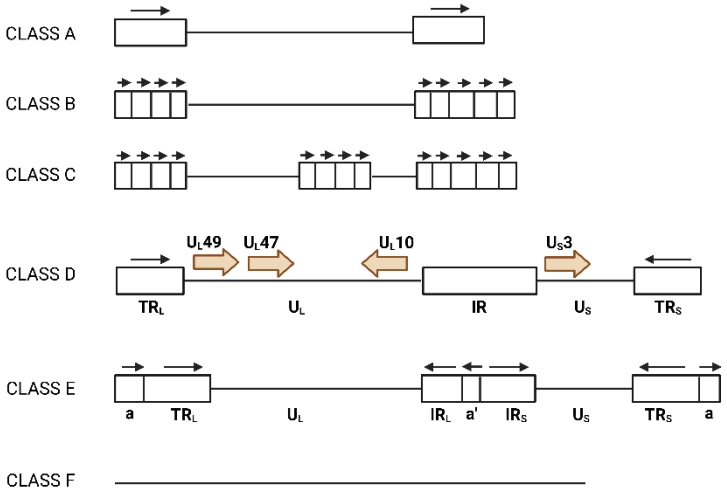

Figure 1.2 Schematic representation of classes of genome organisation of herpesviruses (not to scale). Horizontal lines and rectangles respectively depict unique sequences and repeats. Direction of the arrows indicates the orientation of the repeats and ORFs.

The replication of herpesviruses occurs via a rolling-circle mechanism, and produces concatamers (Schynts, McVoy et al. 2003). The concatamers are cleaved at a palindromic sequence called 'a' sequence by involvement of the pU_L28 protein in HHV-1. Other proteins involved in cleavage and packaging of the HHV-1 genome are pU_L33 and pU_L15 (Poon and Roizman 1993, Lamberti and Weller 1996, Desloges and Simard 2003).

1.2.2 The capsid

The capsid is a proteinaceous icosahedral structure (T=16) and packages the genome of the herpesviruses. The size of the herpesvirus capsid is 125-130 nm. The structural units that form the capsid are called capsomeres (Newcomb, Trus et al. 1993). In HHV-1 the major capsid protein VP5 (pU_L19) forms hexamers at the faces and pentamers at the vertices of icosahedral capsids. Six copies of VP26 (pU_L35) (hexamers) bind to VP5 (pU_L19) to form a cap that sits over the hexons. Between each penton and hexon are triplexes that are formed by VP19 (pU_L38) and VP23 (pU_L18) (Laine, Albecka et al. 2015). The protein pU_L6 forms a portal at one of the 12 vertices of the capsid and acts as a tunnel for the entry and exit of the viral genome into and out of the capsid (Trus, Cheng et al. 2004, Cardone, Winkler et al. 2007, Rochat, Liu et al. 2011).

1.2.3 The tegument

The tegument is a characteristic proteinaceous layer in the virions of the family of herpesviruses, which forms a bridge between the lipid envelope and the proteinaceous capsid, and packages the double-stranded DNA. The tegument is divided into an outer and inner layer on the basis of the association of the proteins with the lipid envelope and the capsid, respectively (Owen, 2015). The tegument is an essential component of the virus particle, as it is responsible for various critical functions required for viral propagation. The tegument of the alpha herpesviruses is made up of approximately 23 proteins. The outer layer of the tegument has a more amorphous arrangement, which is reflected in the asymmetrical arrangement of the glycoproteins. The outer tegument layer is comprised of

pUL7, pU_L11, pU_L14, pU_L16, pU_L17, pU_L21, pU_L24, VP8 (pU_L47), VP16 (pU_L48) and VP22 (pU_L49). The inner tegument has a more icosahedral symmetry because of its close association to the icosahedral capsid of the virus and consists of VP1/2 (pU_L36), pU_L37 and pU_S3 (Mettenleiter 2006, Lee, Vittone et al. 2008, Diefenbach 2015).

1.2.4 Lipid envelope

All members of the alpha herpesviruses, contains an outer lipid envelope. The lipid envelope has embedded virus-encoded proteins, which predominantly play a role in viral attachment, penetration and cell-to-cell spread (Liang, Babiuk et al. 1991, Nakamichi, Ohara et al. 2000). There are 13 envelope proteins in HHV-1, as well as BoHV-1, of which gB (pU_L27), gC (pU_L44), gD (pU_S6), gE (pU_S8), gG (pU_S4), gH (pU_L22), gI (pU_S7), gK (pU_L53), gL (pU_S1), gM (pU_L10) and gN ($pU_L49.5$) are glycosylated and are hence named glycoproteins. Two other viral envelope proteins, pU_L20 and pU_S9, are non-glycosylated (Abaitua, Daikoku et al. 2011). Glycoproteins also help in the maturation of virus and aid in the tropism of the virus to the host tissue and organs during infection (Farnsworth, Wisner et al. 2007).

1.3 Life cycle of alpha herpesviruses

The life cycle of BoHV-1, like that of other members of *Herpesviridae*, is divided into lytic and latent cycles (Esmann 2001).

1.3.1 Lytic Cycle

Generally, this is the immediate life cycle of a virus after it enters the host cell. The lytic cycle focuses on increasing the virus population by replication of the viral genome. The steps involved in the lytic cycle of a herpesvirus include: 1). Entry into the host cell. 2) Genome release into the nucleus. 3) Gene expression and replication. 4). Nuclear egress/primary envelopment of the newly packaged virus particle. 5). Maturation at the Golgi and finally 6). Cellular egress (Weynberg 2018).

1.3.1.1 Entry into the host cells

The immediate first step in the viral life cycle is the entry of the virus particle mediated by the viral glycoproteins. Predominantly two mechanisms of entry into the host cells are proposed. The most widely accepted mechanism proposes a fusion between the viral envelope and host membrane by an interaction between the glycoproteins and the heparin sulfate and nectin-1 or nectin-2 receptors on the host membrane. The second proposed pathway is via endocytosis of the enveloped virus, followed by the fusion of the envelope with intracellular vesicles (Arii, Uema et al. 2009). Both of these pathways are for HHV-1, and BoHV-1, and are cell-specific (Nicola, McEvoy et al. 2003, Pastenkos, Lee et al. 2018).

In HHV-1, gC (pU_L44) and gB (pU_L27) mediate the attachment of the virion to the cell surface receptor, heparin sulfate (Herold, Visalli et al. 1994). Interactions of gB (pU_L27), gD (pU_S6) and gH/gL (pU_L22/pU_S7) with the host cell receptors are critical for the fusion between the virus and the host membrane (Avitabile, Forghieri et al. 2009, Gianni, Amasio et al. 2009). gD (pU_S6) plays a dual role in the fusion process due to interaction with the cellular receptors and triggering of the membrane fusion by interactions with gB (pU_L27) and the gH/gL (pU_L22/pU_S7) complex (Gianni, Amasio et al. 2009). The N-terminal region of gD (pU_S6) interacts with the nectin-1 or nectin-2 receptors of the host cell and releases the C-terminal domain (Arii, Uema et al. 2009, Avitabile, Forghieri et al. 2009). This C-terminal domain interacts and activates gB (pU_L27) and gH/gL (pU_L22/pU_S7) which further triggers the membrane fusion (Zhou, Galvan et al. 2000). In BoHV-1, an involvement of gD (pU_S6) and gB (pU_L27) in the entry of the virus via membrane fusion has also been confirmed (Alves Dummer, Pereira Leivas Leite et al. 2014).

The second pathway involves endocytosis of the virion and eventually the fusion of the viral envelope with the endocytic vesicle in low-pH condition and release of the virus into the cytoplasm (Nicola, McEvoy et al. 2003). Both HHV-1 and BoHV-1 enter some host cells via the endosomal pathway (Nicola, McEvoy et al. 2003, Pastenkos, Lee et al. 2018); however, the factors regulating entry by endosomal pathways are unclear. After membrane

fusion, the capsid surrounded with a layer of some of the tegument proteins is transported to the nuclear pore through the cytoplasm. This transport occurs along microtubules by interaction between tegument protein(s) (HHV-1 VP26 (pUL35)) and the cytoplasmic dynein/dynactin complex (Döhner, Wolfstein et al. 2002, Wolfstein, Nagel et al. 2006). The docking of the nucleocapsid at the nuclear pore complex facilitates the release of the viral genome into the nucleus.

1.3.1.2 Genome release into the nucleus

The release of the herpes viral genome into the host nucleus takes place at the portal formed by the U_L6 viral protein at one capsid vertex (Ojala, Sodeik et al. 2000). The capsid docks over the nuclear pore complex via the portal vertex and the viral genome is injected into the host nucleus (Cardone, Newcomb et al. 2012). In HHV-1 infected cells the tegument proteins VP1/2 (pU_L36) and pU_L37 are essential for the genome release into the host nucleus (Roberts, Abaitua et al. 2009), which is facilitated by the cleavage of VP1/2 (pU_L36) (Cardone, Newcomb et al. 2012).

1.3.1.3 Gene expression and replication

After the release of the herpes viral genome into the nucleus, the viral genome is circularised (Strang and Stow 2005), and the expression of immediate early and early genes is initiated followed by the replication of the viral genome via a rolling circle mechanism (Jackson and DeLuca 2003). Inside the host nucleus expression of viral genes is well conserved and co-ordinated with the viral replication. In HHV-1, the genes are expressed in a chronological ordered cascade of immediate early (IE), early (E) and late (L) genes (Watson and Clements 1980). VP16 (pU_L48) and ICP4 are two transcription factors encoded by HHV-1 (Batterson and Roizman 1983, Campbell, Palfreyman et al. 1984). VP16 (pU_L48), a tegument protein, binds directly to the promoters of the IE genes and activates their transcription (Watson and Clements 1980, Smith, Bates et al. 1993). ICP4 is the product of an IE gene and is the major transcription factor, which is responsible for the

activation of E and L gene expression (Smith, Bates et al. 1993). The E gene products include the proteins essential for the viral genome replication and promote the initiation of replication (Taylor, McNamee et al. 2003).

The replication by a rolling circle mechanism leads to the formation of concatamers, which are later packaged into capsids and cleaved by viral proteins (pU_L15, pU_L28 and pU_L33 for HHV-1) (Rao, Balireddy et al. 2022). In HHV-1 three origins of replication have been identified; OriL is situated within the U_L region of the genome, whereas two OriS are situated within the U_S region of the viral genome (Fig 1.3) (Summers and Leib 2002). The origins of replication are flanked by promoter regions for the key viral factors, which participate in the replication process. For example, OriL of HHV-1 is flanked by genes coding for ICP8 and pU_L30, which play important roles in the replication process (Challberg and Kelly 1989). Seven proteins of HHV-1: pU_L9 (origin binding protein), ICP8 (single-stranded DNA binding protein), pU_L5, pU_L8 and pU_L52 (helicase-primase complex), pU_L30 (DNA polymerase) and pU_L42 (processivity factor) work together to form the core replication machinery (Bruckner, Crute et al. 1991, Fierer and Challberg 1992, Martinez, Shao et al. 1992, Boehmer, Dodson et al. 1993, Dodson and Lehman 1993). pU_L9 and ICP8 interact to recruit the trimeric helicase-primase complex (Tolun, Makhov et al. 2013, Weerasooriya, DiScipio et al. 2019). The helicase-primase complex has a 5' to 3' helicase, ATPase, primase and DNA binding activities (Crute, Mocarski et al. 1988, Dodson, Crute et al. 1989, Boehmer and Lehman 1997, Chattopadhyay, Chen et al. 2006). pU_L8 of the complex is essential for the localisation of the complex at the appropriate site. After the recruitment of the complex pU_L5 further unwinds the double-stranded DNA and pU_L52 adds short RNA primers for complementary DNA synthesis (Falkenberg, Bushnell et al. 1997). The DNA polymerase (pU_L30) and the processivity factor (pU_L42) are recruited next for the extension of DNA and removal of mismatched nucleotides by the 3'-5' exonuclease activities of pU_L30 (Marsden, McLean et al. 1997, Wang, Du et al. 2016). pU_L42 forms a heterodimer with pU_L30 and increases the binding affinity of pU_L30 to the DNA (Gottlieb, Marcy et al. 1990, Johnson, Best et al. 1991).

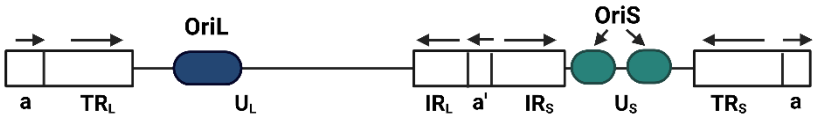

Figure 1.3. Structure of HHV-1 genome with location of origins of replication.

The DNA replication activates the expression of L genes. ICP4 also plays a role in the activation of L gene expression (Smith, Bates et al. 1993). The L gene products are mostly the viral structural proteins like capsid proteins and glycoproteins, which are required for DNA packaging and virion formation (Dembowski, Dremel et al. 2017).

The packaging of the viral genome into the capsids is another simultaneous process inside the host nucleus along with ongoing replication and transcription (Dembowski, Dremel et al. 2017). The simultaneous participation of proteins in different processes indicate a segregation of functions between the proteins to participate in transcription replication, and packaging of the genome. After the genome is appropriately packaged into the preformed capsids the nucleocapsids travel towards the nuclear membrane for egress and further maturation.

1.3.1.4 Nucleocapsid formation and DNA packaging

The proteins essential for the formation of the nucleocapsid are translated in the cytoplasm; however, the assembly of the nucleocapsid occurs inside the nucleus (Brown and Newcomb 2011). The proteins for nucleocapsid formation are conserved between the herpesvirus families (Davison, Eberle et al. 2009). The most extensive studies on the proteins involved and the mechanism of nucleocapsid formation have been carried out in HHV-1 and human cytomegalovirus (HCMV) (Gibson 2008). In HHV-1 (and in other alpha herpesviruses) the formation of the nucleocapsid is initiated by the portal vertex formed by pU_L6; however, the portal vertex is not essential (Chang, Schmid et al. 2007, Nellissery, Szczepaniak et al. 2007). The major capsid protein VP5 (pU_L19), is required for the formation of penton and hexon capsomeres. The triplex proteins VP23 (pUL18) and

VP19 (pUL38), reside in between and connect the capsomeres (Desai, DeLuca et al. 1998, Krautwald, Maresch et al. 2008). The scaffold protein $pU_L26.5$ along with the C-terminal region of pU_L26 are critically important for the capsid formation (Krautwald, Maresch et al. 2008). The scaffold proteins are needed to form a base for the arrangement of the capsid proteins and support the empty capsid. The capsid proteins arrange around the scaffold autocatalytically inside the nucleus (Newcomb, Homa et al. 2005, Huffman, Newcomb et al. 2008, Yang and Baines 2008). The small capsid protein VP26 (pU_L35), situated on tip of the hexons, is not essential for the nucleocapsid formation in alpha herpesviruses (Brown and Newcomb 2011). During the packaging of the genome into the capsid, the scaffold is cleaved by the protease pU_L26 and is forced out of the capsid (Thurlow, Murphy et al. 2006). After the extrusion of the scaffold proteins, the shape of the capsid is maintained by the packaged genome from the inside and by inner tegument proteins pU_L17 and pU_L25 from the outside (Klupp, Granzow et al. 2006). SuHV-1 pU_L17 is an internal nucleocapsid protein, which is necessary forSuHV-1DNA cleavage (Klupp, Granzow et al. 2005). SuHV-1 pUL25 remains associated with the capsid and is not essential for viral DNA cleavage and encapsidation, but plays an essential role in nuclear egress of nucleocapsid (Klupp, Granzow et al. 2006).

The proteins involved in the encapsidation and cleavage of newly replicated concatameric DNA are conserved in the herpesvirus family. In HHV-1, pU_L15, pU_L28 and pU_L33 are responsible for the measurement of unit lengths of the concatameric DNA, and its cleavage, and participate in its packaging (Wills, Scholtes et al. 2006). These proteins form the terminase complex and exist outside the immature capsid. The terminase complex is partially lost during the capsid maturation (Wills, Scholtes et al. 2006). The mechanism of capsid formation around a scaffold protein and the packaging of the DNA into the capsid via a portal complex indicates bacteriophage ancestry of herpesviruses (Agirrezabala, Martín-Benito et al. 2005, Baker, Jiang et al. 2005, Hendrix 2005, Johnson and Chiu 2007). After the packaging of the double-stranded viral DNA into the capsids, the nucleocapsids egress from the nucleus to undergo complete viral morphogenesis.

1.3.1.5 Nuclear egress and primary envelopment

The nuclear egress of the viral capsid into which the newly formed viral genome is packaged occurs by crossing the double membrane of the host nucleus. During this process, the capsid undergoes primary envelopment by budding into inner nuclear membrane and then fusing with the outer nuclear membrane, which leads to de-envelopment (Mettenleiter 2004). The virus-encoded nuclear egress complex plays a crucial role in this vesicle-mediated nucleocytoplasmic transport of the virus (Darlington and Moss 1968). In HHV-1 and related herpesviruses the nucleocapsids inside the nucleus travel with the help of the nuclear actin filaments induced by the viral infections. These filaments enable the nucleocapsids to reach the budding site at the inner nuclear membrane (Lee and Chen 2010). The homologues of HHV-1 tegument proteins pU_L31 and pU_L34 are crucial for the budding of the virus at the inner nuclear membrane in all members of the herpesvirus family (Liang, Tanaka et al. 2004). pU_L34 is a type II membrane protein present in the nuclear membrane as well as in the primary envelope (Mettenleiter 2004, Sam, Evans et al. 2009). pU_L34 interacts with pU_L31 and forms nuclear egress complex, which enables the positioning of the nucleocapsids at the inner nuclear membrane to initiate the budding process (Liang, Tanaka et al. 2004). For the budding of the virus into the inner nuclear membrane, the dissolution of the host nuclear lamina is needed (Hellberg, Paßvogel et al. 2016, Newcomb, Fontana et al. 2017). This dissolution is facilitated by phosphorylation, carried out by viral kinases. The nuclear egress complex also binds directly to proteins lamin A and C to modulate the dissolution of the lamina by interrupting the interaction between the lamin proteins (Bigalke and Heldwein 2017). The $pU_L31/34$ nuclear egress complex recruits protein kinase C, or viral kinases like pU_L97 in HCMV and pU_S3 in HHV-1, to the inner nuclear membrane (Muranyi, Haas et al. 2002, Park and Baines 2006). The lamin proteins are phosphorylated by these kinases, which clears the way for the passage of nucleocapsids into the perinuclear space (Leach, Bjerke et al. 2007). The disruption of the nuclear lamin is essential for the egress of the viral capsid from the nucleus

(Gruenbaum, Margalit et al. 2005). Because of the budding process into the perinuclear region, nucleocapsids acquire the primary envelope.

The host nuclear membrane, rich in phosphatidic acid and phosphatidylinositol, forms the primary envelope of the virus (Funk, Ott et al. 2015). Host protein kinase D regulates vesicle transport and assists both primary envelopment and virion egress by phosphorylation of phosphatidylinositol of the membrane (Lorenz, Vollmer et al. 2015). Tegument proteins are also present in the virion particles after primary envelopment (Guo, Shen et al. 2010). In HHV-1, analysis of the ultrastructure of the primary enveloped virus shows a clearly distinguishable ring-like structure of the tegument closer to the primary envelope. Tegument proteins VP1/2 (pU_L36), pU_L37, VP22 (pU_L49) and VP16 (pU_L48) of HHV-1 are constituents of the early tegument of the primary enveloped virus, where as in SuHV-1 VP1/2 (pU_L36) and pU_L37 are not a part of the primary enveloped virus (Padula, Sydnor et al. 2009).

Two different models explain the nuclear egress of the primary enveloped virion particle. According to the envelopment- de-envelopment model the primary envelope is lost during the fusion with the outer nuclear membrane and the naked capsid, with some surrounding tegument proteins, is released into the cytosol (Hellberg, Paßvogel et al. 2016). Another model is the luminal model, which proposes that the primary enveloped virion egresses by the secretory pathway and maintains its integrity in the cytosol (Roller and Johnson 2021). Viral factors along with host cellular factors play a role in this process (Bigalke and Heldwein 2015). Cellular factors involving p32, CD98 heavy chain and β1 integrin are recruited to the nuclear membrane when the cells are infected with HHV-1 and aid in the de-envelopment process (Hirohata, Arii et al. 2015, Liu, Kato et al. 2015). Viral VP13/14 (pU_L47) and host p32 accumulate at the nuclear membrane and assist in viral de-envelopment (Liu, Kato et al. 2015). CD98 heavy chain/β1 integrin interactions regulate the fusion between the perinuclear virus and the outer nuclear membrane (Ohta, Tsurudome et al. 1994). Viral pU_L34 mediates this interaction, as in absence of pU_L34 the CD98 heavy chain is not redistributed to the nuclear membrane (Maeda, Arii et al. 2017).

During the de-envelopment process, the lamin-associated proteins activate torsins to remodel the outer nuclear membrane for the fusion process and egress of the nucleocapsid from the nucleus (György, Cruz et al. 2018). Torsins are members of the AAA+ ATPase superfamily, which are comprised of enzymes mediating ATP-dependent conformational remodeling of protein (Rampello, Prophet et al. 2020). Torsins are present in the lumen of the endoplasmic reticulum and nuclear membrane (Brown, Zhao et al. 2014), and need the inner nuclear membrane lamin-associated proteins for activation (Sosa, Demircioglu et al. 2014). As the envelopment at the inner nuclear membrane and the fusion at the outer nuclear membrane take place there is a respective decrease and increase in the size of the membrane; this disproportionation of the host nuclear membrane is corrected by the *de novo* synthesis of phospholipids by the virus (Wild, Senn et al. 2009). In case of a dysfunctional nuclear egress complex, an alternative mechanism of egress is the breakdown of the nuclear membrane, which is induced by mitosis related cyclin-dependent kinase and MEK1/2 (Grimm, Klupp et al. 2012). In BoHV-1 infected cells breakdown of the nuclear membrane occurs at later stages of infection (Wild, Engels et al. 2005). After the nuclear egress of the nucleocapsid, incorporation of rest of the tegument and the lipid envelope (termed as secondary envelopment) take place at the Golgi.

1.3.1.6 Tegumentation and Maturation at the Golgi (secondary envelopment)

The assembly of tegument proteins occurs predominantly in the cytoplasm (Mettenleiter 2006). A few tegument proteins of HHV-1, namely VP1/2 (pU_L36), pU_L37, vhs (pU_L41), VP13/14 (pU_L47), VP16 (pU_L48) and VP22 (pU_L49), are present inside the nucleus closely associated with the capsid (Bohannon, Jun et al. 2013, Diefenbach 2015). Of these proteins, VP13/14 (pU_L47), VP16 (pU_L48) and VP22 (pU_L49) are components of the outer tegument, but they also interact with the capsid protein VP5 (pU_L19) (Scholtes, Yang et al. 2010). VP1/2 (pU_L36) is the largest tegument protein of HHV-1 and SuHV-1 (Newcomb, Jones et al. 2012), and forms a complex with the second largest tegument protein, pU_L37; this complex forms a scaffold for the formation of the tegument (Klupp,

Fuchs et al. 2002, Roberts, Abaitua et al. 2009). VP1/2 (pU_L36) interacts very strongly with the major capsid protein VP5 (pU_L19) and is the first tegument protein added to the virion particle (Cardone, Newcomb et al. 2012). The VP1/2(pU_L36)/37 complex acts as the foundation for tegument assembly by providing a link between the capsid and tegument (Vittone, Diefenbach et al. 2005). Absence of the proteins VP1/2 (pU_L36) and pU_L37 prevents the tegument formation (Bucks, O'Regan et al. 2007). The second largest tegument protein of HHV-1, pU_L37, is added to the tegument by a direct interaction with VP1/2 (pU_L36) (Mijatov, Cunningham et al. 2007). In HHV-1, both VP1/2 (pU_L36) and pU_L37 localize at the Golgi, suggesting the final packaging at the Golgi (Desai, Sexton et al. 2008). In absence of VP1/2 (pU_L36), the pU_L37 trafficking of the capsid to the Golgi and its envelopment are blocked (Desai, Sexton et al. 2008). VP11/12 (pU_L46), VP13/14 (pU_L47), VP16 (pU_L48) and VP22 (pU_L49) are conserved proteins among alpha herpesviruses and form the central organizer for the tegument formation (Kelly, Fraefel et al. 2009) by interacting with outer and inner tegument proteins and the glycoproteins of the outer envelope (Mettenleiter 2006). HHV-1 VP16 (pU_L48) interacts with the essential inner tegument protein VP1/2 (pU_L36) and the outer tegument proteins VHS (pU_L41), VP11/12 (pU_L46) and VP22 (pU_L49), as well as gH (pU_L22) (Ko, Cunningham et al. 2010, Fan, Roberts et al. 2015). The arrangement of the tegument in HHV-1 is shown in Figure 1.4.

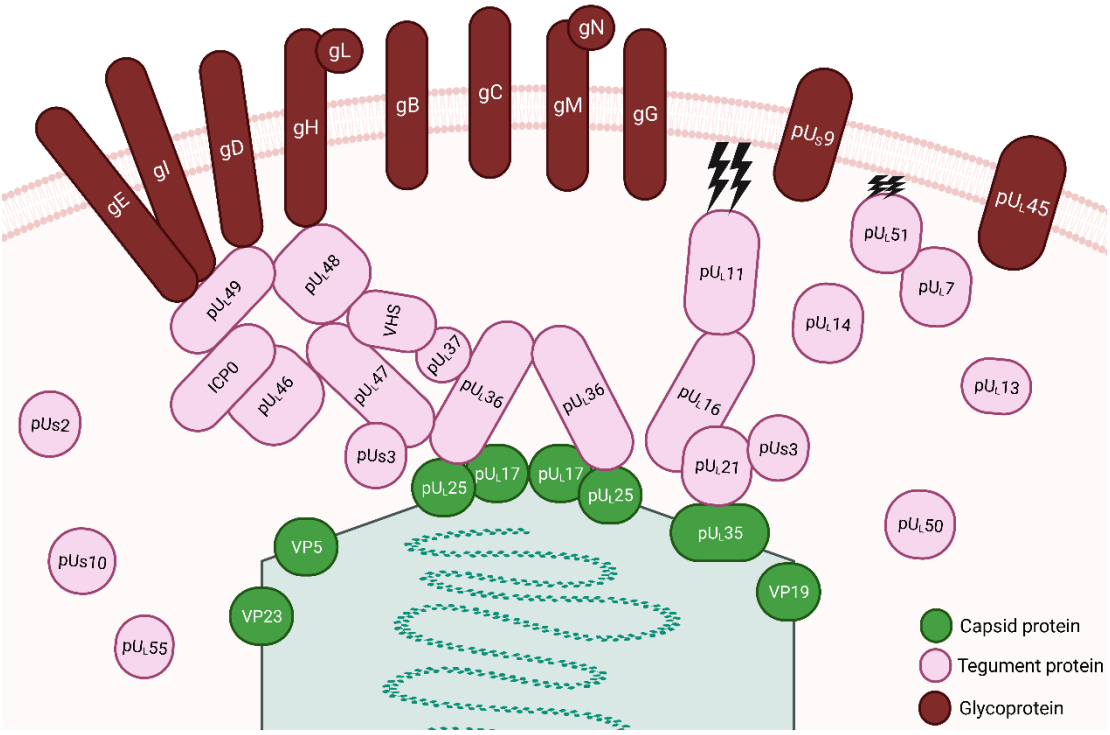

Figure 1.4 Arrangement of tegument proteins in HHV-1. The shape/size of illustrated proteins is not indicative of their true shape/size.

The maturation at the Golgi predominantly involves the addition of tegument proteins (mostly the outer tegument) and the lipid envelope with the glycoproteins to the virus particle (Crump 2018). Proteins added at the Golgi are translocated to the Golgi either by a Golgi localisation signal or via interactions with other glycoproteins or tegument proteins (Lee, Vittone et al. 2008). Outer tegument protein VP22 (pU_L49) is translocated to the trans Golgi network by its interaction with gE and gM, and the presence of either of these two glycoproteins is required for the packaging of VP22 (pU_L49) into the virus (Fuchs, Klupp et al. 2002). In HHV-1 and SuHV-1 VP16 (pU_L48) is essential for the addition of the outer tegument by interaction with outer tegument proteins VHS (pU_L41), VP11/12 (pU_L46), VP13/14 (pU_L47) and VP22 (pU_L49), and with glycoproteins B (pU_L27), D (pU_S6) and H (pU_L22) (Chi, Harley et al. 2005, Vittone, Diefenbach et al. 2005, Mettenleiter 2006). If VP13/14 (pU_L47) and VP22 (pU_L49) are deleted from SuHV-1, the viral assembly is not

blocked, rather the absence of these proteins is compensated by incorporation of a lower molecular weight isoform of VP16 (pU_L48) (Fuchs, Granzow et al. 2003, Michael, Klupp et al. 2006). When VP16 (pU_L46), VP13/14 (pU_L47) or VP22 (pU_L49) were deleted from SuHV-1, incorporation of cellular actin is increased to fill in the gap (Michael, Klupp et al. 2006). In SuHV-1, VP22 (pU_L49) interacts with gE (pU_S8) and gM (pU_L10) and both are sufficient for packaging of VP22 (pU_L49) (Fuchs, Granzow et al. 2003). If BoHV-1 VP8 (pU_L47) is deleted the incorporated tegument is smaller (Lobanov, Maher-Sturgess et al. 2010).

These interactions form the basis for the formation and maintenance of the structurally integral tegument. Apart from the requirement of protein-protein interactions for tegument incorporation, some interactions (tegument-tegument or tegument- glycoproteins) are also involved in the sub-cellular localization of proteins to the site of secondary envelopment (Mettenleiter 2006, Kelly, Fraefel et al. 2009, Diefenbach 2015, Owen, Crump et al. 2015). The site of secondary envelopment is either the Golgi, or the early endosome or autophagosome for most of the herpesviruses (Henaff, Radtke et al. 2012, Lv, Zhou et al. 2019), although for some viruses the site remains unclear (Albecka, Laine et al. 2016, Lebrun, Lambert et al. 2018). The most accepted site for the secondary envelopment in herpesviruses is the Golgi. Accumulation of the majority of the glycoproteins and tegument proteins at the Golgi also supports this notion (Turcotte, Letellier et al. 2005, Sugimoto, Uema et al. 2008, Johnson and Baines 2011). The presence of endocytosis motifs in the cytoplasmic tail of HHV-1 glycoproteins (gE-gI) promotes the incorporation into the virion by sorting the proteins into endosomal compartments (Johnson and Baines 2011). These motifs are not essential for the incorporation of glycoproteins in HHV-1; however, in VZV motif-dependent endocytosis of glycoproteins is essential for the secondary envelopment and addition of the glycoproteins (Wang, Cheng et al. 2015). After the secondary envelopment and final maturation of the virus at the Golgi, the virus proceeds to the next cycle of infection after cellular egress or through cell-to-cell spread (Campadelli-Fiume and Roizman 2006).

1.3.1.7 Cellular egress.

Information about the proteins involved in the cellular egress process, i.e. transport of enveloped virions enclosed in the vesicles to the plasma membrane and their subsequent release from the cell by fusion of the vesicle membrane, is very limited. In HHV-1 and SuHV-1 pU_L20 and glycoprotein K (pU_L53) play a role in its cellular egress (Mettenleiter 2004). pU_L20 and gK (pU_L53) interact to form a functionally active complex (Foster, Chouljenko et al. 2008). However, their precise role and the detailed mechanism of cellular egress is yet to be elucidated.

1.3.2 Latent cycle

Latency is a characteristic feature of herpes viruses, which helps the virus in evading the immune system and prevents clearance from the host. Essentially, when the host immune system responds to the symptomatic or asymptomatic viral shedding during the replicative cycle, the virus is pushed to its latency establishment site to evade the effect of the immune response (Grinde 2013). The site of establishment of latency in alpha herpesviruses is the neuronal trigeminal ganglia (Wilson and Mohr 2012). During the latent cycle of the herpesvirus, the viral genome is circularized and exists as episome. Circularised genome is linearized during resumption of the lytic cycle (Deshmane and Fraser 1989, Wilson and Mohr 2012). During latency, lytic protein expression is supressed and latency-associated transcripts are expressed. These help in suppression of reactivation and maintaining the dormancy of the virus (Phelan, Barrozo et al. 2017). Latency also helps the herpesvirus to co-evolve with the host species (Miszczak, Słońska et al. 2013).

1.3.3 Reactivation from latency

The shift from latency to reactivation happens after the virus and the neighboring neuronal cells recognize some neuronal signals which are induced by stimuli such as exposure to UV light, fever, hormones, stress etc. which leads to an upregulation of the expression of viral lytic genes (Grinde 2013). Stress hormone like gluco-corticoids or

immunosuppressive drugs helps in reactivation by making the episome accessible to transcription factors and hence increase expression of lytic genes (Stucker and Ackermann 2011). The HHV-1 and BoHV-1 proteins bICP0 and VP16 (pU_L48) regulate reactivation of virus from latency in the trigeminal sensory ganglia expressing gluco-corticoid receptors (Kook 2016). After reactivation in the neurons, the virus particles travel from the cell body to the axon in a anterograde manner and travel back to the primary site of infection where they actively replicate and resume the lytic cycle (Grinde 2013).

1.4 Tegument proteins of alpha herpesvirus

1.4.1 Dissociation of tegument proteins from virions

The multifunctional tegument is released from the virus immediately after the infection of the host cell; first, the outer tegument is dissociated, followed by the inner tegument proteins. The dissociation of the tegument leads to the release of the tegument proteins into the host cell enabling them to carry out their designated functions (Heine, Honess et al. 1974). The dissociation of the tegument facilitates the initiation of the viral replication. The initiation of the viral replication also involves the suppression of the host proteins by degrading the mRNAs of host transcription factors. This function is carried out by viral host shut off protein pU_L41 in HHV-1 (Smiley 2004). The tegument proteins also control cell death signalling (Geenen, Favoreel et al. 2005, Geenen, Favoreel et al. 2006, Mori, Goshima et al. 2006, Kelly, Fraefel et al. 2009, Mori 2012) and suppress the host innate immune responses (Bresnahan and Shenk 2000, Lukashchuk, McFarlane et al. 2008, Afroz, Brownlie et al. 2016, Xu, Mallon et al. 2016). The dissociated tegument proteins participate in guiding the viral capsid to the nuclear pore (Kelly, Fraefel et al. 2009, Krautwald, Fuchs et al. 2009, Abaitua, Daikoku et al. 2011, Hennig, Abaitua et al. 2014) and promote the transcription of viral genes (Sivachandran, Wang et al. 2012, Lin, Greco et al. 2013).

The dissociation of the tegument from the virus is an energy-dependent mechanism. Adenosine tri phosphate (ATP), enzymes and magnesium ions (Mg^{2+}) facilitate the tegument dissociation by supplying the required energy and catalysing the reaction

(Morrison, Wang et al. 1998). Heat inactivation of viral and host kinases hindered the dissociation of the tegument, which suggests that the phosphorylation of the tegument proteins triggers their dissociation from the tegument. For example, in HHV-1, the dissociation of the tegument protein VP22 (pU_L49) is promoted by the phosphorylation of the protein by casein kinase-2 (Morrison, Wang et al. 1998). In HHV-1 and BoHV-1, the tegument proteins incorporated in the mature virion are in their non-phosphorylated forms (Meredith, Lindsay et al. 1991, Elliott, O'Reilly et al. 1996, Loomis, Courtney et al. 2006, Zhang, Brownlie et al. 2016). pU_L13 (Kato, Yamamoto et al. 2006) and pU_S3 (Morrison, Wang et al. 1998, Cano-Monreal, Tavis et al. 2008, Labiuk, Lobanov et al. 2010) are viral kinases that are incorporated into the virus and phosphorylate themselves, as well as other proteins like VP11/12 (pU_L46) (Eaton, Saffran et al. 2014).

The dissociation of tegument proteins from viruses occurs according to the order of their requirement during the viral infection and in reverse order of incorporation into the virion. In HHV-1, the first tegument protein to dissociate from the virion immediately after the entry of the virus into the host is VP16 (pU_L48) (Aggarwal, Miranda-Saksena et al. 2012). After dissociation, VP16 (pU_L48) travels into the nucleus and is responsible for immediate early gene expression (Triezenberg, Kingsbury et al. 1988). VP13/14 (pU_L46), a minor tegument protein of HHV-1 also dissociates early after the virus infection. The tegument protein VP22 (pU_L49) of HHV-1 is dissociated after VP16 (pU_L48) and VP13/14 (pU_L47). A few inner tegument proteins, which are in close proximity of the nucleocapsid, for example VP1/2 (pU_L36) and pU_L37, remain associated with the virus during its transport to the nucleus and dissociate very late. VP1/2 (pU_L36) and pU_L37 guide the nuclear capsid towards the nuclear pore complex (Morrison, Wang et al. 1998, Aggarwal, Miranda-Saksena et al. 2012, Schipke, Pohlmann et al. 2012). SuHV-1 shows a similar pattern of dissociation i.e. pU_L11, VP13/14 (pU_L47), VP16 (pU_L48) and VP22 (pU_L49) dissociate early during virus infection compared to VP1/2 (pU_L36), pU_L37 and pU_S3 (Granzow, Klupp et al. 2005). However, specific kinases regulating the phosphorylation of each tegument protein have not been identified.

1.4.2 Functions of tegument proteins of alpha herpesviruses

The tegument proteins of alpha herpesviruses carry out a variety of functions in the viral life cycle. Tegument protein functions include, but are not limited to, dissociation of the other tegument proteins, transport of the capsid to the nucleus, evasion of the host immune response, gene expression, encapsidation of viral DNA, and viral assembly and egress (Campbell, Palfreyman et al. 1984, Smiley 2004, Granzow, Klupp et al. 2005, Kelly, Fraefel et al. 2009).

The entry of the virus particle into the host cell is facilitated by glycoproteins, and this entry takes place by either a pH-dependent endocytic pathway or pH-independent (direct) fusion of the viral envelope and host membrane. At the site of primary infection, direct fusion of the viral envelope and the host membrane by glycoprotein-receptor binding is predominant; however, in some cases virus internalisation into endocytic vesicles has been observed, but the reason is unclear (Granzow, Klupp et al. 2005, Delboy and Nicola 2011). After the cellular entry of the virus the capsids associate with the host microtubules for their transport to the nucleus and around the cell (Lyman and Enquist 2009). For HHV-1, the tegument proteins involved in the transport of the viral capsid to the nucleus are the capsid associated tegument proteins VP1/2 (pU_L36) (Hennig, Abaitua et al. 2014) and pU_L37 (Krautwald, Fuchs et al. 2009). VP1/2 (pU_L36) is critically important for the navigation of the capsid to the nucleus in association with pU_L37. As VP1/2 (pUL36) remains attached to the capsid, the nuclear localisation signal (NLS) of VP1/2 (pU_L36) is responsible for the movement of the capsid towards the nucleus (Kelly, Fraefel et al. 2009). pU_L37 mimics the host transport machinery due to the structural similarity of its N-terminal region to the host protein complex controlling cellular protein trafficking; and thus, plays a role in the transport of the virions to the nucleus. However, it is not essential for the docking of the capsid at the nuclear pore complex (Roberts, Abaitua et al. 2009, Pitts, Klabis et al. 2014). On arrival of the capsid at the nuclear pore complex, the cleavage of VP1/2 (pU_L36) promotes the release of the viral DNA into the nucleus (Jovasevic, Liang et al. 2008). A similar involvement of VP1/2 (pU_L36) and pU_L37 in the transport of capsid

to the nucleus was confirmed in SuHV-1 (Krautwald, Fuchs et al. 2009), which supports a conservative mechanism in alpha herpesviruses. ICP0 is another early tegument protein, which has E3 ubiquitin ligase activity and is involved in promoting host 26S proteasomal degradation to enable efficient capsid delivery in HHV-1- infected cells (Delboy and Nicola 2011). Other tegument proteins involved in the transport of the capsid in the HHV-1 infected cells are pU_S11, which plays a role in anterograde transport of the capsid during the establishment of latency (Diefenbach, Miranda-Saksena et al. 2002), and VP26 (pU_L35), which interacts with dynein to aid in the transport of the capsid to the nucleus (Douglas, Diefenbach et al. 2004).

Tegument proteins regulate the viral gene expression during the infection cycle. The gene expression is divided into immediate early, early and late expression; late gene expression starts after the initiation of viral DNA replication. The initiation of the transcription of immediate early genes of HHV-1 is regulated by VP16 (pU_L48), which is an α trans-inducing factor and is critical for a lytic infection of HHV-1(Post, Mackem et al. 1981, Campbell, Palfreyman et al. 1984, Pellett, McKnight et al. 1985). Following the dissociation from the tegument, VP16 (pU_L48) induces the formation of a transcription complex involving host cell factor-1 and Oct-1 (Gerster and Roeder 1988, Stern, Tanaka et al. 1989, Herr 1998, Wysocka and Herr 2003). This complex binds to the viral immediate early gene promoters and results in expression of ICP0, ICP4, ICP22 and ICP27 proteins (Roizman, Gu et al. 2005, Gu and Roizman 2007). HHV-1 VP11/12 (pU_L46) and VP13/14 (pU_L47) enhance the activity of VP16 (pU_L48). pU_L14 enhances the nuclear localisation of VP16 (pU_L48) and hence these proteins (VP11/12 (pU_L46), VP13/14 (pU_L47) and pU_L14) play an indirect role in the expression of immediate early genes (Zhang and McKnight 1993, Vittone, Diefenbach et al. 2005, Yamauchi, Kiriyama et al. 2008). The tegument proteins ICP0 and ICP4 promote the transition of the transcription from immediate early genes to early genes (Quinlan and Knipe 1985, Härle, Sainz et al. 2002, Gu and Roizman 2007). The tegument protein ICP4 has the ability to function both as the activator of early gene transcription and repressor of immediate early gene transcription (DeLuca, McCarthy

et al. 1985, Sampath and Deluca 2008). HHV-1 tegument protein pU_L37 interacts with the TNF receptor associated factor-6 to activate NF-κβ signalling pathway (Liu, Fitzgerald et al. 2008). This regulation of NF-κβ activity also plays a role in transcriptional activation of ICP0 (Amici, Rossi et al. 2006). HHV-1 VHS (pU_L41) degrades host mRNA, and consequently the amount of viral mRNA in host cells is increased (Smiley 2004, Taddeo and Roizman 2006).

Tegument proteins play a role in the packaging of the newly synthesised viral genome into the capsid, i.e. the encapsidation of the viral DNA (Weller and Coen 2012). After the replication of the viral DNA the first step is the recognition of the unit length of the genome to be packaged. In HHV-1 the unit length is characterised by the palindromic 'a' sequence flanked by unique b (Ub) and unique c (Uc) sequences (Stow, McMonagle et al. 1983, Hodge and Stow 2001). The nuclease tegument proteins pU_L12 (Goldstein and Weller 1998) and $pU_L12.5$ (Bronstein, Weller et al. 1997) of HHV-1 facilitate a change in the conformation of the viral genome to enable its encapsidation. 12 copies of the pU_L6 protein form the portal vertex through which the DNA enters the capsid for the process of encapsidation (Newcomb, Juhas et al. 2001, Albright, Nellissery et al. 2011). pU_L32 participates in the appropriate formation of the portal vertex and hence enables proper packaging of the viral genome (Albright, Kosinski et al. 2015). pU_L25, exposed on the capsid, plays a role in the stabilisation of the viral capsid and is necessary for retaining the genome in the capsid (Newcomb, Homa et al. 2006, Cockrell, Huffman et al. 2011). pU_L25 forms a bridge between the tegument and the capsid by interacting with the capsid proteins VP5 (pU_L19) and VP19 (pU_L38) and the tegument protein pU_L17, which also helps the stabilisation of pU_L25 in the capsid (Ogasawara, Suzutani et al. 2001, Cockrell, Sanchez et al. 2009). The tegument protein VP16 (pU_L48) also promotes the encapsidation of the viral DNA, which is suggested by reduced viral DNA encapsidation in VP16 (pU_L48)-deleted virus (Mossman, Sherburne et al. 2000, Albright, Kosinski et al. 2015).

Tegument proteins also play a major role in the egress of the virus from the nucleus. During the process of nuclear egress the capsid has to cross two nuclear membranes for

which it goes through envelopment and de-envelopment (Fig 1.5) (Skepper, Whiteley et al. 2001). After the viral capsid assembly, the nucleocapsids bud at the inner nuclear membrane by disrupting the rigid nuclear lamina. As a result of budding into the perinuclear space, the inner nuclear membrane is acquired as envelope and the process is termed as primary envelopment and the immature virus is primary enveloped virus (Scott and O'Hare 2001, Muranyi, Haas et al. 2002, Leach, Bjerke et al. 2007, Mou, Wills et al. 2008). In HHV-1 and SuHV-1, pU_L31 and pU_L34 form a complex at the inner nuclear membrane and play a role in disrupting the nuclear lamina. The $pU_L31/34$ complex also promotes the localisation of the nucleocapsids adjacent to the inner nuclear membrane thereby facilitating the budding process (Reynolds, Wills et al. 2002, Reynolds, Liang et al. 2004, Simpson-Holley, Baines et al. 2004, Simpson-Holley, Colgrove et al. 2005, Bjerke and Roller 2006, Bigalke and Heldwein 2017). The C-terminal region of pU_L31 forms a bridge between the nuclear membrane and the virus capsid inside the nucleus, which enables the formation of the budding vesicle by the inner nuclear membrane (Funk, Ott et al. 2015). HHV-1 serine/threonine kinase pU_S3 phosphorylates lamins and lamin receptor emerin resulting in destabilisation of nuclear lamina (Leach, Bjerke et al. 2007, Morris, Hofemeister et al. 2007, Mou, Wills et al. 2008). pU_S3 also phosphorylates pU_L31 and pU_L34 in both HHV-1 and SuHV-1 and regulates the nuclear localisation of the $pU_L31/34$ complex (Purves, Spector et al. 1991, Ryckman and Roller 2004, Kato, Yamamoto et al. 2005, Mou, Wills et al. 2009). In HHV-1, another viral kinase, pU_L13, is also likely to play a role in regulation of localisation of $pU_L31/34$ complex, either directly or indirectly through phosphorylation of pU_S3 (Kato, Yamamoto et al. 2006, Kato, Tanaka et al. 2008).

In the de-envelopment process, the primary envelope of the virus fuses with the outer nuclear membrane and releases the nucleocapsid into the cytoplasm. In HHV-1 infected cells a host cellular component, P32, moves to the nuclear egress complex to interact with the pU_L31-pU_L34 complex and enhances the process of de-envelopment of the virus particle (Guan, Guo et al. 2014, Liu, Kato et al. 2015). The viral tegument protein ICP22 or VP13/14 (pU_L47) forms a bridge between the host P32 and the viral pU_L31-pU_L34

complex (Maruzuru, Shindo et al. 2014). At the outer nuclear membrane, the de-envelopment of HHV-1 and SuHV-1 virions involves the tegument protein pU_S3 (Mou, Wills et al. 2008). pU_S3 phosphorylates the cytoplasmic tail of gB (pU_L27) in the primary enveloped virion to enhance gB (pU_L27)-mediated fusion of the primary envelope and the outer nuclear membrane (Wisner, Wright et al. 2009).

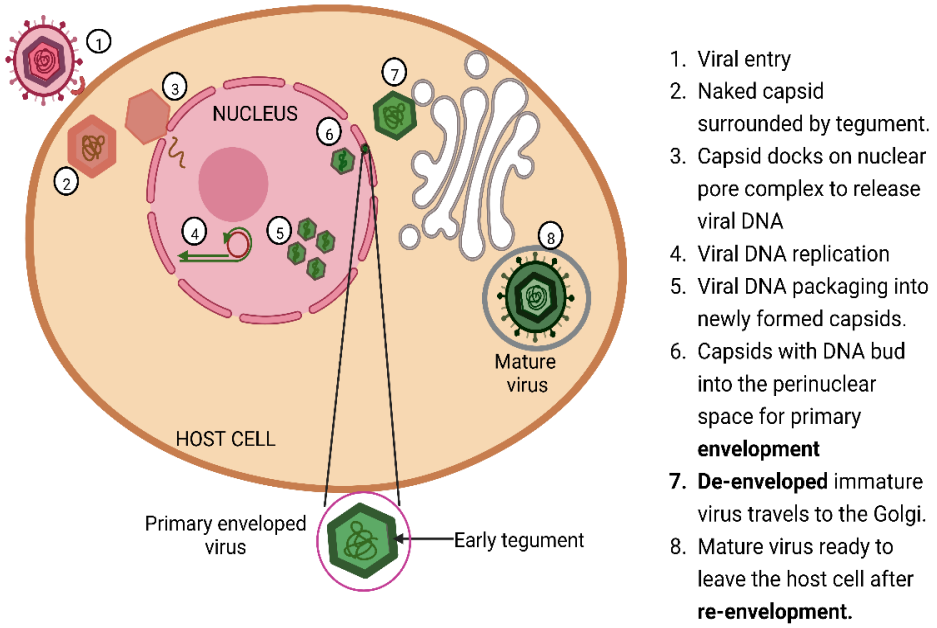

Figure 1.5. Virus maturation showing viral envelopment, de-envelopment and re-envelopment.

Recruitment and incorporation of most of the tegument proteins takes place in the cytoplasm. This process of tegument incorporation is accompanied by the secondary envelopment of the virion. Molecular interactions involving viral membrane proteins, tegument proteins and capsid proteins play pivotal roles in the secondary envelopment and tegument incorporation (Mettenleiter 2004, Mettenleiter 2006). Tegument protein pU_L16 is conserved across *Herpesviridae* and plays a critical role in virion morphogenesis in the cytoplasm. HHV-1 and SuHV-1 pU_L16 binds to the capsid indirectly by interacting with pU_L21, which is a capsid-associated tegument protein (Takakuwa, Goshima et al. 2001,

Klupp, Böttcher et al. 2005). pU$_L$16 also binds to pU$_L$11, which is a conserved membrane-associated tegument protein. This interaction of pU$_L$16 and pU$_L$11 is conserved among *Herpesviridae* (Loomis, Courtney et al. 2003). Another HHV-1 tegument protein, VP16 (pU$_L$48), interacts with inner tegument proteins (VP1/2 (pU$_L$36)), outer tegument proteins (VHS (pU$_L$41), VP11/12 (pU$_L$46), VP13/14 (pU$_L$47) and VP22 (pU$_L$49)) or the cytoplasmic tail of glycoproteins (gH (pU$_L$22)) (Vittone, Diefenbach et al. 2005). These interactions contribute to forming a bridge between the viral capsid and envelope during virus morphogenesis. The tegument protein VP22 (pUL49) interacts with membrane protein gE (pU$_L$47) (Mettenleiter 2006). These interactions (Fig 1.6) play an important role in the incorporation as well as maintenance of the respective proteins as well as the viral morphology (Owen, Crump et al. 2015).

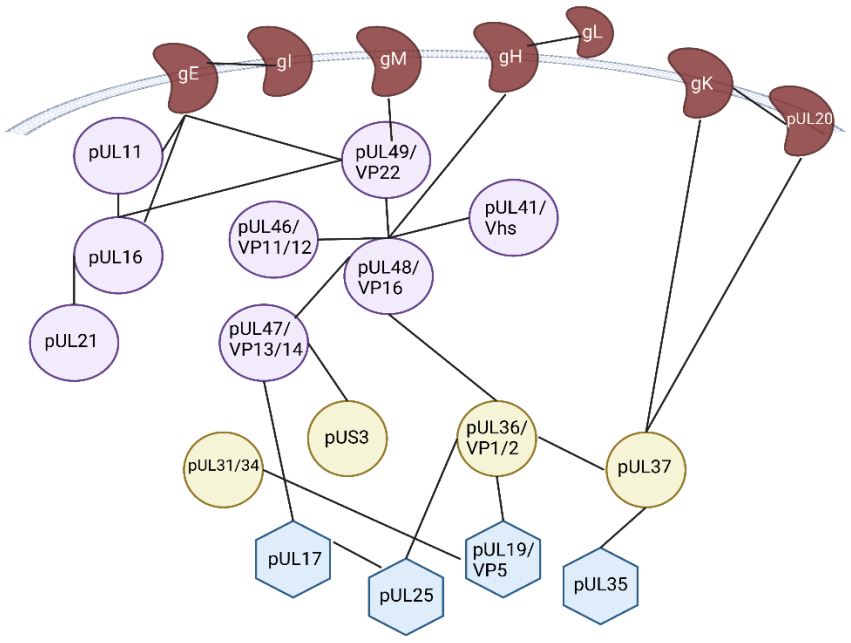

Figure 1.6. Schematic representation of protein-protein interactions playing a role in herpesvirus tegument incorporation. Hexagons (blue) represent capsid proteins. Circles in yellow represent inner tegument proteins. Circles in purple represent outer tegument proteins. Bean shaped structures (red) represent glycoproteins and solid lines depict interaction.

1.4.3 Major tegument protein VP8 of BoHV-1

VP8 (pU_L47) is the most abundant tegument protein of BoHV-1 and is a translation product of the UL47 gene (Carpenter and Misra 1991). VP8 (pU_L47) is phosphorylated and glycosylated and conserved among the alpha herpesviruses (Meredith, Lindsay et al. 1991). In absence of VP8 (pU_L47) from BoHV-1, a considerable reduction in the amount of tegument protein VP22 (pU_L49) was observed. The deletion of VP8 (pU_L47) from the virus also led to a substantial change in the morphology of the mature BoHV-1 (van Drunen Littel-van den Hurk, Garzon et al. 1995, Lobanov, Maher-Sturgess et al. 2010).

VP8 (pU_L47) contains two NLSs, NLS1 and NLS2, near the N-terminus of the protein (Fig 1.7). These NLSs are responsible for the nuclear localisation of VP8 (pU_L47) in BoHV-1 infected cells (Zheng, Brownlie et al. 2004, Verhagen, Hutchinson et al. 2006). NLS1 (11RPRR15) is critical for the nuclear localisation of VP8 (pU_L47); however, it is not sufficient (Zheng, Brownlie et al. 2004). NLS1 requires the involvement of a 9-amino acid stretch, NLS2 ($R^{48}PRVRRPR^{54}$) for optimal nuclear localisation of VP8 (pU_L47). NLS2 is essential for the complete function of NLS1 (Verhagen, Donnelly et al. 2006). In cells transfected with VP8 (pU_L47), translocation of VP8 (pU_L47) from one nucleus to the nucleus of another cell has been observed, indicating shuttling of VP8 (pU_L47) between nucleus and cytoplasm. The cytoplasmic export of VP8 (pU_L47) occurs at later stages of infection (Zheng, Brownlie et al. 2004). The export of VP8 (pU_L47) out of the nucleus is controlled by the presence of nuclear export signals (NES). VP8 (pU_L47) contains two NESs, NES1 and NES2. NES1 (^{485}LSAYLTLFYAL^{495}) is constituted of hydrophobic leucine-rich sequences (Fig 1.7). NES1 is a chromosomal maintenance 1 (CRM-1) dependent sequence and is weak, as it does not optimally transport VP8 (pU_L47) from the nucleus to the cytoplasm (Zheng, Brownlie et al. 2004, Verhagen, Donnelly et al. 2006, Williams, Verhagen et al. 2008). NES2, situated between 95 -123 amino acids of VP8 (pU_L47), is a CRM-1 independent sequence as it remains unaffected by a CRM-1 inhibitor (Verhagen, Donnelly et al. 2006). NES2 has stronger nuclear export activity compared to NES1 (Verhagen, Donnelly et al. 2006). Nevertheless, nuclear export of VP8 (pU_L47) is

observed in the absence of both NES1 and NES2 (Verhagen, Donnelly et al. 2006), indicating the presence of other mechanisms of export of VP8 (pU_L47) from the nucleus to the cytoplasm.

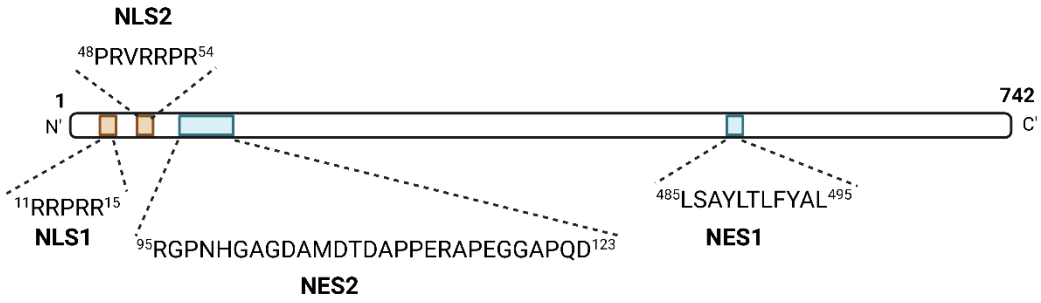

Figure 1.7. Schematic representation of position of NLS and NES in VP8 (pU_L47) protein.

VP8 (pU_L47) has diverse functions and is important for the replication of BoHV-1. A 100-fold and 1000-fold reduction was reported in the intracellular and extracellular viral titre, respectively, in cells infected with VP8 (pU_L47)-deleted BoHV-1, suggesting a critical yet dispensable role of VP8 (pU_L47) in BoHV-1 replication in cell culture. In infected cattle no viral replication was observed in the absence of VP8 (pU_L47) (van Drunen Littel-van den Hurk, Garzon et al. 1995, Lobanov, Maher-Sturgess et al. 2010). BoHV-1 VP8 (pU_L47) interacts with the host cellular kinase Casein kinase-2 (CK2) and the viral kinase pU_S3 (Labiuk, Babiuk et al. 2009). These kinases phosphorylate VP8 (pU_L47) in the cytoplasm and in the nucleus, respectively, at different serine/threonine residues. Host kinase CK2 phosphorylates VP8 (pU_L47) at 7 residues, T^{65}, S^{66}, S^{79}, S^{80}, S^{82}, S^{88} and T^{107}. Viral kinase pUS3 phosphorylates VP8 (pU_L47) at S^{16} and S^{32} residues. The phosphorylation of S^{16} is required for the subsequent phosphorylation at S^{32} (Zhang, Afroz et al. 2015). The phosphorylation of VP8 (pU_L47) by viral kinases regulates multiple functions of VP8 (pU_L47) in the nucleus, but does not affect the nuclear import of the protein. The functions regulated by phosphorylation include DNA encapsidation in the virus and distribution of

promyelocytic leukemia bodies (Labiuk, Babiuk et al. 2009, Zhang, Afroz et al. 2015, Zhang, Donovan et al. 2019). Phosphorylation of VP8 (pU_L47) in the nucleus by pU_S3 is also required for the cytoplasmic localisation of VP8 (pU_L47), as the non-phosphorylated VP8 (pU_L47) remains nuclear (Zhang, Donovan et al. 2019). VP8 (pU_L47) incorporated into the mature virus is in a non-phosphorylated state; however, the mechanism and site of de-phosphorylation remains unclear. VP8 (pU_L47) also interacts with DNA-damage binding proteins and regulate apoptosis (Vasilenko, Snider et al. 2012). VP8 (pU_L47) modulates the immune response by down regulating the entry of STAT-1 into the nucleus (Afroz, Brownlie et al. 2016). VP8 (pU_L47) shows RNA binding activities and binds to the RNAs of glycoproteins gB (pU_L27), gC (pU_L44) and gD (pU_S6) (Donnelly, Verhagen et al. 2007, Islam, Schulz et al. 2015). The significance of these RNA binding activities are yet to be studied.

1.4.4 VP8 (pU_L47) homologues in other alpha herpesviruses

In HHV-1, the protein encoded by *U_L47* is known as VP13/14 and is homologous to BoHV-1 VP8 (pU_L47). ICP8 activates the expression of VP13/14 (pU_L47) (Chen and Knipe 1996) and its stimulation is mediated by a tyrosine kinase (Yura, Kusaka et al. 1997). Similar activation of BoHV-1 VP8 (pU_L47) by ICP8 homologue is unknown. BoHV-1 ICP8 is a major DNA binding protein (Hammerschmidt, Conraths et al. 1988). Regulation of alpha-transducing factor (alpha TIF) or VP16 (pU_L48)-mediated transactivation of immediate early genes is carried out by VP13/14 (pU_L47) (Chen and Knipe 1996). Any such role of BoHV-1 VP8 (pU_L47) is not studied yet.

Similar to BoHV-1 VP8 (pU_L47), HHV-1 VP13/14 (pU_L47) is post-translationally modified and is phosphorylated and glycosylated. In contrast to BoHV-1 VP8 (pU_L47), host CK2 does not phosphorylate HHV-1 VP13/14 (pU_L47) however; HHV-1 pU_S3 phosphorylates VP13/14 (pU_L47) at S^{77}. Amino acid sequence of host protein kinase A and Akt are similar to HHV-1 viral kinase pU_S3, which suggests a possible role in phosphorylation of VP13/14 (pU_L47). Such a sequence similarity for BoHV-1 is unknown.

The phosphorylated state of VP13/14 (pU_L47) was only found in HHV-1 infected cells; however, VP13/14 (pU_L47) incorporated into the virus is de-phosphorylated (Meredith, Lindsay et al. 1991). The phosphorylation status of BoHV-1 VP8 (pU_L47) is similar to that of HHV-1 VP13/14 (pU_L47) in infected cells and in mature virus (van Drunen Littel-van den Hurk, Garzon et al. 1995, Zhang, Afroz et al. 2015).

Predominant nuclear localisation of HHV-1 VP13/14 (pU_L47), is governed by N-terminally situated NLSs (Donnelly and Elliott 2001). The phosphorylation of S^{77} by pU_S3 near the NLS enhances the activity of the NLS, which enables VP13/14 (pU_L47) to stay in the nucleoplasm. In the absence of pU_S3 VP13/14 (pU_L47) accumulates in the nuclear membrane (Kato, Liu et al. 2011). In BoHV-1, pUS3 phosphorylates S^{16} and S^{32}, of which S^{16} is adjacent to NLS1; however, this phosphorylation is not critical for nuclear localisation of VP8 (pU_L47) (Zhang, Afroz et al. 2015). The egress of VP13/14 (pU_L47) from the nucleus into the cytoplasm is regulated by the presence of two NESs similar to BoHV-1 VP8 (pU_L47). The NES-1 functions in a CRM-1-dependent manner and NES-2 is CRM-1-independent during the navigation of VP13/14 (pU_L47) from the nucleus into the cytoplasm (Donnelly and Elliott 2001, Williams, Verhagen et al. 2008). HHV-1 VP13/14 (pU_L47) interacts with the nuclear egress complex formed by pU_L31-pU_L34 to facilitate the egress of the newly formed nucleocapsids from the nucleus (Liu, Kato et al. 2014). The interaction between BoHV-1 VP8 (pU_L47) and the nuclear egress complex of BoHV-1, however, has not been investigated.

VP13/14 (pU_L47) associates with polyadenylated RNA, which indicates its RNA binding activity. VP13/14 (pU_L47) expressed in *Escherichia coli* (*E.coli*) also binds to RNA; however, post-translational modification of BoHV-1 VP8 (pU_L47) is required for it's RNA binding activity (Donnelly, Verhagen et al. 2007, Islam, Schulz et al. 2015). VP13/14 (pU_L47) co-localises with ICP4, which functions as an activator of viral transcription. The co-localisation of VP13/14 (pU_L47) and ICP4 indicates an involvement of HHV-1 VP13/14 (pU_L47) in the viral translation (Donnelly, Verhagen et al. 2007). VP13/14 (pU_L47) maintains the stability of the RNA via an interaction with ICP27

(Dobrikova, Shveygert et al. 2010, Shu, Taddeo et al. 2013). Similar roles of BoHV-1VP8 (pU_L47) have not been identified yet. Although HHV-1 VP13/14 (pU_L47) interacts with the nuclear egress complex, it is not incorporated into primary enveloped virions (Padula, Sydnor et al. 2009, Liu, Kato et al. 2014).

The protein encoded by the *U_L47* gene of Marek's disease virus-1 (MDV-1), an avian herpesvirus, is also homologous to BoHV-1 VP8 (pU_L47). However, MDV-1 pU_L47 is not essential, although deletion of pUL47 impairs virus growth (Dorange, Tischer et al. 2002). pU_L47 of infectious laryngotracheitis virus (ILTV), an other avian alpha herpesvirus, is also not critical for the viral growth, although it is needed for virulence (Helferich, Veits et al. 2007). In SuHV-1, impaired virion morphogenesis and reduction in virus titer was observed in *U_L47* deletion mutant-infected cells, which suggests a function in the virion assembly (Kopp, Klupp et al. 2002).

In all the studies on alpha herpesviruses, the *U_L47* gene product is essential for virulence (Lobanov, Maher-Sturgess et al. 2010); however, the detailed virulence mechanism for each virus remains unknown. In all alpha herpesviruses, a defect in viral morphology is also observed in absence of *U_L47* gene product (Lobanov, Maher-Sturgess et al. 2010), but the specific role in viral morphogenesis is unknown. BoHV-1 VP8 (pU_L47) plays a role in regulation of apoptosis as well as induction of immunity in the host (Afroz, Brownlie et al. 2016, Afroz, Garg et al. 2018). Such a role is not known in other alpha herpesviruses. HHV-1 VP13/14 (pU_L47) plays a role in nuclear egress (Liu, Kato et al. 2014) but a similar role of BoHV-1 VP8 (pU_L47) is yet to be investigated. A comprehensive list of similarities and differences in characteristics of BoHV-1 pU_L47 and HHV-1 pU_L47 is presented in Table 1.1

Table 1.1 Comprehensive list of similarities and differences of BoHV-1, HHV-1, SuHV-1 and MDV pU$_L$47

Properties	**BoHV-1 pU$_L$47**	**HHV-1 pU$_L$47**	**SuHV-1 pUL47**	**MDV pUL47**
Gene	*U$_L$47*	*U$_L$47*	*U$_L$47*	*U$_L$47*
Protein Name	VP8	VP13/14	VP13/14	pUL47
Phosphorylated	Yes, by viral pUS3 and host CK2	Yes, by viral pUS3	Not Known	Not Known
Glycosylated	Yes	Yes	Not Known	Not Known
Activated by	Not Known	ICP8	Not Known	Not Known
NLS	Present, Two	Present, Two	Not Known	Not Known
NES	Present, Two (CRM dependent and independent)	Present, Two (CRM dependent and independent)	Not Known	Not Known
Essential/ non-essential	Non-essential	Non-essential	Non-essential	Non-essential
Role in virion morphogenesis	Yes	Yes	Yes	Yes
Requirement in virulence	Yes	Yes	Yes	No
Role in nuclear egress	Not Known	Yes, interacts with nuclear egress complex pU$_L$31/34	Not Known	Not Known
Role in apoptosis	Yes	Not Known	Not Known	Not Known

Immune response modulation	Yes, downregulates STAT1	Not Known	Not Known	Not Known
mRNA binding activity	Present after post-translational modification	Present without post-translational modification	Not Known	Not Known
Status in mature virus	De-phosphorylated	De-phosphorylated	Not Known	Not Known

1.4.5 Tegument protein VP22 (pU_L49) of BoHV-1

The tegument protein VP22 (pU_L49) in BoHV-1 is encoded by the *U_L49* gene and consists of 258 amino acids. VP22 (pU_L49) is phosphorylated by the viral kinase pU_S3 (Labiuk, Lobanov et al. 2010) and is not essential for the growth of BoHV-1 in tissue culture. The viral titre of VP22 (pU_L49)-deleted BoHV-1 is ~10 fold reduced compared to WT BoHV-1, and a 40% reduction of plaque diameter suggests a role for VP22 (pU_L49) in viral cell-to-cell spread (Liang, Chow et al. 1995). VP22 (pU_L49)-deleted BoHV-1 is less virulent in cattle, which suggests a functional role of VP22 (pU_L49) in viral replication of BoHV-1 in the host (Elliott, Hafezi et al. 2005) . VP22 (pU_L49) associates with histone proteins and nucleosomes (Ren, Harms et al. 2001), however, the mechanism by which VP22 (pU_L49) plays a role in viral replication is not known. *In-silico* studies on BoHV-1 VP22 (pU_L49) did not detect a basic amino acid-rich classical NLS sequence (Zheng, Brownlie et al. 2005). Localisation of transiently expressed VP22 (pU_L49) with a fluorescent tag in the nucleus suggests that nuclear transport is independent from viral factors, and also indicates the presence of an atypical NLS, which is found in proteins that shuttle between the nucleus and the cytoplasm (Harms, Ren et al. 2000). A few studies have reported that the C-terminal region of BoHV-1 VP22 (pU_L49) is important for the nuclear localisation (Zhu, Qiu et al. 2005). Amino acids 130-232 of VP22 (pU_L49) bind to the H_4

histone, which facilitates nuclear retention of VP22 (pU$_L$49) (Zhu, Qiu et al. 2005). Studies also demonstrated the presence of nuclear targeting signals at amino acids 121-139 and a non-classical NLS at amino acids 130-133. The NES of BoHV-1 VP22 (pU$_L$49) is formed by amino acids 204-216 (Zheng, Brownlie et al. 2005). The position of the NLS and NES in BoHV-1 VP22 (pU$_L$49) is shown in Fig 1.8. VP22 (pU$_L$49) also contains a mitochondrial targeting signal in the C-terminal 49 amino acids. The mitochondrial targeting signal overlaps with the sequence required for nuclear targeting (Zhu, Qiu et al. 2005). Mitochondrial localisation suggests a role in apoptosis, but this is yet to be confirmed.

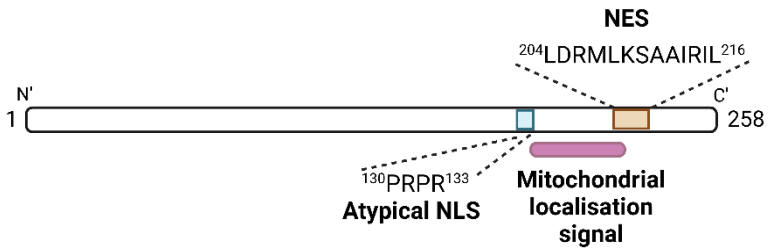

Figure 1.8. Schematic representation of the position of the NLS and NES in BoHV-1 VP22 (pU$_L$49).

VP22 (pU$_L$49) possesses a protein transport function and can carry proteins across the cell membrane without any other assistance (Yu, Xiao et al. 2012). VP22 (pU$_L$49) also plays an important role in the cell-to-cell spread of BoHV-1 and the gM (pU$_L$10)-independent incorporation of gN (pU$_L$49.5) into the mature virus during secondary envelopment of the virus (Pannhorst, Wei et al. 2018). An interaction between VP22 (pU$_L$49) and gM (pU$_L$10) has been confirmed; however further interactions with glycoproteins are yet to be elucidated (Pannhorst, Wei et al. 2018).

In cells transiently expressing BoHV-1 VP22 (pU$_L$49), an upregulation in the expression ratio of Bax to Bcl-2 was observed (more upregulation of Bax compared to Bcl-2), which suggests a function of VP22 (pU$_L$49) in induction of apoptosis (Qiu, Zhu et al. 2005, Kalthoff, Granzow et al. 2008) and in viral spread at the late stages of infection.

Additionally, in cells infected with VP22 (pU_L49)-deleted BoHV-1, a significant reduction in the plaque diameter was observed, which further supports the role of VP22 (pU_L49) in the cell-to-cell spread of BoHV-1 (Kalthoff, Granzow et al. 2008).

1.4.5.1 VP22 (pU_L49) homologues in other alpha herpesviruses

VP22 (pU_L49) is one of the most highly expressed and highly conserved tegument proteins in the alpha herpesviruses. However, VP22 (pU_L49) is absent from the beta and gamma herpesviruses (Kelly, Fraefel et al. 2009). The reason for the absence of VP22 (pU_L49), or a protein with similar functions, is unknown. The interactions of VP22 (pU_L49) with viral proteins, cellular proteins and chromatins play important roles in the viral life cycles. VP22 is encoded by the *U_L49* gene across most of the alpha herpesviruses (Wu, Cheng et al. 2012). In HHV-1, the *U_L49* gene is located between *U_L48* and *U_L 49.5*. *U_L49* in the same orientation as *U_L48* and is opposite to *$U_L49.5$* (Elliott and Meredith 1992). However, in BoHV-1 the orientation of these three genes is the same. In VZV, MDV and EHV-1, the homologue of VP22 (pU_L49) is encoded by *ORF9* and is designated ORF9p (Kennedy, Grinfeld et al. 2005).

In HHV-1 nearly 2000 copies of VP22 (pU_L49) are present in each virion particle (Heine, Honess et al. 1974). VP22 (pU_L49) is post-translationally modified (Knopf and Kaerner 1980, Hutchinson, Whiteley et al. 2002) and is phosphorylated by different kinases in different viruses. The phosphorylation of HHV-1 VP22 (pU_L49) is carried out by host CK2 and viral pU_L13 (Elliott, O'Reilly et al. 1996, Asai, Ohno et al. 2007), while the protein encoded by *ORF47* is responsible for the phosphorylation of VZV VP22 (pU_L49) (Riva, Thiry et al. 2013). In BoHV-1, viral pUS3 (and not pUL13) phosphorylates VP22 (pU_L49). HHV-1 VP22 (pU_L49) is present in both phosphorylated and non-phosphorylated forms in infected cells and is hypo-phosphorylated in the mature virus (Geiss, Tavis et al. 2001). For BoHV-1 the phosphorylation status of VP22 (pU_L49) in the mature virus is not known yet;

however, several forms of phosphorylated VP22 (pU_L49) are found in the infected cells (Lobanov, Zheng et al. 2010).

HHV-1 VP22 (pU_L49) is predominantly cytoplasmic in the early stages of infection and becomes nuclear at the late stages where it is highly phosphorylated (Meredith, Lindsay et al. 1991). When EHV-1 VP22 (pU_L49) is overexpressed in transfected cells it localises both in the nucleus and in the cytoplasm, but in EHV-1-infected cells the localisation of VP22 (pU_L49) is predominantly nuclear, which suggests an involvement of other viral factors (Okada, Kodaira et al. 2014). The NLS in EHV-1 VP22 (pU_L49) is between amino acids 154-188 (Okada, Kodaira et al. 2014). However, the subcellular localisation of MDV VP22 (pU_L49) is more diffuse in the cytoplasm as well as the nucleus (Dorange, El Mehdaoui et al. 2000). BoHV-1 VP22 (pU_L49) is a shuttling protein between the nucleus and cytoplasm and hence is nuclear as well as cytoplasmic (Lobanov, Zheng et al. 2010).

Like BoHV-1 VP22, HHV-1 and SuHV-1 VP22 (pU_L49) are not essential for virus replication in cell culture; however, the homologue of VP22 (pU_L49) (ORF9p) is indispensable for viral replication of VZV, MDV and EHV-1. ORF9p has histone and nucleosome binding activity, but its exact involvement in viral replication remains undiscovered (Dorange, Tischer et al. 2002, Che, Reichelt et al. 2008, Okada, Izume et al. 2015). In HHV-1 (similar to BoHV-1), the virulence of U_L49-deleted virus is impaired (Pomeranz and Blaho 2000, Elliott, Hafezi et al. 2005). In infected cells the Virion Host Shutoff protein encoded by *U_L41* is critically dependent on the presence of VP22 (pU_L49) for its translation (Su and Zheng 2017, He, Wang et al. 2020). Such an interaction between BoHV-1 VP22 (pU_L49) and pU_L41 is not known. Also, a drastic reduction in the amount of gD (pU_S6) and gB (pU_L27) was observed in U_L49-deleted HHV-1 (Duffy, Mbong et al. 2009). HHV-1 VP22 (pU_L49) forms a complex with gE (pU_S8) and gM (pU_L10) via a glycoprotein binding region. This glycoprotein-binding region is found to be conserved across alpha herpesviruses, which indicates an important role of the gE-VP22-gM complex (Mouzakitis, McLauchlan et al. 2005). Similar effect of BoHV-1 UL49 gene deletion on amount of glycoproteins is not clear. An interaction between BoHV-1 VP22 (pU_L49) with

gM (pU_L10) and gE (pU_S8) is also known (Pannhorst, Wei et al. 2018, Yezid, Pannhorst et al. 2020). VZV ORF9p accumulates around the trans-Golgi network, which suggests a role in the secondary envelopment of the virus (Che, Oliver et al. 2013, Riva, Thiry et al. 2013). EHV-1 VP22 (pU_L49) regulates the expression of the ICP4 early gene (Okada, Suganuma et al. 2018).

HHV-1 VP22 (pU_L49) plays a role in the cell-to-cell transmission of the virus by stabilising the microtubule system (Elliott and O'Hare 1998). A similar role of BoHV-1 VP22 (pU_L49) in cell-to-cell spread is known, but not clearly established for SuHV-1 or VZV. HHV-1 VP22 (pU_L49) also plays a role in the evasion of the host innate immune system by downregulation of IFN-β production (Huang, You et al. 2018). Participation of BoHV-1 VP22 (pU_L49), or SuHV-1 pUL49 or VZV ORF9p in evasion of the host immune system has not been studied yet.

1.4.6 Glycoproteins of BoHV-1

BoHV-1 encodes 11 glycoproteins, which are identified as gB (*U_L27*), gC (*U_L44*), gD (*U_S6*), gE (*U_S8*), gG (*U_S4*), gH (*U_L22*), gI (*U_S7*), gK (*U_L53*), gL (*U_L1*), gM (*U_L10*) and gN (*$U_L49.5$*) (Baranowski, Keil et al. 1996, Jones and Chowdhury 2010).

Glycoproteins gB (pU_L27), gC (pU_L44) and gD (pU_S6) play an essential role in viral attachment and entry into the host (Liang, Babiuk et al. 1991). gB (pU_L27) is a type 1 membrane protein, exists as a complex with gD (pU_S6) and is a major component of the BoHV-1 envelope. gB (pU_L27) has an approximate MW of 130 kDa and is cleaved into 74 kDa and 55k Da proteins, which are linked by di-sulfide bonds (van Drunen Littel-van den Hurk and Babiuk 1986). The 74-kDa subunit of gB (pU_L27) binds to heparin sulphate on the host cell surface and facilitates BoHV-1 entry (Li, Liang et al. 1996). gC (pU_L44) is a type 1 transmembrane protein, with an apparent MW of 91 kDa in its mature form, and exists as a homodimer in infected cells (van Drunen Littel-van den Hurk and Babiuk 1986, Liang, Babiuk et al. 1991). gC (pU_L44) also participates in viral entry by binding to heparin sulphate. Although gC (pU_L44) is not essential, deletion of gC (pU_L44) significantly

reduces viral replication (Liang, Babiuk et al. 1991). gD (pU_S6), a type 1 membrane protein, however, binds to non-heparin sulphate surface receptors and is essential for viral entry. gB (pU_L27) and gD (pU_S6) are involved in fusion of proteins during viral entry rendering both essential for BoHV-1 (Liang, Babiuk et al. 1991, Fehler, Herrmann et al. 1992). The cleavage of gB (pU_L27) contributes to cell-to-cell spread of BoHV-1. Blocking of the cleavage site of gB (pU_L27) results in the reduction of the plaque size of BoHV-1 (Kopp, Blewett et al. 1994). Studies have shown that intramuscular immunization with gB (pU_L27), gC (pU_L44) and/or gD (pU_S6) leads to production of high levels of neutralizing antibodies which protect cattle from BoHV-1 infection (van Drunen Littel-van den Hurk, Gifford et al. 1990, Alves Dummer, Pereira Leivas Leite et al. 2014).

gE (pU_S8) and gI (pU_S7) are conserved in all alpha herpesviruses and are type 1 membrane proteins. These are non-essential glycoproteins as they are not required in the replication of virus in cell cultures (Whitbeck, Knapp et al. 1996). gE (pU_S8) and gI (pU_S7) form a complex as soon as they are expressed, and this complex is retained as a heterodimer during its incorporation into BoHV-1. The gE (pU_S8)-gI (pU_S7) complex is required for cell to cell spread as the plaque size of gE (pU_S8)-gI (pU_S7) deletion mutants is reduced (Yoshitake, Xuan et al. 1997). Inspite of the complex formation by gE (pU_S8) and gI (pU_S7), the incorporation of gE (pU_S8) does not require gI (pU_S7). In contrast, gE (pU_S8) is essential for the packaging of gI (pU_S7) into BoHV-1, as extracellular release of un-incorporated gI (pU_S7) into the medium was detected during infection with gE (pU_S8)-deleted BoHV-1 (Whitbeck, Knapp et al. 1996). On reactivation from latency, the C-terminal region of gE plays a crucial role in the anterograde transport of BoHV-1 (Brum, Coats et al. 2009, Yezid, Pannhorst et al. 2020). Calves vaccinated with gE-deleted virus do not shed virus in the nasal secretions after latency re-activation due to a defective anterograde transport (Romera, Puntel et al. 2014). High immunogenicity of gE (pU_S8) and attenuation of virus in its absence make gE (pU_S8) a potent antigenic marker (Kaashoek, Rijsewijk et al. 1998). A gE (pU_S8)-deleted virus serves as a DIVA or marker vaccine, because the vaccinated animals lack antibody against gE, Therefore, they are easily

distinguishable from cattle infected with wild-type virus (Strube, Abar et al. 1995, Turin and Russo 2003, Romera, Puntel et al. 2014). The gE-deleted marker vaccine elicits protective immunity against the virus, and hence is the only vaccine (not live modified) allowed in European countries where BoHV-1 has been eradicated (Nuotio, Neuvonen et al. 2007, Raaperi, Orro et al. 2014).

gG (pU_S4) of BoHV-1 is a secreted glycoprotein and blocks the interaction of chemokines to their receptors by binding to the chemokine itself (Nakamichi, Matsumoto et al. 2002, Bryant, Davis-Poynter et al. 2003), so gG (pU_S4) plays a role in immune evasion of BoHV-1. Consequently, a gG (pU_S4)-deleted BoHV-1 is less virulent. gG (pU_S4) also participates in construction of a junctional adherence between BoHV-1 infected cells and hence aids in cell-to-cell spread (Nakamichi, Ohara et al. 2000, Nakamichi, Matsumoto et al. 2002). gG (pU_S4) participates in establishment of infection by functioning as an anti-apoptotic viral factor. Similar to gE (pU_S8), gG (pU_S4) also has great potential to be employed as a selective marker in live vaccines (Kaashoek, Rijsewijk et al. 1998).

gH (pU_L22), a type 1 membrane protein is an essential glycoprotein as it functions in entry of BoHV-1 (Nakamichi, Kuroki et al. 2001). Proper processing and transport of gH (pU_L22) requires heterodimer formation with another type 1 transmembrane glycoprotein, gL (pU_L1) (Khattar, van Drunen Littel-van den Harke et al. 1996). The gH (pU_L22)-gL (pU_L1) complex induces a neutralizing antibody response and facilitates the anchoring of gL (pU_L1) to the plasma membrane (van Drunen Littel-van den Hurk, Khattar et al. 1996). Is critical for fusion between the envelope of BoHV-1 and the plasma membrane of the host cell (Fitzpatrick, Zamb et al. 1990, Baranowski, Dubuisson et al. 1993). gK (pU_L53) is another type III transmembrane essential glycoprotein for BoHV-1 with a molecular mass of 36 kDa. gK (pU_L53) contains four transmembrane domains and a cleavable signal at the N-terminal end. gK (pU_L53) interacts with U_L20 and is critical for virus production and spread (Robinson, Meers et al. 2008). Table 1.2 shows a comprehensive list of BoHV-1 glycoproteins and their functions

Table 1.2 Comprehensive list of BoHV-1 glycoproteins and their functions.

Glycoproteins	**Essential/ Non-essential**	**Functions**	**References**
gB (pU_L27)	Essential	Binds to heparin sulfate and facilitates viral entry.	(Li, Liang et al. 1996)
gC (pU_L44)	Non- essential	Binds to heparin sulfate and facilitates viral entry	(Liang, Babiuk et al. 1991)
gD (pU_S6)	Essential	Facilitates viral entry by binding to nectin 1 and 2.	(Liang, Babiuk et al. 1991, Fehler, Herrmann et al. 1992)
gE (pU_S8)	Non- essential	Forms a complex with gI and plays a role in cell-to-cell spread.	(Yoshitake, Xuan et al. 1997)
gG (pU_S4)	Non- essential	Plays a role in immune evasion.	(Bryant, Davis-Poynter et al. 2003)
gH (pU_L22)	Essential	Forms a heterodimer with gL and plays a role in viral entry.	(Fitzpatrick, Zamb et al. 1990, Baranowski, Dubuisson et al. 1993)
gI (pU_S7)	Non- essential	Forms a complex with gE and plays a role in cell-to-cell spread.	(Yoshitake, Xuan et al. 1997)
gK (pU_L53)	Essential	Critical for virus production and spread.	(Robinson, Meers et al. 2008)
gL (pU_S1)	Essential	Forms a heterodimer with gH and plays a role in viral entry.	(Fitzpatrick, Zamb et al. 1990, Baranowski, Dubuisson et al. 1993)
gM (pU_L10)	Non- essential	Forms a complex with gN and plays a role in cell-to-cell spread and virus maturation.	(Pannhorst, Wei et al. 2018, Li, Wang et al. 2021)
gN ($pU_L49.5$)	Non- essential	Forms a complex with gM and plays a role in cell-to-cell spread and virus maturation.	(Pannhorst, Wei et al. 2018, Li, Wang et al. 2021)

1.4.6.1 Glycoprotein M (pU_L10) of BoHV-1

Glycoprotein M (pU_L10) is a surface virion component with multiple trans-membrane domains and is a type III membrane protein (Fig 1.7), which is conserved among alpha herpesviruses. Glycoprotein M (pU_L10) of BoHV-1 is a 46 kDa protein, which is translated by the *U_L10* gene and is dispensable for the replication of the virus *in vitro* as well as *in vivo* (Wu, Zhu et al. 1998). During the intermediate stages of infection, gM (pU_L10) recruits other proteins to the appropriate compartments to facilitate primary and secondary envelopment of the virus, and later during infection, gM (pU_L10) promotes viral egress (Li, Wang et al. 2021). Glycoprotien M (pU_L10) also plays a role in the maturation of the virus as it plays a role in the incorporation of other glycoproteins or tegument proteins (Jahn and Scheller 2006, van den Bogaart, Holt et al. 2010, Pannhorst, Wei et al. 2018). The proper processing of gM (pU_L10) requires the formation of a complex with gN ($pU_L49.5$) (9 kDa) through di-sulfide bonds. The leucine zipper motifs present in the transmembrane region of gM (pU_L10) is critical for the maturation of the gM (pU_L10) /gN ($pU_L49.5$) complex in the endoplasmic reticulum (Graul, Kisielnicka et al. 2019).

1.4.6.2 Glycoprotein M (pU_L10) of other alpha herpesviruses

Glycoprotein M (pU_L10), conserved across alpha herpesviruses, plays a role in the modulation of virus-induced membrane fusion during virus infection. gM (pU_L10) also facilitates the transport of proteins to the appropriate intracellular membranes for the virus maturation, as well as virus egress. gM (pU_L10) interacts with various viral and cellular proteins and plays a role in the viral replication cycle by a complex mechanism (Li, Wang et al. 2021). gM (pU_L10) is encoded by different ORFs in different viruses. In HHV-1 (McGeoch, Dalrymple et al. 1988), HHV-2 (Dolan, Jamieson et al. 1998), ILTV (Veits, Lüschow et al. 2003), MDV (Cai, Jang et al. 1999) and BoHV-1 (Wu, Zhu et al. 1998) gM is encoded by the U_L10 gene, whereas ORF50 and ORF52 encode gM in VZV (Davison and Scott 1986, Sadaoka, Yanagi et al. 2010) and EHV-1 (Telford, Watson et al. 1992), respectively. In different alpha herpesviruses gM (pU_L10) homologues play certain similar

roles in the viral life cycle, but differ in importance (Browne, Bell et al. 2004). In HHV-1 (Baines and Roizman 1991), SuHV-1 (Dijkstra, Visser et al. 1996), EHV-1 (Osterrieder, Neubauer et al. 1996, Shakya, O'Callaghan et al. 2017), ILTV (Fuchs and Mettenleiter 1999) and BoHV-1 (König, Giesow et al. 2002) gM (pU_L10) is non-essential for viral replication while in MDV gM (pU_L10) is essential for viral replication (Tischer, Schumacher et al. 2002). In HHV-1, gM (pU_L10) and gE (pU_S8) both are non-essential glycoproteins, which function redundantly in viral secondary envelopment; hence, a simultaneous deletion of both of these genes is detrimental for the virus (Mettenleiter, Müller et al. 2013).

HHV-1 gM (pU_L10) is predicted to consist of eight membrane-spanning regions (Fig 1.9) (Van Cleemput, Poelaert et al. 2020). It is expressed as a 47-kDa precursor in the endoplasmic reticulum. gM (pU_L10) exists as a complex with gN ($pU_L49.5$) connected by covalent di-sulfide bonds in HHV-1 (Baines and Roizman 1991), SuHV-1 (Li, Zhuang et al. 2021) and BoHV-1 (Pannhorst, Wei et al. 2018). During maturation, gM (pU_L10) is modified with complex N-linked oligosaccharides resulting in a glycosylated protein with an approximate molecular weight of 53-63 kDa (Baines and Roizman 1991, Baines and Roizman 1993). During viral entry gM (pU_L10) promotes membrane fusion induced by gB (pU_L27), gC (pU_L44), gD (pU_S6) and gH (pU_L22) /gL (pU_S1) in order to release the nucleocapsid into the cytoplasm, but gM (pU_L10) is not an essential protein for virus entry (El Kasmi and Lippé 2015). The C-terminus of gM (pU_L10) contains trafficking motifs responsible for transfer of viral proteins to the proper sites within the cell (Striebinger, Zhang et al. 2015, Striebinger, Funk et al. 2016). Similar trafficking motifs are present in gM (pU_L10) of VZV (Zerboni, Sung et al. 2018); however, such motifs in BoHV-1 are yet to be identified. The N-terminal region of gM (pU_L10) plays a role in virus maturation (Crump, Bruun et al. 2004). HHV-1 gM (pU_L10) is recruited to the nuclear membrane by pU_L31 and pU_L34 (Wills, Mou et al. 2009, Zhang, Nagel et al. 2009) and later interacts with exportin XPO6 for the nuclear egress. Eventually, the localisation of gM (pU_L10) is at the Golgi (Zhang, Nagel et al. 2009). In HHV-1 infected cells the packaging of gM

(pU$_L$10) takes place at the nuclear membrane during primary tegumentation (Baines, Wills et al. 2007). The gM (pU$_L$10)/gN (pU$_L$49.5) complex also interacts with soluble N-ethylmaleimide-sensitive factor attachment protein receptors (v-SNARE) and vesicle-associated membrane protein-3 (VAMP3) to facilitate the transport of the protein complex between membrane-enclosed organelles (Kawabata, Serada et al. 2014, Wang, Li et al. 2017).

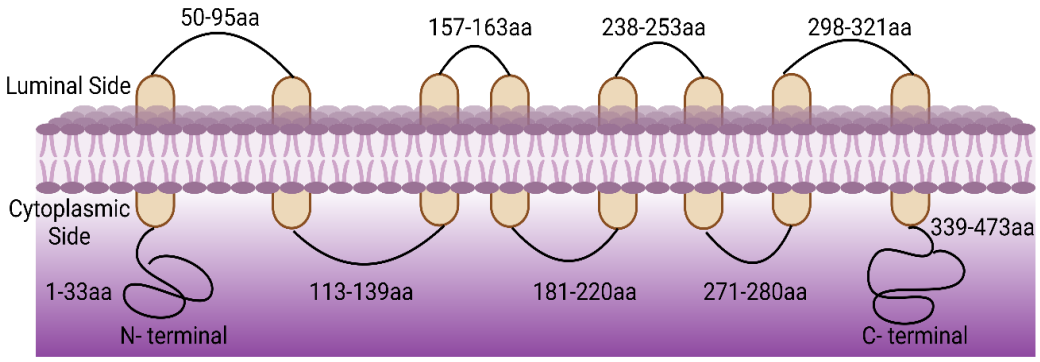

Figure 1.9. Membrane spanning motifs of HHV-1 glycoprotein M (pU$_L$10) as predicted by PSORT- II

During HHV-1 infection gM (pU$_L$10) interacts with various cellular proteins to promote virus-induced membrane fusion for its entry (El Kasmi and Lippé 2015). However, the role of BoHV-1 gM (pU$_L$10) in viral entry is unknown. gM (pU$_L$10) also participates in the transport of the viral proteins to the appropriate site for their incorporation into the virus (El Kasmi and Lippé 2015). For example, transiently expressed HHV-1 gN (pU$_L$49.5) was retained in the endoplasmic reticulum in the absence of expression of gM (pU$_L$10). However, in cells transiently expressing both gM (pU$_L$10) and gN (pU$_L$49.5), localisation of gN (pU$_L$49.5) to the Golgi was observed (El Kasmi and Lippé 2015, Striebinger, Funk et al. 2016). This suggests a requirement of gM (pU$_L$10) -gN (pU$_L$49.5) complex formation for the proper processing and incorporation of both gM (pU$_L$10) and gN (pU$_L$49.5). The

expression of gM (pU_L10) in HHV-1 infected cells leads to the incorporation of proteins, such as gD (pU_S6) and the gH (pU_L22) /gL (pU_S1) complex, which do not contain transport signal sequences, into the virus particle (Zhang, Nagel et al. 2009, Ren, Bell et al. 2012, Lau and Crump 2015). This indicates that gM (pU_L10) is critical for efficient morphogenesis of HHV-1 and recruitment of proteins (devoid of transport signals) to the site of incorporation. SuHV-1 gM (pU_L10) also to plays a role in the secondary envelopment of the virus (Brack, Dijkstra et al. 1999). Overall, gM (pU_L10) is conserved within the alpha herpesvirus family and plays an important role in virus morphogenesis by recruiting viral proteins to the site of incorporation (Golgi). This suggests that BoHV-1 gM (pU_L10) may also play a role in recruitment of proteins to the site of incorporation or secondary involvement (Golgi).

The herpesvirus tegument proteins are important to the virus and are often multifunctional. Therefore, an understanding of the incorporation of the tegument proteins at different stages of viral morphogenesis is important. VP8 (pU_L47) plays multiple roles in the viral replication process of BoHV-1. The phosphorylation status of VP8 (pU_L47) and its regulation of the functions of VP8 (pU_L47) are also well understood. However, the site(s) and mechanisms of VP8 (pU_L47) incorporation into the virion are unclear. In this study, the focus is on elucidating the stages of VP8 (pU_L47) incorporation into the virion, as well as protein-protein interactions (tegument-tegument or tegument-glycoprotein) regulating the process of VP8 (pU_L47) incorporation.

CHAPTER-2

2. HYPOTHESIS AND OBJECTIVES

2.1 Rationale and Hypothesis

VP8 (pU_L47) is the most abundant tegument protein of BoHV-1. It is essential for BoHV-1 infection in cattle and plays various roles in replication of the virus in cell culture. The virus titer is significantly reduced when cells are infected with a virus lacking VP8 (pU_L47). VP8 (pU_L47) contains NLSs and hence localises into the nucleus. Later during infection, pU_S3-phosphorylated nuclear VP8 (pU_L47) is translocated to the cytoplasm from where it localises to the Golgi and is packaged into the mature virus. However, if VP8 (pU_L47) is not phosphorylated, it remains in the nucleus, and hence does not localize to the Golgi and is not packaged into the virions at the Golgi. Despite staying nuclear, approximately 30% VP8 (pU_L47) ends up in the mature virus of BoHV-1 encoding non-phosphorylated VP8 (pU_L47). This indicates a partial packaging of VP8 (pU_L47) at early stages of maturation in the perinuclear region.

In cells co-transfected with a plasmid expressing viral kinase pU_S3 and a plasmid expressing VP8 (pU_L47), the nuclear VP8 (pU_L47) acquires the ability to become cytoplasmic, but is unable to localise to the Golgi. This suggests an involvement of a viral factor in the translocation of VP8 (pU_L47) from the cytoplasm to the Golgi for its final packaging.

We therefore hypothesise a partial packaging of VP8 (pU_L47) at early stages of viral maturation in the perinuclear space and involvement of a viral factor (presumably a viral glycoprotein) in the transport of VP8 (pU_L47) from the cytoplasm to the Golgi, which aids in the final packaging of VP8 (pU_L47) into the mature virus.

2.2 Objectives

- To investigate the incorporation of VP8 (pU_L47) during the primary envelopment process and to identify the protein-protein interactions critical for this process.

- To identify the viral factor (presumably a glycoprotein) involved in the Golgi localisation and hence final packaging of VP8 (pU_L47).

CHAPTER- 3

3. VP8 (pU_L47), THE MAJOR TEGUMENT PROTEIN OF BOVINE HERPESVIRUS-1, IS PARTIALLY PACKAGED DURING EARLY TEGUMENT FORMATION IN A VP22 (pU_L49)-DEPENDENT MANNER.

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Keywords: BoHV-1; major tegument protein; early tegument; tegument formation; virus maturation

3.1 Permission to use

3.2 Author's Contribution

All of the experiments were planned, performed, analyzed, and interpreted by Soumya Sucharita . The BoHV-1YmVP8 used in Fig 3.8 and 3.9 were constructed by Dr. Kuan Zhang. The manuscript is written and edited by Ms. Soumya Sucharita and Dr Sylvia van den Hurk.

3.3 Abstract

Bovine herpesvirus-1 (BoHV-1) is a major cause of rhinotracheitis and vulvovaginitis in cattle. VP8 (pU_L47), the major tegument protein of BoHV-1, is essential for viral replication in the host. VP8 (pU_L47) is phosphorylated by the viral kinase pU_S3, mediating its translocation to the cytoplasm. VP8 (pU_L47) remains nuclear when not phosphorylated. Interestingly, VP8 (pU_L47) has a significant presence in mature BoHV-1YmVP8, in which the VP8 (pU_L47) phosphorylation sites are mutated. This suggests that VP8 might be packaged during primary envelopment of BoHV-1. This was investigated by mass spectrometry and Western blotting, which showed VP8 (pU_L47), as well as VP22 (pU_L49), to be constituents of the primary enveloped virions. VP8 (pU_L47) and VP22 (pU_L49) interaction is shown via co-immunoprecipitation experiments, in both BoHV-1-

infected and VP8 (pU_L47)-transfected cells. VP8 (pU_L47) and VP22 (pU_L49) also co-localised with one another and with nuclear lamin-associated protein 2 in BoHV-1-infected cells, suggesting an interaction between VP8 (pU_L47) and VP22 (pU_L49) in the perinuclear region. In cells infected with VP22 (pU_L49)-deleted BoHV-1 (BoHV-1ΔU_L49), VP8 (pU_L47) was absent from the primary enveloped virions, implying that VP22 (pU_L49) is critical for the early packaging of VP8 (pU_L47). In conclusion, a novel VP22 (pU_L49)-dependent mechanism for packaging of VP8 (pU_L47) was identified, which may be responsible for a significant amount of VP8 (pU_L47) in the viral particle.

3.4 Introduction

Bovine herpesvirus-1 (BoHV-1) belongs to the family Alphaherpesvirinae and is a major cause of bovine rhinotracheitis and vulvovaginitis (Jones 2019); it also causes reduced milk production as well as infertility in cows and, hence, adversely affects the dairy industry (Lucchese, Benkirane et al. 2016). The structure of BoHV-1 includes a proteinaceous 125 nm diameter capsid, which packages the double-stranded DNA of about 135 kb; the capsid is covered by a tegument, which consists of approximately 20 proteins (Haanes, Thomsen et al. 1995). A lipid envelope forms the outermost shell of the virus, and contains glycoproteins (Lobanov, Maher-Sturgess et al. 2010). Tegument proteins are responsible for diverse functions, and around 11 tegument proteins are conserved across alphaherpesviruses along with their interactions (Owen, Crump et al. 2015, Metrick, Koenigsberg et al. 2020). The life cycle of human herpesvirus-1 (HHV-1) and BoHV-1 starts by entry into the host cell via receptor-mediated endocytosis, which involves fusion of the viral envelope and the endocytic membrane due to interaction between the host cell molecules and glycoproteins (Mettenleiter, Klupp et al. 2006, Muylkens, Thiry et al. 2007).

During entry into the host cell, HHV-1 and BoHV-1 lose the lipid envelope, and shed some of their tegument proteins. The capsid then releases the viral DNA into the nucleus through the nuclear pore complex, where the DNA replicates via a rolling circle mechanism (Homa and Brown 1997, Muylkens, Thiry et al. 2007). The newly formed DNA

is packaged into the capsid inside the nucleus, which is ready to exit the nucleus via a budding process (Muylkens, Thiry et al. 2007, Crump 2018). During egress, the capsid gains a primary envelope from the inner nuclear membrane, and buds into the perinuclear space (Muylkens, Thiry et al. 2007, Mettenleiter, Klupp et al. 2009). It is known for HHV-1 that a few tegument proteins are also packaged at this stage of primary envelopment (Granzow, Klupp et al. 2001). Subsequently, the primary enveloped virus particle fuses with the outer nuclear membrane and loses the primary envelope, while retaining some of the tegument proteins, and buds out of the nucleus (Mettenleiter 2004). As the virus particle proceeds through the cytoplasm towards its site of maturation at the Golgi, the tegument undergoes changes in its composition due to loss and/or addition of proteins (Granzow, Klupp et al. 2001). At the Golgi, the final maturation of the virus takes place, after the addition of the lipid envelope and the glycoproteins (Mettenleiter, Klupp et al. 2009).

VP8 (pU_L47), the major tegument protein of BoHV-1, is a product of the *U_L47* gene (Carpenter and Misra 1991), and plays a versatile role in viral replication (Lobanov, Maher-Sturgess et al. 2010), induction of humoral and cell-mediated immunity (van Drunen Littel-van den Hurk, Garzon et al. 1995), alteration of host defense mechanisms (Zhang, Afroz et al. 2015), and cell death or apoptosis (Afroz, Garg et al. 2018). It is a late protein, and is conserved throughout the alphaherpesvirus family (Carpenter and Misra 1991). The requirement for VP8 (pU_L47) during BoHV-1 replication has been inferred from a 1000-fold reduction in viral titre in cells, and the total inability of a VP8-deleted BoHV-1 (BoHV-1ΔU_L47) to replicate in cattle upon infection. In addition, the amounts of glycoprotein D (gD) (pU_S6) and tegument protein VP22 (pU_L49) were significantly reduced in the mature BoHV-1ΔU_L47 (Lobanov, Maher-Sturgess et al. 2010). Early during infection with HHV-1 and BoHV-1, the *U_L47*-encoded protein localises to the nucleus (Donnelly and Elliott 2001, Verhagen, Hutchinson et al. 2006), which is mediated by nuclear localisation signals (NLSs). At later stages of infection, BoHV-1 VP8 (pU_L47) and HHV-1 VP13/14 (pU_L47) are exported, which is consistent with the presence of nuclear export signals (NESs) (Donnelly and Elliott 2001, Zheng, Brownlie et al. 2004). VP8 (pU_L47) undergoes

phosphorylation in the cytoplasm by host casein kinase 2 (CK2), and in the nucleus by a viral kinase the unique short protein 3 (pU_S3). This phosphorylation aids in many cellular functions of VP8 (pU_L47), including host defense modulation and export of VP8 (pU_L47) from the nucleus to the cytoplasm. When a mutant (BoHV-1YmVP8), in which the phosphorylation sites were replaced by alanine, was studied, it was found that VP8 (pU_L47) remained nuclear (Zhang, Donovan et al. 2019), and in the mature BoHV-1YmVP8 virus particles VP8 (pU_L47) was present in a reduced, but still significant, amount (Zhang, Brownlie et al. 2016). There was also a 100-fold reduction in the titer of BoHV-1YmVP8, which might be due to a substantial reduction in VP8 (pU_L47) in the mature virus.

The fact that YmVP8 remained nuclear, and yet was present in the mature virion, led us to hypothesize that early packaging of VP8 (pU_L47) occurs during the primary tegumentation process. We determined that VP8 (pU_L47) is a part of the primary tegument of the primary enveloped virus, along with VP22 (pU_L49), a small tegument protein translated by the *U_L49* gene (Liang, Chow et al. 1995). During infection, BoHV-1 and HHV-1 VP22 (pU_L49) localise into the nucleus with the help of an NLS present at the C-terminus (Ren, Harms et al. 2001, Schlegel and Blaho 2009); however, other subcellular localisations are slightly different in HHV-1 and BoHV-1 (Harms, Ren et al. 2000). We demonstrated that VP8 (pU_L47) and VP22 (pU_L49) interact and co-localize in the perinuclear region in cells infected with WT BoHV-1 or BoHV-1-YmVP8. VP22 (pU_L49) was shown to be critical for the packaging of VP8 (pU_L47) at the early tegumentation stage, as in VP22 (pU_L49)-deleted virus (BoHV-1ΔU_L49)-infected cells the primary enveloped virions were found to be devoid of VP8 (pU_L47).

3.5 Materials and Methods

3.5.1 Cell Lines and Viruses

COS-7 and Madin–Darby bovine kidney (MDBK) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and Eagle's minimum essential medium (MEM; Sigma-Aldrich Canada Ltd.), respectively,

supplemented with 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, Gibco, Life Technologies, Burlington, ON, Canada), 1% nonessential amino acids (Gibco, Life Technologies), 50 µg/mL gentamycin (Gibco, Life Technologies), and 10% fetal bovine serum (FBS; Gibco, Life Technologies), with 5% CO2 in a 37°C incubator.

WT BoHV-1, BoHV-1YmVP8, in which all of the phosphorylation sites were mutated (Zhang, Brownlie et al. 2016), and BoHV-1ΔU_L49, a VP22 (pU_L49)-deleted mutant (Liang, Chow et al. 1995) were propagated and titred on MDBK cells in 24-well plates, followed by overlay with 0.8% UltraPure low melting-point agarose (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) in MEM, and stocks were maintained at -80°C.

For viral infections, 85–90% confluent monolayers of MDBK cells were infected with BoHV-1, BoHV-1-YmVP8, or BoHV-1ΔU_L49 at different multiplicities of infection (MOIs) in MEM. Virus was replaced after 1.5 h with MEM containing 2% FBS. Cells were collected at different time points post-infection.

3.5.2 Plasmids and Antibodies

The plasmid pFLAG-VP8 was previously described in (Zhang, Afroz et al. 2015). pHA-VP22 was constructed by amplifying the *U_L49* gene from the BoHV-1 genome using 3'ATTGAATTCATGGCCCGGTTCCACAGG 5' and 3'ATTTCTAGAATTTTCATACTAGCA CAGCA 5' primers, followed by ligation with an XbaI- and EcoRI-digested pcDNA3.1HA (Addgene, Watertown, MA, USA). Plasmid DNA was sequenced at the NRC Plant Biotechnology Institute (Saskatoon, SK, Canada).

Antibodies including mouse monoclonal VP8 (pU_L47)-specific antibody (clone 1G4 2G2), rabbit polyclonal VP22 (pU_L49)-specific antibody, and rabbit polyclonal VP5-specific antibody were described previously in (Liang, Chow et al. 1995, van Drunen Littel-van den Hurk, Garzon et al. 1995). FLAG tag-specific mouse monoclonal antibodies (Sigma- Aldrich Canada Ltd.) and HA tag-specific rabbit polyclonal antibodies (Cell Signaling Technology, Danvers, MA, USA) were used to detect the respective proteins. Mouse monoclonal antibodies specific to lamin-associated proteins A and C (LAP2) were

purchased from Abcam Inc. (Toronto, ON, Canada). Mouse monoclonal antibodies specific to nucleolin, and rabbit polyclonal antibodies specific for tubulin, were purchased from Sigma-Aldrich Canada Ltd. For Western blotting, IRDye 680RD goat anti-rabbit IgG, IRDye 680RD goat anti-mouse IgG, IRDye 800RD goat anti-rabbit IgG, and IRDye 800RD goat anti-mouse IgG (Li-Cor Biosciences, Lincoln, NE, USA) were used. Alexa 488-conjugated goat anti-mouse IgG, Alexa 488-conjugated goat anti-rabbit IgG, Alexa 633-conjugated goat anti-mouse IgG, and Alexa 633-conjugated goat anti-rabbit IgG (Invitrogen/Thermo Fisher Scientific) were used for immunofluorescent staining.

3.5.3 Isolation of Nuclear Membrane

The procedure for the isolation of the nuclear membrane was derived from a procedure applied to HHV-1-infected COS-7 cells (Padula, Sydnor et al. 2009). MDBK cells were infected at an MOI of 10 with WT BoHV-1 or BoHV-1ΔU_L49 and incubated at 37 °C for 8 h. Cells were collected in sucrose buffer A (10 mM Tris-HCl pH 7.4, 0.32 M sucrose, 3 mM MgCl2) and centrifuged at 1700×g for 10 min, followed by resuspension of the pellets in fresh sucrose buffer A with 60 µL/mL of protease inhibitor cocktail (Sigma-Aldrich Canada Ltd.). Cells were manually disrupted using a 25G × 5/8”-gauge needle; the resulting suspension was termed the total cell extract (TCE). The TCE was centrifuged for 10 min at 700× g, and the nuclei in the pellet were resuspended in sucrose buffer B (10 mM Tris-HCl pH 7.4, 2.4 M sucrose, 3 mM MgCl2) and centrifuged at 50,000× g for 1 h at 4°C. The resulting pellet contained the purified nuclei, which were washed and resuspended in 1 mM DNase and digestion buffer (10 mM Tris-HCl pH 8.5, β-mercaptoethanol, 0.3 M sucrose), and then incubated for 20 min at room temperature. The washed nuclei were centrifuged at 40,000× g for 15 min, and the digestion was repeated for 1 h, followed by centrifugation at 40,000× g for 15 min. The resulting pellet contained the nuclear membrane and was called the nuclear membrane preparation (NMP) and resuspended in sucrose buffer C (10 mM Tris-HCl pH 7.4, 0.25 M sucrose, 3 mM MgCl2).

3.5.4 Isolation of Primary Enveloped Virions

The NMP was sonicated (5 pulses of 10 s on and 20 s off) in order to release the primary enveloped virions from the perinuclear space. The suspension was incubated with 50 µg/mL of proteinase K (Sigma-Aldrich, Canada Ltd.) for 1 h on ice, and quenched with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, Canada Ltd.). The suspension was then overlaid on a 30% sucrose cushion (Sigma-Aldrich, Canada Ltd.) (in 10 mM Tris-HCl, (pH 7.4)) and centrifuged at 40,000× g for 1 h at 4 °C. The pellet containing the primary enveloped virions was resuspended in sucrose buffer C.

3.5.5 Purification of Mature Virus

MDBK cells were infected with WT BoHV-1 or BoHV-1ΔU_L49 at an MOI of 10, and culture medium was collected 18 h post-infection. The medium was clarified at 3000× g for 20 min to remove any cell debris. The supernatant was collected and overlaid over 30% sucrose (in TNE (10 mM Tris-HCl pH 7.4, 1.5 mM NaCl, 1 mM EDTA)), and was centrifuged for 2 h at 25,000× g and 4 °C. The resulting viral pellet was solubilised at 4 °C in TNE overnight. The virus was layered over a 20–60% sodium potassium tartrate gradient and centrifuged for 90 min at 28,000× g and 4 °C; this step was performed twice. The virus band was collected, diluted in TNE, and centrifuged at 25,000× g and 4°C for 1 h. The resulting pellet was resuspended in TNE and stored at -80 °C until further use.

3.5.6 Gel Electrophoresis

Twenty microliters of both the primary enveloped virus particles and the purified mature virions were run on an 8% SDS-polyacrylamide gel after being boiled in SDS loading dye at 95 °C for 3 min. The gel was stained with ProtoBlue Safe (National Diagnostic, Thermo Fisher Scientific) and de-stained in distilled water.

3.5.7 Mass Spectrometry Sample Preparation and Analysis

The viral samples purified from culture medium (mature) and the perinuclear space (primary enveloped) were precipitated using -20°C cold acetone. Two sets of samples from two different infections were sent to the Mass Spectrometry Facility at the University of Missouri (Columbia, MI, USA). Acetone-precipitated samples from the mature and primary enveloped virus particles were washed and resuspended with 6 M urea, 2 M thiourea, and 100 mM ammonium bicarbonate. In-solution digestion was carried out overnight and then stopped after 16 h. The samples were then zip-tipped, lyophilised, resuspended, and loaded into the LTK Orbitrap XL (Thermo Fisher Scientific). Subsequently, proteins were identified using computer software against the NCBI Herpesvirus Database.

3.5.8 Transfections

Monolayers of COS-7 cells at 80–90% confluency were transfected with a total of 3 µg of plasmid (pFLAG-VP8, pVP22-HA, or pFLAG-VP8 + pVP22-HA) using Lipofectamine LTX transfection reagent (Invitrogen/Thermo Fisher Scientific), and cells were collected 24–48 h post-transfection.

3.5.9 Preparation of Cell Lysates

Infected MDBK cells or transfected COS-7 cells were collected using ice-cold phosphate-buffered saline at pH 7.4 (PBS) (Gibco, Life technologies) after various time intervals, and then centrifuged at 8000× g for 10 min. Cell pellets were subjected to lysis in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1% Triton-X100, pH 7.4) and protease inhibitor cocktail (Sigma-Aldrich Canada Ltd.) at a 10:1 v/v ratio for 40 min on ice. Cell lysates were clarified by centrifugation at 12,000× g for 10 min at 4 °C. The supernatants were collected and stored at -20°C for further use.

3.5.10 Immunoprecipitation and Western Blotting

Infected cell lysates (prepared as described above) were incubated with VP8 (pU_L47)-specific or VP22 (pU_L48)-specific antibodies overnight at 4°C, followed by Protein G Sepharose Fast Flow beads (GE HealthCare, Niskayuna, NY, USA) for 3 h at 4°C. Transfected cell lysates were incubated with Anti-FLAG M2 Affinity Gel (Sigma-Aldrich Canada Ltd.) or anti-HA agarose (Pierce/Thermo Fisher Scientific) and incubated at 4°C overnight. Lysates were washed 3–4 times with wash buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 8) and eluted with SDS loading dye by boiling at 95°C for 2 min. SDS loading dye was also added to a fraction of the whole cell lysates, which were then boiled at 95 °C for 2 min. These samples were subjected to SDS-polyacrylamide gel electrophoresis on 8% gels, and transferred to 0.45 µm nylon membranes (Bio-Rad, Hercules, CA, USA). This was followed by blocking the membrane with 3% blotto (3 g milk powder in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% w/v Tween 20, pH 7.4)) for 1 h, and incubation with the corresponding antibodies overnight. The next day, the membranes were washed with TBST and incubated with IRDye 680RD goat anti-mouse IgG or IRDye 800RD goat anti-rabbit IgG (LI-COR Bioscience, Lincoln) for 2 h. An Odyssey Infrared Imaging System and Odyssey 3.0.16 application software (LI-COR Biosciences) were used for detection and analysis.

3.5.11 Confocal Microscopy

Cells were seeded in 2-well Permanox Lab-Tek chamber slides (Nunc, Thermo Fisher), and were infected at 80–90% confluency with BoHV-1, BoHV-1YmVP8, or BoHV-1ΔU_L49 at various MOIs. Cells were fixed at different times post-infection with 4% paraformaldehyde (Sigma-Aldrich Canada Ltd.) and washed with phosphate-buffered saline (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS)). After washing, the cells were blocked overnight with 1% goat serum. The following day, the cells were treated with 0.1% Triton X-100 to permeabilize them. Subsequently, they were incubated with the corresponding primary antibodies prepared in 1% goat serum for

2–3 h, followed by Alexa 488-conjugated goat anti-rabbit or goat anti-mouse IgG, Alexa 633-conjugated goat anti-rabbit or goat anti-mouse IgG, or both Alexa 488- and Alexa 633-conjugated IgG (Invitrogen, Thermo Fisher), for 1–2 h at room temperature. Prolong Gold with DAPI (Invitrogen, Thermo Fisher) was used as a mounting medium and for staining of the nuclei of the cells after the antibody incubations. These cells were analysed with a Leica SP8 confocal microscope (Leica Microsystems,Wetzlar, Germany), and Leica Application Suite X software was used to enlarge cells to indicate localisations in detail. Perinuclear localization of VP8 and/or VP22 was enumerated in 10 fields with 30–100 cells each.

3.6 Results

3.6.1 VP8 (pU_L47) and VP22 (pU_L49) Are Packaged at the Early Tegumentation Stage

When not phosphorylated, VP8 (pU_L47) remains nuclear during viral infection, but is present in a significant amount in the mature BHV-1YmVP8 virus particles. In order to examine the possibility of VP8 (pU_L47) being packaged at the early tegumentation stage, primary enveloped virions were isolated from the perinuclear region and tested for the presence of VP8 (pU_L47) by mass spectrometry and Western blotting. Prior to the extraction of the primary enveloped virus from the perinuclear membranes, the purity of the NMP was tested. Both TCE and NMP were analysed for the presence of tubulin, a cytoskeletal protein; nucleolin, a nuclear protein; and lamin-associated protein 2 (LAP2), a protein specific to the nuclear membrane. The NMP was found to be devoid of tubulin and nucleolin; only LAP2 was found in the NMP, confirming it to be free from other cellular components (Figure 3.1). To determine their purity, the mature and primary enveloped viruses were analysed via SDS-PAGE. Figure 3.2 shows the stained polyacrylamide gel, which indicates distinct bands of VP8 (pU_L47) (91 kD) and VP22 (pU_L49) (29 kD) in both mature and primary enveloped virus samples. The detection of VP5 (pU_L19) (the major capsid protein; 105 kD) confirms the presence of virus particles.

Viruses purified from the culture medium and the perinuclear membrane were submitted for mass spectrometry analysis. Table 3.1 shows a comprehensive list of the mass spectrometry results. As hypothesised, VP8 (pU_L47) was found to be a part of the primary enveloped virus, suggesting its packaging at the early stage of tegument formation. Interestingly, another tegument protein, VP22 (pU_L49), was also found to be a part of the early tegument. In HHV-1, VP22 (pU_L49) is known to play a critical role in viral protein packaging, while in BoHV-1 it aids in the packaging of gN ($pU_L49.5$), so VP22 (pU_L49) might play a role in VP8 (pU_L47) packaging. The presence of both VP8 (pU_L47) and VP22 (pU_L49) in primary enveloped virus particles was confirmed by Western blotting (Figure 3.3)

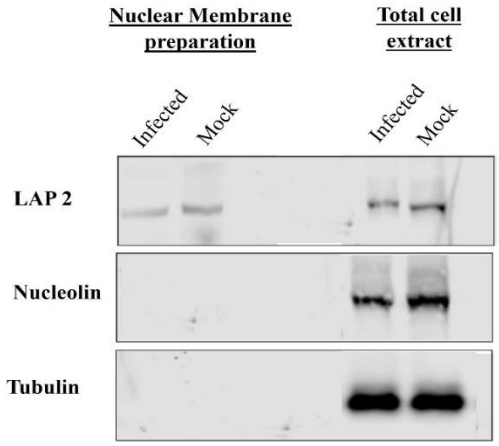

Figure 3.1 Nuclear membrane preparations from BoHV-1-infected and mock-infected MDBK cells were separated by SDS-PAGE on 8% reducing gels and subjected to Western blotting to check for the absence of nucleolin and tubulin, and the presence of LAP2, in the nuclear membrane. The cellular markers were detected by mouse monoclonal anti-LAP2, mouse monoclonal anti-nucleolin, and rabbit polyclonal anti-tubulin IgG, followed by IRDye 680RD goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG, respectively.

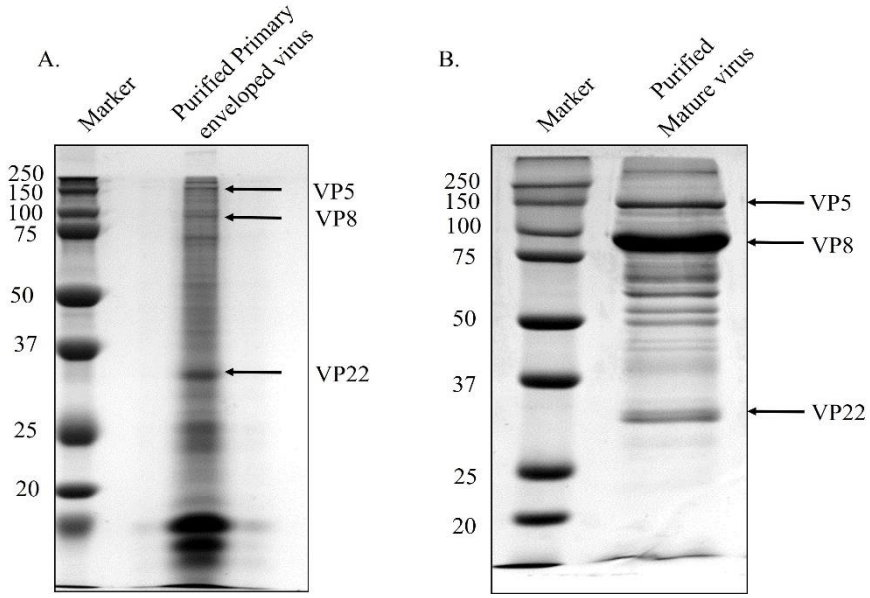

Figure 3.2. Purified primary enveloped virus (**A**) and mature virus (**B**). MDBK cells were infected with BoHV-1, and virus was purified from the perinuclear membrane at 8 h post-infection, and from the culture medium at 18 h post-infection. The proteins were separated by SDS-PAGE on 8% reducing gels and stained with ProtoBlue Safe. The positions of the capsid protein VP5 (pUL19) and tegument proteins VP8 (pUL47) and VP22 (pUL49) are indicated with arrows. Molecular weight markers (kDa) are shown in the left margin.

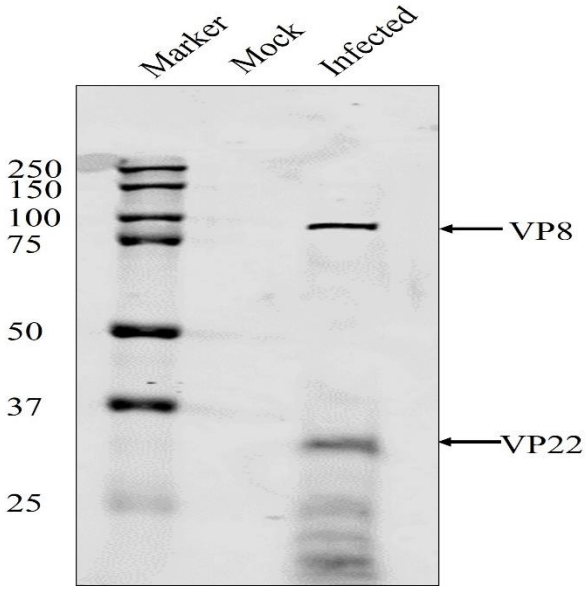

Figure 3.3. Identification of VP8 (pUL47) and VP22 (pUL49) in primary enveloped virus purified from the NMP of MDBK cells infected with WT BoHV-1 at an MOI of 10 and harvested 7 h post-infection. The proteins were separated by SDS-PAGE on 8% reducing gels, and VP8 and VP22 (pUL49) were identified by Western blotting with mouse VP8-specific monoclonal antibody and rabbit VP22-specific polyclonal antibody, followed by IRDye 680RD goat anti-mouse IgG and IRDye 800RD goat anti-rabbit IgG, respectively. The positions of the tegument proteins VP8 (pUL47) and VP22 (pUL49) are indicated with arrows. Molecular weight markers (kDa) are shown in the left margin.

Table 3.1 Comprehensive list of proteins detected by mass spectrometry analysis

Gene	Protein	Present in Primary Enveloped Virus	Present in Mature Virus	Molecular Weight (kDa)	Number of Unique Peptides	Peptide Coverage (%)
			Glycoproteins			
UL27	Glycoprotein B (gB)	x	√	101.1	200	63
UL44	Glycoprotein C (gC)	x	√	55.3	134	60
US6	Glycoprotein D (gD)	x	√	44.5	19	73
US8	Glycoprotein E (gE)	x	√	61.1	65	46
UL22	Glycoprotein H (gH)	x	√	88.3	82	57
UL1	Glycoprotein L (gL)	x	√	17.1	8	23
US4	Glycoprotein G (gG)	x	√	46.5	67	48
UL10	Glycoprotein M (gM)	x	√	45.5	6	13
UL49.5	Glycoprotein N (gN)	x	√	10.2	5	25
UL53	Glycoprotein K (gK)	x	√	35.8	5	25
US7	Glycoprotein I (gI)	x	√	39.9	100	45
			Envelope Proteins			
UL20	pUL20	x	√	25.6	5	37
UL34	pUL34	x	√	27.1	55	54
UL43	pUL43	x	√	36.9	9	25
			Capsid Proteins			
UL6	Capsid portal protein	√	√	75.1	30	86
UL18	Capsid triplex subunit	√	√	33.3	37	46
UL19	VP5 major capsid protein	√	√	105.1	106	52
			Tegument Proteins			
UL2	pUL2	√	x	33.8	8	18
UL7	pUL7	x	√	32.5	21	63
UL11	pUL11	x	√	9.7	15	96
UL14	pUL14	x	√	23.2	31	76
UL16	pUL16	x	√	36.4	12	25
UL17	Cleavage protein	x	√	72.6	59	83
UL21	pUL21	×	√	60.2	47	48
UL25	Cleavage protein	×	√	63.1	62	63
UL26	Maturational protease	√	√	63.7	14	46
UL29	Major DNA binding protein	√	√	78.9	13	29
UL31	pUL31	x	√	39.5	35	72
UL32	pUL32	x	√	62.9	4	23
UL36	Egress regulating protein	√	x	327.2	79	57
UL37	Egress regulating protein	√	x	105.9	86	67
UL39	pUL39	√	√	86.1	27	58
UL40	pUL40	√	√	31.1	15	57
UL41	Host shut off protein	x	√	50.1	41	65
UL42	DNA processivity protein	x	√	42.6	67	63
UL46	VP11/12	x	√	77.6	26	38
UL47	VP8	√	√	80.7	47	65
UL48	VP16	x	√	54.1	55	58

Table 3.1 Contd.

Gene	Protein	Present in Primary Enveloped Virus	Present in Mature Virus	Molecular Weight (kDa)	Number of Unique Peptides	Peptide Coverage (%)
UL49	VP22	√	√	26.8	148	84
UL50	pUL50	x	√	34.1	18	63
UL51	pUL51	x	√	24.9	16	86
UL54	bICP27	x	√	43.3	26	63
US1.67	pUS1.67	x	√	27.2	28	44
US3	Serine/threonine kinase	x	√	49.9	27	48
			Host Proteins			
	HSP70	x	√	70.8	4	18
	Eukaryotic initiation factor 4H	x	√	30.8	26	18
	Annexin	x	√	38.9	15	75
	Alpha tubulin	x	√	50	5	23
	Beta actin	x	√	40.3	9	13
	Histone	√	√	13.9	5	29
	PCNA	√	√	28.8	11	26

√ Indicates presence and x indicates absence.

3.6.2 VP8 (pU_L47) Interacts with VP22 (pU_L49) in the Perinuclear Region and in Mature Virus in BoHV-1-Infected Cells and in BoHV1-YmVP8-Infected Cells

In view of the coexistence of VP8 (pU_L47) and VP22 (pU_L49) in the primary enveloped virus, and the fact that the VP22 (pU_L49) levels were reduced in the mature virus when the cells were infected with BoHV-1ΔU_L47, we studied the interaction between VP8 (pU_L47) and VP22 (pU_L49) in the mature and primary enveloped virus particles. The viruses collected from the culture medium and the NMP of MDBK cells infected with WT BoHV-1 were lysed and incubated overnight with antibodies specific to VP8 (pU_L47) or VP22 (pU_L49), followed by incubation with Protein G Sepharose. After the bound proteins were eluted and subjected to Western blotting, it was found that VP22 (pU_L49) interacted with VP8 (pU_L47) and vice versa in the primary enveloped virions obtained from the NMP (Figure 3.4A). Similarly, in the mature virus, VP22 (pU_L49) interacted with VP8 (pU_L47), and vice versa (Figure 3.4B).

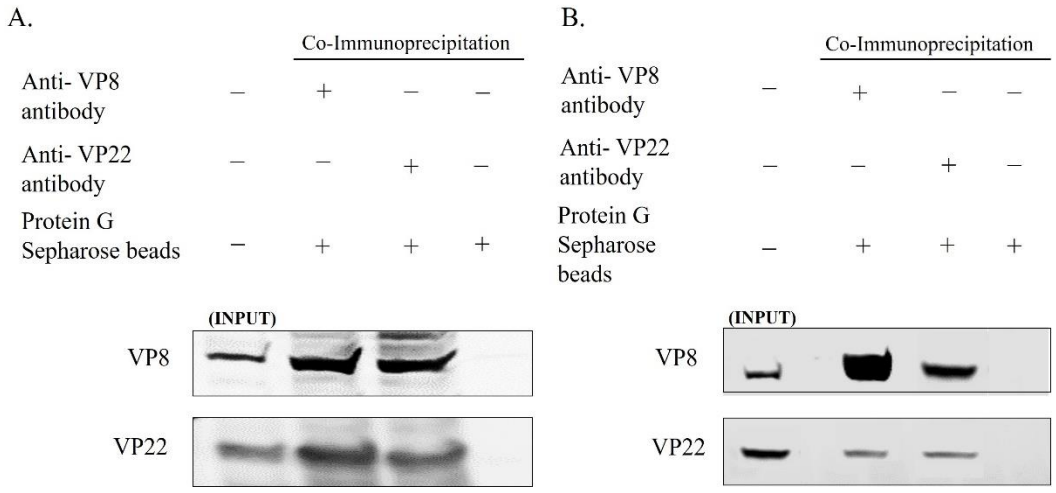

Figure 3.4 VP8 (pU_L47) and VP22 (pU_L49) interact during BoHV-1 infection. MDBK cells were infected with WT BoHV-1 at an MOI of 3 and harvested 8 h post-infection (primary enveloped virus; (A)) or 18 h post infection (mature virus; (B)). Lysates were generated and incubated with mouse VP8-specific monoclonal antibodies or rabbit VP22-specific polyclonal antibodies, followed by Protein Sepharose G beads. VP8 (pU_L47) and VP22 (pU_L49) were detected by Western blotting with monoclonal anti-VP8 and rabbit anti-VP22 antibodies, followed by IRDye 680RD goat anti-mouse IgG and IRDye 800RD goat anti-rabbit IgG, respectively. Immunoprecipitation of VP8 (pU_L47) with VP22 (pU_L49), and vice versa, is shown for both primary enveloped (A) and mature (B) virus.

In order to determine whether VP8 (pU_L47) and VP22 (pU_L49) interact in cells infected with BoHV-1-YmVP8, in which VP8 (pU_L47) is not phosphorylated and remains nuclear, MDBK cells were infected with BoHV-1-YmVP8 at an MOI of 0.5 and collected at 18 h post-infection to purify the perinuclear membrane, and at 30 h post-infection to isolate mature virus. The cell lysates were incubated overnight with VP8 (pU_L47)-specific or VP22 (pU_L49)-specific antibodies, followed by binding to Protein G Sepharose beads and elution with SDS loading dye. Western blot analysis showed co-precipitation of VP22 (pU_L49) with VP8 (pU_L47), and vice versa, in the NMP (Figure 3.5A). Similarly, in the mature virus, VP22 (pU_L49) interacted with VP8 (pU_L47), and vice versa (Figure 3.5B).

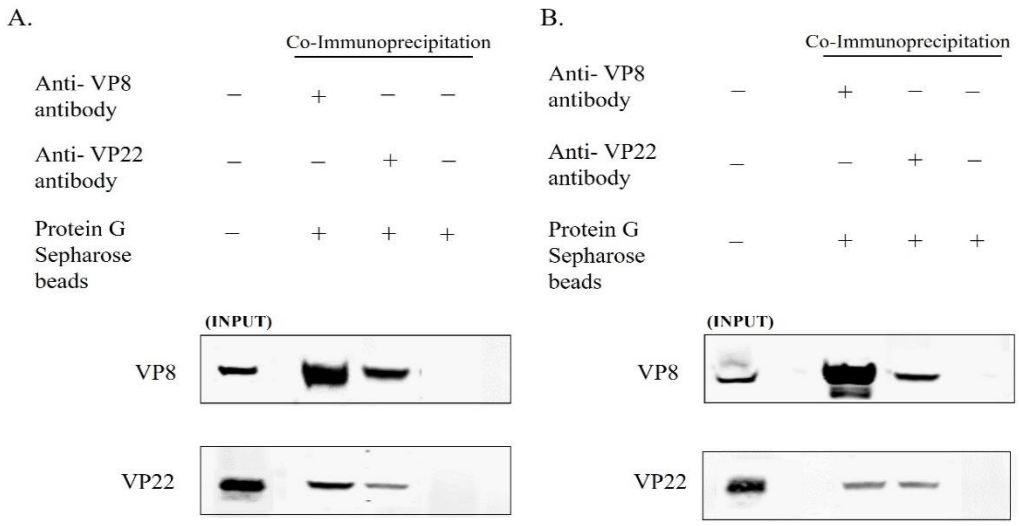

Figure 3.5 VP8 (pU_L47) and VP22 (pU_L49) interact during BoHV-1YmVP8 infection. MDBK cells were infected with BoHV-1YmVP8 at an MOI of 0.5 and harvested 18 h post-infection (primary enveloped virus; (**A**)) or 30 h post-infection (mature virus; (B)). Lysates were generated and incubated with mouse VP8-specific monoclonal antibodies or rabbit VP22-specific polyclonal antibodies, followed by Protein Sepharose G beads. VP8 (pU_L47) and VP22 (pU_L49) were detected by Western blotting with monoclonal anti-VP8 and rabbit anti-VP22 antibodies, followed by IRDye 680RD goat anti-mouse IgG and IRDye 800RD goat anti-rabbit IgG, respectively. Immunoprecipitation of VP8 (pU_L47) with VP22 (pU_L49), and vice versa, is shown for primary enveloped (A) and mature (B) virus.

These data suggest that VP8 (pU_L47) and VP22 (pU_L49) interact with one another in the NMP, and possibly at a later stage during final envelopment at the Golgi. The interaction with VP22 (pU_L49) does not depend on phosphorylation of VP8 (pU_L47), as it occurred with both the WT VP8 (pU_L47) and the non-phosphorylated form of VP8 (pU_L47).

3.6.3 VP8 (pU_L47) is absent in the primary enveloped virus in the absence of VP22 (pU_L49)

Since an interaction was found between VP8 (pU_L47) and VP22 (pU_L49), the dependence of VP8 (pU_L47) on VP22 (pU_L49) for its packaging into the primary tegument was examined. MDBK cells were infected with WT BoHV-1 or BoHV-1ΔU_L49, and the virions extracted from the NMP were tested for the presence of VP8 by Western blotting. VP8 (pU_L47) was not detected in the NMP of the BoHV-1ΔU_L49-infected cells, suggesting that VP22 (pU_L49) is required for packaging of VP8 (pU_L47) during primary envelopment.

VP5 (pU_L19) (major capsid protein) was used as a control to confirm the presence of virus particles (Fig 3.6).

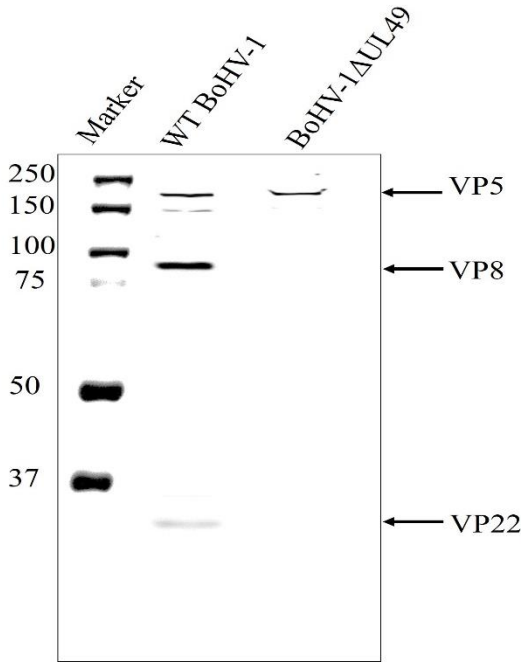

Fig. 3.6 VP8 (pU_L47) is absent in primary enveloped virus in BoHV-1ΔUL49-infected cells. Primary enveloped virus was purified from the NMP of MDBK cells infected with BoHV-1ΔUL49 at an MOI of 3 and harvested 18 hr post-infection. The proteins in the primary enveloped virus were separated by SDS-PAGE on 8% gels, and VP8 (pU_L47) and VP22 (pU_L49) were identified by Western blotting with mouse VP8-specific monoclonal antibody and rabbit VP22-specific polyclonal antibody, followed by IRDye 680RD goat anti-mouse IgG and IRDye 800RD goat anti-rabbit IgG, respectively. The positions of the tegument proteins VP8 (pU_L47) and VP22 (pU_L49) are indicated with arrows. Capsid protein VP5 (pU_L19) indicates presence of viral particles. Molecular weight markers (kDa) are shown in the left margin.

3.6.4 VP8 (pU_L47) and VP22 (pU_L49) interact with each other outside the context of infection

In order to determine whether interaction between VP8 (pU_L47) and VP22 (pU_L49) is independent of other viral factors, COS-7 cells were co-transfected with both VP8-FLAG and VP22-HA plasmids (and individually with VP8-FLAG and VP22-HA as controls). At 48 h after transfection the cells were collected and lysed. The lysates were incubated with anti-FLAG beads or anti-HA beads and the proteins were eluted with SDS loading dye.

When these samples were subjected to Western blotting it was found that VP8-FLAG was precipitated along with VP22-HA when the lysate was incubated with anti-HA beads, and that VP22-HA was precipitated along with VP8-FLAG when the lysate was incubated with anti-FLAG beads. The specificity of the VP8-FLAG and VP22-HA interaction was demonstrated by the absence of VP8-FLAG when the lysate from VP8 -FLAG-transfected cells was incubated with anti-HA agarose and similarly, the absence of VP22-HA when the lysate from VP22-HA-transfecetd cells was incubated with Anti-FLAG M2 Affinity Gel (Fig.3.7).

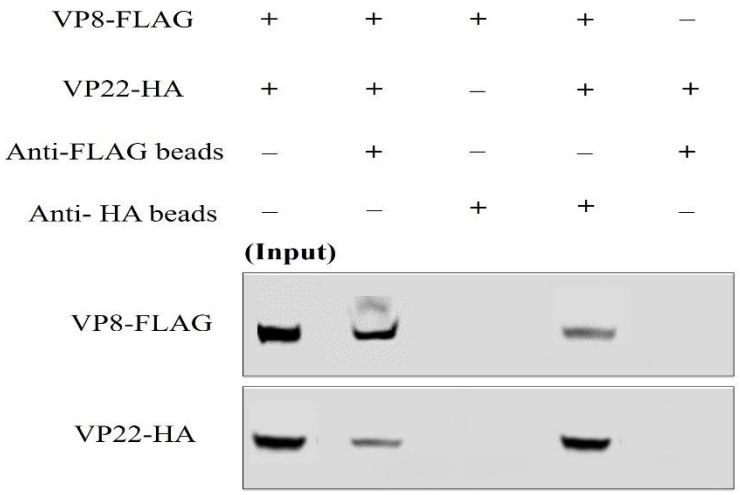

Fig. 3.7. VP8 (pU_L47) and VP22 (pU_L49) interact outside the context of infection in transfected cells. COS-7 cells were transfected with VP8-FLAG and/or VP22-HA plasmids. The cells were lysed at 24 h post-transfection, and incubated with Anti-FLAG M2 Affinity Gel or anti-HA agarose. VP8 (pU_L47) and VP22 (pU_L49) were detected by Western blotting with mono-clonal mouse anti-FLAG and polyclonal rabbit anti-HA antibodies, followed by IRDye 680RD goat anti-mouse IgG and and IRDye 800RD goat anti-rabbit IgG, respectively. VP22 (pU_L49) was precipitated with VP8 (pU_L47) and Anti-FLAG M2 Affinity Gel and VP8 (pU_L47) was precipitated with VP22 (pU_L49) and anti-HA agarose.

3.6.5 VP8 (pU_L47) and VP22 (pU_L49) localise in the perinuclear region in cells infected with WT BoHV-1 or BoHV-1YmVP8

To confirm the presence of VP8 (pU_L47) and VP22 (pU_L49) in the perinuclear region at early stages of infection, MDBK cells were infected with WT BoHV-1 or BoHV-1-

YmVP8 at an MOI of 5 and fixed at 4 or 5 hr post infection. Antibodies specific for LAP2 followed by Alexa 633-conjugated goat anti-mouse IgG were used to identify the nuclear membranes and antibodies specific for VP8 (pU_L47) and VP22 (pU_L49) followed by Alexa 488-conjugated goat anti-rabbit IgG were used to identify the respective proteins. In both WT BoHV-1- and BoHV-1YmVP8-infected cells, the presence of VP8 (pU_L47) in the perinuclear nuclear region was confirmed by the yellow fluorescence. No signal was observed in mock-infected cells. Fig. 3.8 shows co-localisation of VP8 (pU_L47) with LAP2 in the perinuclear region of MDBK cells infected with WT BoHV-1 or BoHV-1YmVP8 at 4 and 5 hr post infection. At 4 h post infection VP8 (pU_L47) was present at low levels and was mostly nuclear in both WT BoHV-1- and BoHV-1YmVP8-infected cells. After infection with WT BoHV-1, 2.6% of the cells showed presence of VP8 (pU_L47) in the perinuclear region. In BoHV-1YmVP8-infected cells, less co-localisation of VP8 (pU_L47) and LAP2 (< 2%) was observed when compared to the WT BoHV-1-infected cells. At 5 h post-infection, VP8 (pU_L47) expression increased; in WT BoHV-1-infected cells a low proportion of VP8 (pU_L47) was cytoplasmic, while 37.5% of the observed cells showed presence of VP8 (pU_L47) in the perinuclear region. Meanwhile in BoHV-1YmVP8-infected cells, VP8 (pU_L47) remained nuclear due to the absence of phosphorylation, in 36.74% of the cells a distinct co-localisation of VP8 (pU_L47) with LAP2 was observed in the perinuclear region. These localization patterns continued through the later stages of infection (data not shown). This supports the hypothesis of a certain amount of VP8 (pU_L47) being packaged during the early envelopment process in both BoHV-1- and BoHV-1YmVP8-infected cells.

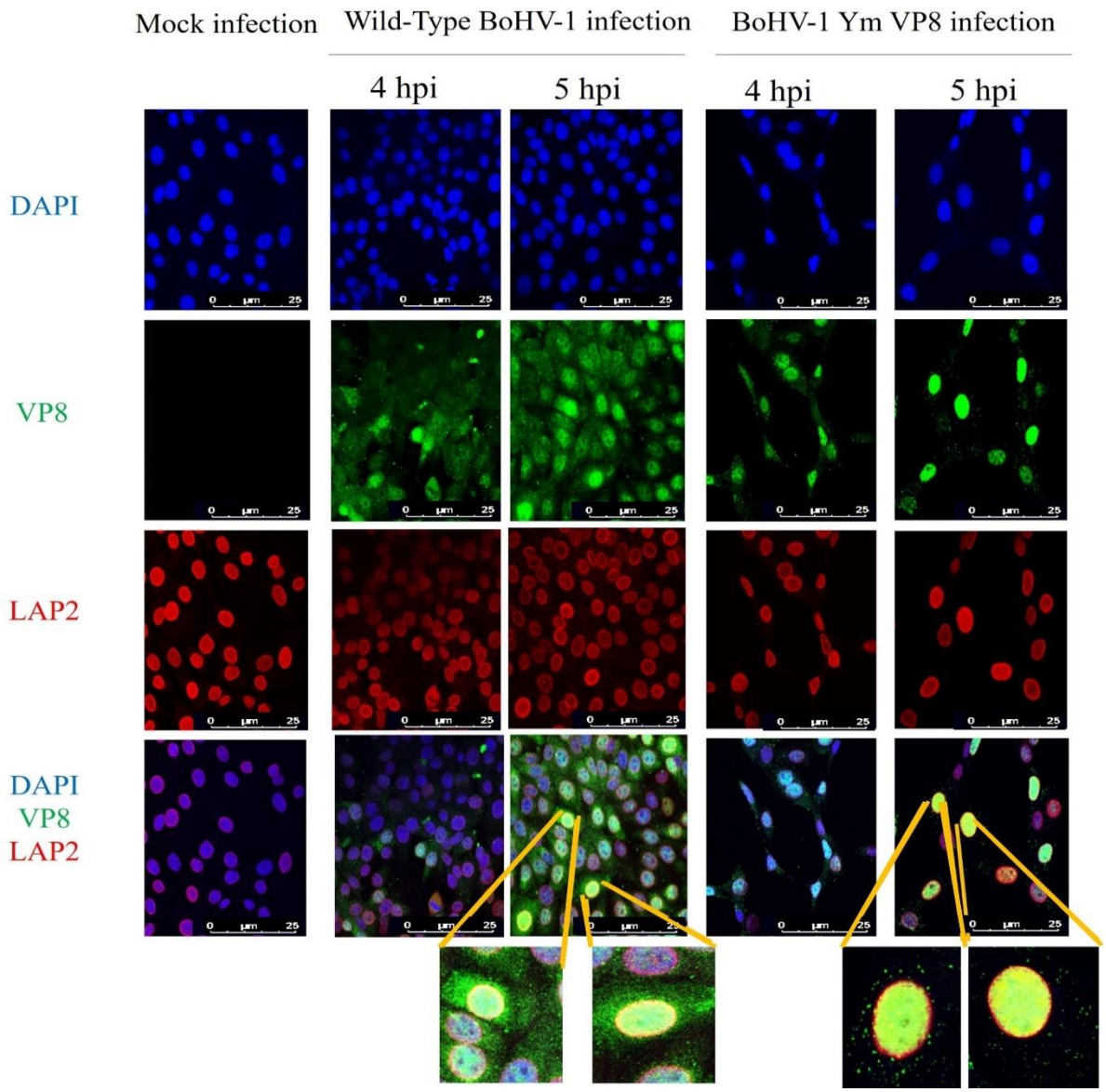

Fig. 3.8. Co-localisation of VP8 (pU_L47) and LAP2. MDBK cells were infected with WT BoHV-1 or BoHV-1YmVP8 at an MOI 5, and fixed and permeabilized at 4 or 5 hr post infection. VP8 (pU_L47) was identified with rabbit VP8-specific antibody and Alexa-488-conjugated goat anti-rabbit IgG. LAP2 was detected with monoclonal LAP2-specific antibody and Alexa-633 conjugated anti-mouse IgG. The yellow ring around the cells shows VP8 (pU_L47) co-localized with LAP2 in the perinuclear region. Selected cells were observed at 10× magnification to show more details. 1 cm represents 5µm in the scale.

Fig 3.9 shows similar results for the presence of VP22 (pU_L49) in the perinuclear region. VP22 (pU_L49) was mostly cytoplasmic at 4 h post-infection in both BoHV-1- and BoHV-1YmVP8-infected cells. However, at 4 hr post infection 9% of the BoHV-1-infected cells were found to have VP22 (pU_L49) present in the perinuclear region. In BoHV-1YmVP8-infected cells 15% of the cells showed VP22 (pU_L49) localisation in the perinuclear region. Localisation of VP22 (pU_L49) in the perinuclear region was evident at 5 h post-infection in both BoHV-1 and BoHV-1YmVP8-infected cells. After WT BoHV-1 infection, 31.6% of the cells showed VP22 (pU_L49) in the perinuclear region, whereas in BoHV-1YmVP8 infected cells 38.5% showed a clear presence of VP22 (pU_L49) in the perinuclear region. No signal was observed in mock-infected cells. VP22 (pU_L49) is predominantly cytoplasmic during early stages of infection (Wang and Brattain 2007, Lobanov, Zheng et al. 2010); however due to its small size VP22 (pU_L49) (29kDa) can passively diffuse into the nucleus via the nuclear pores (Wang and Brattain 2007, Wu, Cheng et al. 2020) which is probably responsible for some nuclear VP22 (pU_L49).

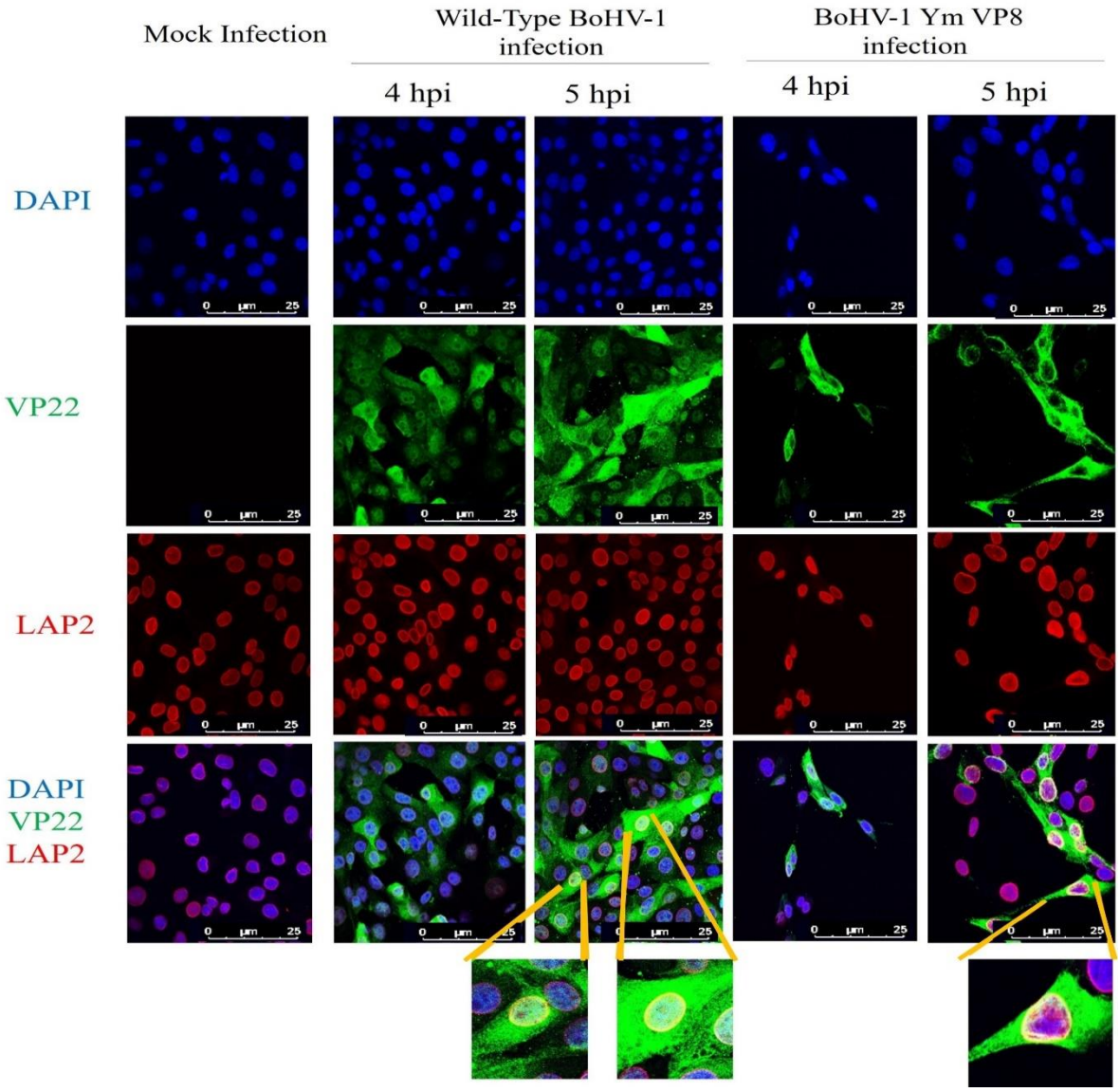

Fig 3.9. Localisation of VP22 (pU_L49) in the perinuclear region. MDBK cells were infected with WT BoHV-1 or BoHV-1YmVP8 at MOI 5, and fixed and permeabilized 4 and 5 h post-infection. VP22 (pU_L49) was identified with rabbit VP22-specific antibody and Alexa-488-conjugated goat anti-rabbit IgG. LAP2 was detected with monoclonal LAP2-specific antibody and Alexa-633 conjugated anti-mouse IgG. The yellow ring around the cells shows VP22 (pU_L49) co-localized with LAP2 in the perinuclear region. Selected cells were observed at 10× magnification to show more details. 1 cm represents 5µm in the scale.

3.6.6 VP22 (pU_L49) co-localises with VP8 (pU_L47):

After demonstrating the interaction of VP8 (pU_L47) and VP22 (pU_L49) and the presence of VP8 (pU_L47) and VP22 (pU_L49) in the perinuclear region, co-localisation of VP8 (pU_L47) and VP22 (pU_L49) was investigated in WT BoHV-1-infected cells at MOI of 5. Fig. 3.10 shows co-localisation of VP8 (pU_L47) and VP22 (pU_L49) proteins at 4 h and 5 h post-infection. At 4 h post-infection co-localisation of VP8 (pU_L47) and VP22 (pU_L49) was observed in 15% of the cells, mostly around the perinuclear region, with some in the cytoplasm. At 5 h post-infection more co-localisation of VP8 (pU_L47) and VP22 (pU_L49) was observed in the cytoplasm than in the perinuclear region, but 38.5% cells showed co-localisation of VP8 (pU_L47) and VP22 (pU_L49) in the perinuclear region.

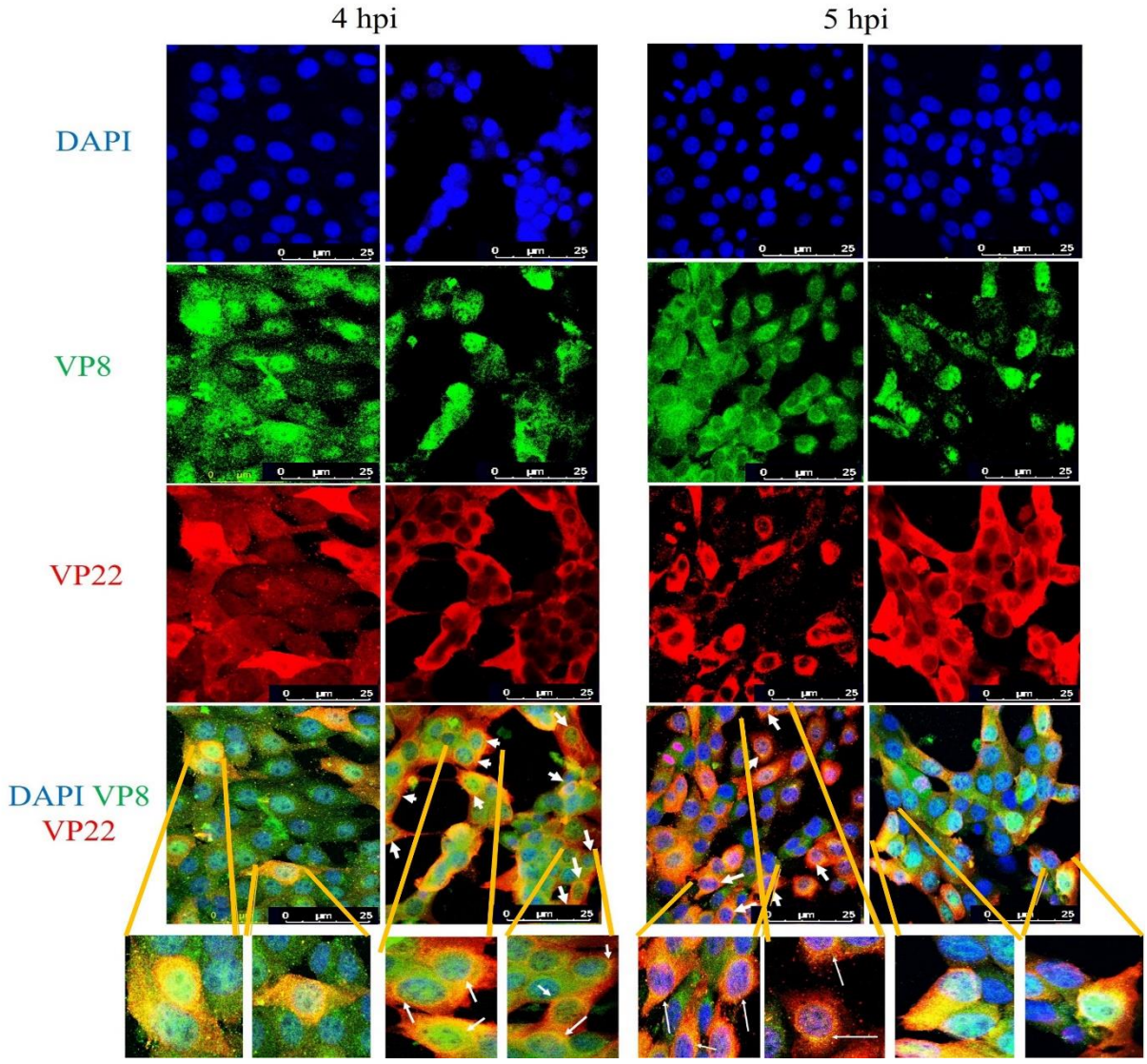

Fig 3.10. Co-localisation of VP22 (pU_L49) and VP8 (pU_L47). MDBK cells were infected with WT BoHV-1, and fixed and permeabilized 4 and 5 h post-infection. VP8 (pU_L47) was identified with monoclonal VP8-specific antibody and Alexa 488-conjugated goat anti-mouse IgG. VP22 (pU_L49) was detected with rabbit VP22-specific antibody and Alexa 633-conjugated anti-rabbit IgG. The yellow ring (as indicated by the arrows) shows co-localisation of VP8 (pU_L47) and VP22 (pU_L49) proteins in the perinuclear region. Selected cells were observed at 10× magnification to show more details. 1 cm represents 5µm in the scale.

3.6.7 VP8 (pU_L47) is not localized to the perinuclear region in BoHV-1ΔU_L49-infected cells

To further confirm the absence of VP8 (pU_L47) in the perinuclear region of BoHV-1ΔU_L49-infected cells, as shown in Fig. 3.6, MDBK cells were infected with BoHV-1ΔU_L49 at MOI 5 and fixed 4 or 5 h post-infection. LAP2-specific antibodies followed by Alexa 633-conjugated goat anti-mouse IgG were used to indicate the nuclear membranes and antibodies specific for VP8 (pU_L47) followed by Alexa 488-conjugated goat anti-rabbit IgG were used to identify VP8 (pU_L47). In BoHV-1ΔU_L49-infected cells no nuclear VP8 (pU_L47) was detected at 4 h post-infection, which may be due to a slower infection rate of BoHV-1ΔU_L49 infection, whereas at 5 and 6 h post-infection VP8 (pU_L47) was partially nuclear and partially cytoplasmic, similar to WT BoHV-1 infected cells. No VP8 was localised in the perinuclear region at 4, 5 or 6 h post-infection (Fig.3.11). From these results it can be inferred that VP22 (pU_L49) is required for the perinuclear localisation and hence packaging of VP8 (pU_L47) during the early tegumentation process.

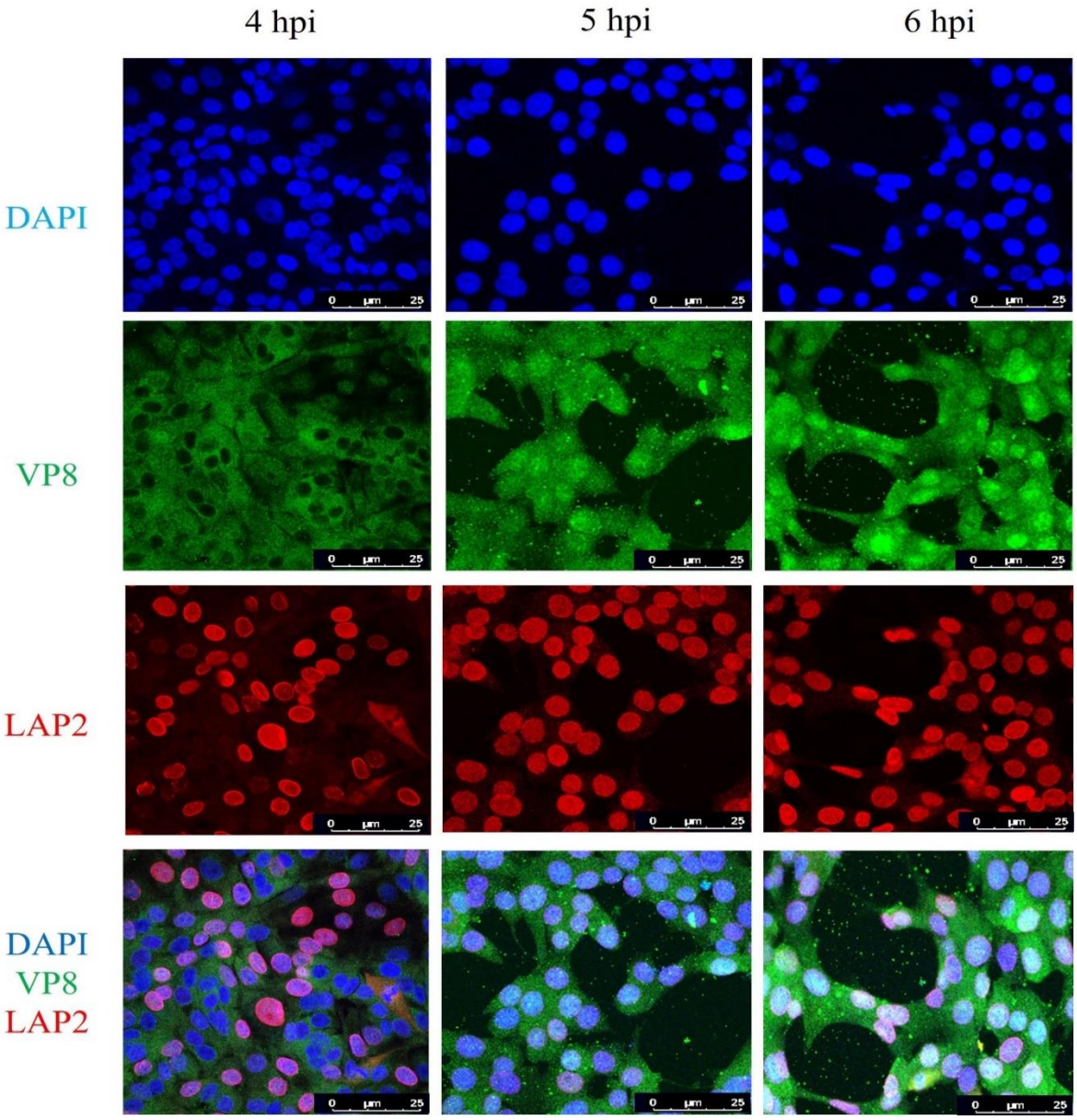

Fig. 3.11. VP8 (pU_L47) does not localise in the perinuclear region in BoHV-1ΔUL49- infected cells. MDBK cells were infected with BoHV-1ΔUL49 at MOI 5, and fixed and permeabilized 4, 5 or 6 hr post infection. VP8 (pU_L47) was identified with rabbit VP8-specific antibody and Alexa 488-conjugated goat anti-rabbit IgG. LAP2 was detected with monoclonal LAP2-specific antibody and Alexa 633-conjugated anti-mouse IgG. No yellow and hence no presence of VP8 (pU_L47) in the perinuclear region was seen. 1 cm represents 5μm in the scale.

3.7 Discussion

In this study a novel, VP22 (pU_L49)-dependent, packaging mechanism of VP8 (pU_L47) during primary envelopment was identified. During the maturation process of alpha herpesviruses, tegument proteins are added at different stages, namely during primary envelopment, i.e. when virus moves from the nucleus into the perinuclear region during its egress (Granzow, Klupp et al. 2001), in the cytoplasm, after it exits the nucleus and travels towards the Golgi, and at the Golgi where the final maturation takes place (Mettenleiter, Klupp et al. 2006, Mettenleiter, Klupp et al. 2009). VP8 (pU_L47) contains phosphorylation sites and gets phosphorylated by host kinase CK2 in the cytoplasm and viral kinase pU_S3 in the nucleus (Labiuk, Babiuk et al. 2009). Previous studies showed that the phosphorylation of VP8 (pU_L47) by pU_S3 is necessary for its translocation into the cytoplasm and for various other functions (Zhang, Brownlie et al. 2016). Tegument proteins such as VP8 (pU_L47) may be either completely packaged during the primary envelopment process, completely packaged at the Golgi, or partially packaged during primary envelopment and partially in the Golgi. A similar pattern of packaging during the early tegumentation process has been shown in previous studies for HHV-1 VP22 (pU_L49) (Loret, Guay et al. 2008). When MDBK cells were infected with BoHV-1YmVP8 and the mature virus was tested for the amount of VP8 (pU_L47), a significant amount of VP8 (pU_L47) is present in the BoHV-1YmVP8 virions, despite the fact that VP8 (pU_L47) remains nuclear (Zhang, Brownlie et al. 2016). This suggested that VP8 (pU_L47) might be packaged during early tegumentation; however, due to the fact that the amount of VP8 in the mature BoHV-1YmVP8 virions was significantly reduced, it can be inferred that the early packaging of VP8 (pU_L47) at the nucleus is only partial and that VP8 (pU_L47) is further packaged in the Golgi.

In this study, mass spectrometry analysis performed on duplicate biological samples of primary enveloped virions extracted from the NMP and the mature virus extracted from the culture medium identified the proteins specific to these fractions. Based on the purity of the fractions it can be inferred that the NMP was devoid of any nuclear or cytoplasmic

contaminants and the identified proteins are solely from the primary enveloped virus present in the perinuclear region. The comprehensive list of identified proteins is similar to the proteins present in HHV-1 for both primary enveloped and mature virus (Mocarski Jr 2007, Loret, Guay et al. 2008, Padula, Sydnor et al. 2009). In the BoHV-1 mass spectrometry analysis, 11 glycoproteins, gB (pU_L27), gC (pU_L44), gD (pU_S6), gE (pU_S8), gH (pU_L22), gL (pU_S1), gG (pU_S4), gM (pU_L10), gN ($pU_L49.5$), gK (pU_L53) and gI (pU_S7), are a part of the mature virus as glycoproteins are acquired at the Golgi along with the lipid envelope; this was consistent with previous studies on protein composition of BoHV-1 (Barber, Daugherty et al. 2017). The absence of glycoproteins from the primary enveloped virus further confirms the purity of the virus extracted from the perinuclear region. Major and minor capsid proteins are a part of both the primary enveloped and the mature virus, which demonstrates the presence of the viral capsid in these regions. Most of the tegument proteins are a part of only the mature virus, i.e. they are packaged outside the nucleus in the cytoplasm or at the Golgi, except for pU_L2, VP1/2 (pU_L36), and pU_L37, which were only present in the primary enveloped virions; this means they are packaged during the early tegumentation process and are lost as they travel from the nucleus to the Golgi. In HHV-1 VP1/2 (pU_L36) and pU_L37 play a critical role in viral egress and hence are only needed at the early stages (Granzow, Klupp et al. 2001). pU_L26, pU_L29, pU_L39, pU_L40, VP8 (pU_L47) and VP22 (pU_L49) are a part of primary enveloped virus as well as mature virus, which suggests that they are packaged during early tegument formation and retained and/or lost and added during travel to the Golgi. Host proteins, namely HSP70, annexin, tubulin, actin, PCNA and histones, form an integral part of herpesviruses (Mocarski Jr 2007), were also found in the BoHV-1 mature virus samples, and in the case of histones and PCNA also in the primary enveloped virus particles. The absence of annexin from the primary enveloped BoHV-1 does not agree with HHV-1, in which annexin 2 is an integral part of the primary enveloped virus (Padula, Sydnor et al. 2009). Overall, fewer host proteins were found in the BoHV-1 virus particles when compared to HHV-1, which may be related to the purity of the samples or specific characteristics of these herpesviruses.

VP8 (pU_L47) and VP22 (pU_L49) are both a part of primary enveloped virus i.e. a certain amount of both proteins is packaged during the early tegumentation process. In HHV-1 and BoHV-1 VP22 (pU_L49) is a critical tegument protein, which plays a role in the packaging of several other tegument proteins as well as glycoproteins (Pannhorst, Wei et al. 2018, DuRaine, Wisner et al. 2020); it is also responsible for the shuttling of viral proteins in the host cells (Zheng, Babiuk et al. 2005, Yu, Xiao et al. 2012, Xue, Huang et al. 2014). The viral titer was also 10-fold reduced in BoHV-1ΔU_L49-infected cells when compared to WT BoHV-1 infected cells (Liang, Chow et al. 1995). A reduction in the amount of VP22 (pU_L49) in BoHV-1ΔU_L47-infected cells (Lobanov, Maher-Sturgess et al. 2010) suggests interdependence between VP8 (pU_L47) and VP22 (pU_L49). Partial packaging of critical proteins like VP8 (pU_L47) and VP22 (pU_L49) into the early tegument might also be a survival strategy of the virus in conditions which oppose the transportation or translocation of these proteins to the Golgi. In this study, an interaction between VP8 (pU_L47) and VP22 (pU_L49) was confirmed in both the primary enveloped virions and the mature virus. This interaction was independent of the phosphorylation status of VP8 (pU_L47) (as they were found to interact in BoHV-1 YmVP8-infected cells) and also of other viral factors (as they interact outside the context of infection). The presence of VP8 (pU_L47) and VP22 (pU_L49) was shown in the perinuclear region at early time points, which supports the probability of both proteins being packaged during early tegumentation and assisting each other in the process.

Because VP22 (pU_L49) aids in packaging of proteins and interacts with VP8 (pU_L47), the require-ment of VP22 (pU_L49) for VP8 (pU_L47) packaging at the early tegumentation stage was investigated. The fact that in BoHV-1ΔU_L49-infected cells VP8 (pU_L47) was absent from the perinuclear region (Fig 3.6 and Fig 3.11) confirms that VP22 (pU_L49) is needed for VP8 (pU_L47) packaging into the primary enveloped virus particles.

In summary, our results support the hypothesis that VP8 (pU_L47) is partially packaged during the early tegumentation process along with VP22 (pU_L49) and that VP22 (pU_L49), an interaction partner of VP8 (pU_L47), is required for the early packaging of VP8

(pU$_L$47) into the early tegument. In the absence of phosphorylation, when VP8 (pU$_L$47) remains mostly nuclear, the limited quantity of VP8 (pU$_L$47) packaged during the early tegumentation process helps the virus to obtain a certain amount of this critical and multifunctional protein. However, in WT virus-infected cells, most of the VP8 (pU$_L$47) is translocated into the cytoplasm and hence gets packaged at the Golgi along with the partial packaging during the early tegumentation. In addition to suggesting a novel mechanism for VP8 (pU$_L$47) packaging at the early tegumentation process, this study also supports the correlation of the amount of VP8 (pU$_L$47) in the virions and the replication efficiency in cell culture. Based on previous studies and the current study it can be safely said that VP8 (pU$_L$47) plays a critical role in viral replication and its reduction in mature virus causes the replication efficiency and virulence to be significantly reduced.

3.8 Conclusion

The presence of both VP8 (pU$_L$47) and VP22 (pU$_L$49) as a part of the early tegument indicates early packaging of these proteins. This explains why a substantial, though reduced, amount of VP8 (pU$_L$47) is incorporated in the virus particles when it is not phosphorylated and remains nuclear. In cells infected with a VP22 (pU$_L$49) deletion mutant VP8 (pU$_L$47) was not present in the early tegument, which suggests that VP8 (pU$_L$47) depends on VP22 (pU$_L$49) for its early packaging.

3.9 Acknowledgments

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3.10 Authors' Contribution

Conceptualization, S.S. and S.v.D.L.-v.d.H.; methodology, S.S. and S.v.D.L.-v.d.H.; software, S.S.; validation, S.S. and S.v.D.L.-v.d.H.; formal analysis S.S.;

investigation, S.S.; resources, S.S., K.Z. and S.v.D.L.-v.d.H.; data curation, S.S.; writing—original draft preparation, S.S. and S.v.D.L.-v.d.H.; writing—review and editing, S.S., K.Z. and S.v.D.L.-v.d.H.; visualization, S.S., K.Z. and S.v.D.L.-v.d.H.; supervision, S.v.D.L.-v.d.H.; project administration, S.v.D.L.-v.d.H.; funding acquisition, S.v.D.L.-v.d.H. All authors have read and agreed to the published version of the manuscript.

CHAPTER 4

4. LINKER BETWEEN CHAPTER 3 AND 5

In Chapter 3 we determined early packaging of VP8 (pU_L47) in the perinuclear space with the involvement of another early packaged tegument protein, VP22 (pU_L49). An interaction between VP8 (pU_L47) and VP22 (pU_L49) was established in cells infected with WT BoHV-1 and BoHV-1YmVP8 in the perinuclear region as well as in the mature virus. VP8 (pU_L47) and VP22 (pU_L49) were also shown to co-localise in the perinuclear space. In absence of VP22 (pU_L49), no VP8 (pU_L47) was present in the perinuclear region. These results indicate a partial early packaging of VP8 (pU_L47) which explains why purified mature BoHV-1YmVP8 contains 30% of the amount of VP8 (pU_L47) present in WT BoHV-1.

In Chapter 5 we investigated the involvement of a viral factor in the localisation of VP8 (pU_L47) from the cytoplasm to the Golgi and hence final packaging of VP8 (pU_L47) into the mature virus. Based on the results in Chapter 3, which suggest early packaging of VP8 (pU_L47) in the perinuclear region, the investigations in Chapter 5 would contribute to providing the complete sequence of incorporation of the major tegument protein VP8 (pU_L47) into the mature BoHV-1.

CHAPTER 5

5. BOVINE HERPESVIRUS-1 GLYCOPROTEIN M (pU_L10) MEDIATES THE TRANSLOCATION TO THE GOLGI APPARATUS AND PACKAGING OF VP8 (pU_L47)

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Keywords: bovine herpesvirus-1; tegument; VP8; glycoprotein M; Golgi localization

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5.2 Author's contribution

All of the experiments were planned and performed by Soumya Sucharita. Analysis and interpretation of the results were carried out by Soumya Sucharita. The anti-rabbit gM specific antibody used in this study was manufactured by Dr Suresh Tikkoo. The manuscript was written and edited by Soumya Sucharita and Dr Sylvia van den Hurk.

5.3 Abstract

VP8 (pU_L47), the most abundant tegument protein of bovine herpesvirus-1 (BoHV-1), plays an important role in viral replication. According to our previous studies, VP8 (pU_L47) localizes to the Golgi apparatus of BoHV-1-infected cells where it can be packaged into the virus; however, Golgi localization of VP8 (pU_L47) does not occur outside of the context of infection. The goal of this study was to identify the viral factor(s) involved in the tropism of VP8 (pU_L47) towards the Golgi. VP8 (pU_L47) was shown to interact with glycoprotein M (gM) (pU_L10), and the VP8 (pU_L47) and gM (pU_L10) domains that are essential for this interaction were identified. VP8 (pU_L47) and gM (pU_L10) co-localized to the Golgi apparatus in BoHV-1-infected cells. In cells co-transfected with VP8 (pU_L47)- and gM (pU_L10)-encoding plasmids, VP8 (pU_L47) was also shown to localise at the Golgi, suggesting gM (pU_L10) to be sufficient. The localization of VP8 (pU_L47) to the Golgi was lost in cells infected with a gM (pU_L10) deletion mutant, and the amount of VP8 (pU_L47) incorporated into mature virus was significantly reduced. However, with the restoration of gM (pU_L10) in a revertant virus, the localization to the Golgi and the amount of VP8

(pU_L47) incorporated in the virions were restored. These results indicate that gM plays a critical role in VP8 (pU_L47) subcellular localization to the Golgi and packaging into mature virions.

5.4 Introduction

Bovine herpesvirus-1 (BoHV-1) of the subfamily *Alphaherpesvirinae* is an important pathogen in cattle. It causes infectious bovine rhinotracheitis, conjunctivitis, balanoposthitis, and vulvovaginitis (Levings and Roth 2013), which may lead to infertility, abortion, and reduced milk production (Lucchese, Benkirane et al. 2016). Structurally, BoHV-1 is composed of an icosahedral proteinaceous capsid with a diameter of 125 nm. The 135 kb double-stranded DNA genome is packaged inside the capsid (Haanes, Thomsen et al. 1995). Approximately 20 proteins create a layer known as the tegument around the capsid, which is characteristic of herpesviruses. The viral envelope is made up of lipids and glycoproteins (Lobanov, Maher-Sturgess et al. 2010). The tegument is an essential layer because tegument proteins can perform numerous vital roles in the life cycle of herpesviruses, including replication, virus assembly, and immune evasion. However, the tegument of many of the herpesviruses remains poorly characterized (Kelly, Fraefel et al. 2009).

VP8 (pU_L47), encoded by the *U_L47* gene, is the most abundant tegument protein in BoHV-1 and is critical for virus replication and involved in induction of immune responses in the host (van Drunen Littel-van den Hurk, Garzon et al. 1995, Lobanov, Maher-Sturgess et al. 2010). It also plays a role in the alteration of the host defense responses and apoptosis of host cells (Afroz, Garg et al. 2018). A recombinant BoHV-1 that does not express VP8 (pU_L47) does not replicate in cattle; in vitro replication of this deletion mutant is also impaired leading to a 100-fold reduction of intracellular virus and a 1000-fold reduction of extracellular virus in cell culture (Lobanov, Maher-Sturgess et al. 2010). Previously, we reported that phosphorylation of VP8 (pU_L47) by viral as well as host kinases plays an

indispensable role in the functions and subcellular localization of VP8 (pU_L47) (Zhang, Donovan et al. 2019).

Due to the presence of nuclear localization signals (NLS) (Zheng, Brownlie et al. 2004), VP8 (pU_L47) enters into the nucleus at the early stages of infection. Inside the nucleus, VP8 (pU_L47) causes recruitment and redistribution of promyelocytic leukemia protein, which in turn inhibits host antiviral responses (Zhang, Afroz et al. 2015). In transfected cells, the nuclear VP8 (pU_L47) causes an accumulation of lipid droplets and increases cellular cholesterol (Zhang, Donovan et al. 2019). VP8 (pU_L47) moves to the cytoplasm at later stages of infection after phosphorylation by viral kinase pU_S3 and to translocate to the Golgi, where the final packaging into the mature virus occurs. In cells co-transfected with VP8 (pU_L47) and pU_S3, VP8 (pU_L47) also moves from the nucleus to the cytoplasm, but is not transported to the Golgi in the absence of other viral factors (Zhang, Donovan et al. 2019).

The aim of this study was to identify the viral factor(s) responsible for the subcellular localization of VP8 (pU_L47) to the Golgi. Glycoprotein M (gM) (pU_L10) was shown to interact with VP8 (pU_L47), and the domains of gM (pU_L10) and VP8 (pU_L47) that are involved and essential for the interaction of these two proteins were identified. The localisation of VP8 (pU_L47) at the Golgi was found to be mediated by gM, both within and outside the context of infection. In cells infected with a gM-deleted virus (ΔgM BoHV-1), VP8 (pU_L47) remained cytoplasmic, and the amount of VP8 (pU_L47) incorporated into mature virus was significantly reduced. This further supports a critical role of gM (pU_L10) in the localisation of VP8 (pU_L47) at the Golgi apparatus and packaging into the virions.

5.5 Materials and Methods

5.5.1 Cells and Viruses

Madin–Darby bovine kidney (MDBK) cells, embryonic bovine tracheal (EBTr) cells, and fetal bovine testicular (FBT) cells were grown in Eagle's minimum essential medium (MEM; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), supplemented with 10 mM

N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, Gibco, Life Technologies, Burlington, ON, Canada), 1% nonessential amino acids (Gibco, Life Technologies), 50 µg/mL gentamycin (Gibco, Life Technologies), and 10% fetal bovine serum (FBS; Life Technologies). The cells were grown in a 37 °C incubator supplied with 5% CO2.

WT BoHV-1, ΔgM-BoHV-1 (BoHV-1 devoid of the *U_L10* gene), and gM revertant BoHV-1 (ΔgM Rev BoHV-1) were propagated in MDBK cells. The titers were determined in MDBK cells in 24-well plates after overlay with 0.8% UltraPure low-melting agarose (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) in MEM, and the virus stocks were stored at −80 °C.

Cell monolayers at 85–90% confluency were infected with WT BoHV-1, ΔgM-BoHV-1, or ΔgM Rev BoHV-1 at different multiplicities of infection (MOIs) in MEM. Virus was removed and replaced with MEM containing 2% FBS after 1.5 h. Cells and culture media were collected at different time points and centrifuged at 3000× g for 30 min at 4 °C.

5.5.2 Plasmids

pFLAG-VP8, pFLAG-ΔNLS VP8, and plasmids encoding truncated versions of VP8 (pU_L47) with FLAG tag were used as described previously (Labiuk, Babiuk et al. 2009). pHA-gM was constructed by amplification of the *U_L10* gene from the BoHV-1 genome using 3′ATTGAATTCATGGCGGGCTCCGCGCAGCCTG 5′ and 3′ ATTTCTAGATTTGAC-GTGCGCGGGGGGTGGG 5′ primers, and ligation into XbaI- and EcoRI-digested pcDNA3.1HA (Addgene, Water-town, MA, USA).

pHA-gM deletion mutants were constructed similarly by amplifying desired segments of the *U_L10* gene from the BoHV-1 genome using primers shown in Table 5.1. The amplified segments were ligated into pcDNA3.1HA (Addgene) digested with XbaI and EcoRI.

Table 5.1. Primer list for construction of plasmids encoding HA-tagged gM truncated versions.

Plasmid	Forward Primer	Reverse
pHA-gM (70–438aa)	3′ATTTCTAGAATGGGCGCGCGCCACCCGGCGCT5′	3′ATGATATCTTTGACGTGCGCGGGGGGTGGG5′
pHA-gM (140–438aa)	3′ATTTCTAGAATGACCGCCGGGCTGCCCGGCGC5′	3′ATGATATCTTTGACGTGCGCGGGGGGTGGG5′
pHA-gM (210–438aa)	3′ATTTCTAGAATGCTGGGGCTGTCGCTGGCACA5′	3′ATGATATCTTTGACGTGCGCGGGGGGTGGG5′
pHA-gM (300–438aa)	3′ATTTCTAGAATGGCCCCCCGGGCTGCCGCTAG5′	3′ATGATATCTTTGACGTGCGCGGGGGGTGGG5′
pHA-gM (250–438aa)	3′ATTTCTAGATGGTCGCCGGCGTGACGG5′	3′ATGATATCTTTGACGTGCGCGGGGGGTGGG5′
pHA-gM (140–264aa)	3′ATTTCTAGATGGCGCTGGCGGCCT5′	3′ATGATATCTTTCAAAAAGAGCACGGCGG5′

5.5.3 Antibodies

Antibodies, including mouse monoclonal VP8-specific antibody (clone 1G4 2G2) (Labiuk, Babiuk et al. 2009) and rabbit polyclonal VP5-specific antibody (Lobanov, Maher-Sturgess et al. 2010), were used as described previously. Antibodies specific for glycoproteins B, C, D (Li, van Drunen Littel-van den Hurk et al. 1995), and H (Marshall, Israel et al. 1988) were used as described. Antibodies specific for glycoproteins E, N, and L were produced in-house by using synthetic peptides according to the protocol described by Labiuk et al. (Labiuk, Babiuk et al. 2009). To produce rabbit gM-specific antibodies, a blunt end repaired (MScI-PpuMI) 265 bp DNA fragment of the U_L10 Orf (containing C-terminal 80 amino acids) was ligated to XmaI-digested (blunt-end repaired) plasmid pGEX-2T DNA creating plasmid pGEX-U_L10. GST-U_L10 fusion protein was produced by induction of plasmid pGEXUL10 transformed E.coli with 0.1 mM isopropyl -b-D-thiogalactoside (IPTG) and purified by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Rabbits were immunized subcutaneously with 300 µg of fusion protein in Freund's complete adjuvant. The rabbits were boosted with 100 µg of fusion protein in Freund's incomplete adjuvant at 4, 8, and 12 weeks after primary injection. The rabbits were bled 10 days after the last boost and sera were collected.

HA tag-specific rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) and FLAG tag-specific mouse monoclonal antibody (Sigma-Aldrich Canada Ltd.)

were used to detect HA-tagged and FLAG-tagged proteins, respectively. Mouse monoclonal antibody Golgi 58K and rabbit polyclonal antibody GolgB1 specific for Cis-Golgi (Sigma-Aldrich) were used to detect the Golgi apparatus. For Western blotting, IRDye 680RD goat anti-rabbit IgG, IRDye 680 RD goat anti-mouse IgG, IRDye 800RD goat anti-rabbit IgG, and IRDye 800RD goat anti-mouse IgG (Li-Cor Biosciences, Lincoln, NE, USA) were used. Alexa 488-conjugated goat anti-mouse IgG and Alexa 633-conjugated goat anti-rabbit IgG (Invitrogen/Thermo Fisher Scientific) were used for immunofluorescent staining.

5.5.4 Transfection

Monolayers of EBTr cells at 80–90% confluency were transfected with a total amount of 3 µg plasmid(s) (pFLAG-VP8, pHA-gM, pFLAG-VP8 deletion mutants, pHA-gM deletion mutants, pFLAG-ΔNLS VP8) using Lipofectamine 3000 transfection reagent (Invitrogen/Thermo Fisher Scientific), and cells were collected at 24–48 h post transfection.

5.5.5 Preparation of Cell Lysates

Infected MDBK cells and transfected EBTr cells were collected using ice-cold phosphate-buffered saline, pH 7.4 (PBS) (Gibco, Life technologies), at different time intervals and centrifuged at 8000× g for 10 min. Cell pellets were lysed for 40 min on ice in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, pH 7.4) and protease inhibitor cocktail (Sigma-Aldrich Canada Ltd.) in a 10:1 v/v ratio. Cell lysates were clarified by centrifugation at 12,000× g for 10 min at 4°C. The supernatants were collected and stored at −20 °C for further use.

5.5.6 Immunoprecipitation and Western Blotting

Infected cell lysates (prepared as described above) were incubated with VP8-specific or gM-specific antibodies overnight at 4 °C, followed by Protein G Sepharose Fast Flow beads (GE HealthCare, Niskayuna, NY, USA) for 5 h at 4 °C. Transfected cell lysates were

incubated with Anti-FLAG M2 Affinity Gel (Sigma-Aldrich Canada Ltd.) or anti-HA agarose (Pierce/Thermo Fisher Scientific) and incubated at 4 °C overnight. Lysates were washed 3 to 4 times with wash buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 8) and eluted with SDS loading dye at 95 °C for 2 min. SDS loading dye was also added to a fraction of the whole cell lysates, which were then treated at 95 °C for 2 min before being used as input control. These samples were subjected to SDS-polyacrylamide gel electrophoresis on 8% or 15% gels or 4–15% gradient gels and transferred to 0.45 µm nylon membranes (Bio-Rad, Hercules, CA, USA) at 100 Volts for 1 h. This was followed by blocking the membrane with 3% blotto (3 g milk powder in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% w/v Tween 20, pH 7.4)) for 1 h and incubation with respective antibodies overnight. The following day, membranes were washed with TBST and incubated with IRDye 680RD goat anti-mouse IgG or IRDye 800RD goat anti-rabbit IgG (LI-COR Biosciences, Lincoln, NB, USA) for 2–3 h. An Odyssey Infrared Imaging System and Odyssey 3.0.16 application software (LI-COR Bio-sciences) were used for detection and analysis. Quantification of the protein bands was performed by using Image Studio Lite version 5.2. Intensities of the protein bands were reported in comparison to the intensities of the protein bands of WT BoHV-1.

5.5.7 Construction of Recombinant Virus

Plasmids were constructed by use of primers listed in Table 2. A pEGFP-N1 plasmid was cut with *NheI* and *AgeI* and self-ligated using T4 polymerase to remove the multiple cloning site. The GFP cassette from the resulting plasmid was amplified by primer 1 and primer 2 and cloned into pUC57 cut with *BamHI* and *KpnI* to construct pUC-GFP. Three prime regions of the *U_L10* gene were amplified from the BoHV-1 genome and cloned into pUC-GFP, which was then digested with *KpnI* and *EcoRI* to construct pGFP-U_L10. To mutate the *U_L10* start codon, two segments were amplified using pairs of primers 5, 6 and primers 7, 8. These segments were later joined together using primers 5 and 7. The joined segment was cloned into the pUC-U_L10 vector digested with *HindIII* and *BamHI*, which

resulted in pGFP-U_L10del. Primers 3 and 5 were used to amplify the modified *U_L10* region from pGFP-U_L10del, and the resultant PCR fragment was transfected with the WT BoHV-1 genome for homologous recombination in FBT cells using Lipofectamine 3000 (Invitrogen). Viral plaques expressing GFP were plaque-purified and ΔgM BoHV-1 stocks were prepared. A schematic for the construction of ΔgM BoHV-1 is shown in Figure 5.1

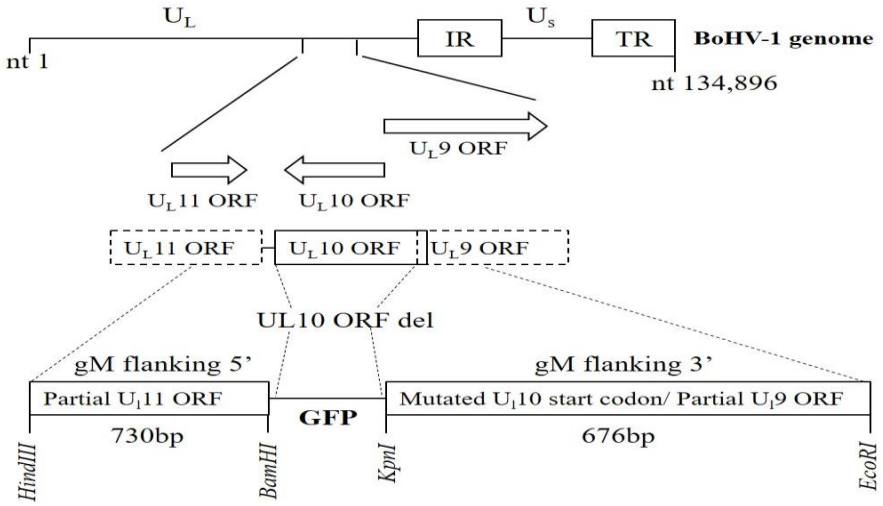


Figure 5.1. Schematic of the plasmid constructed for deletion of the *U_L10* gene. On top, the BoHV-1 genome consisting of unique long (U_L) and unique short (U_S) regions along with internal repeats (IR) and terminal repeats (TR) is depicted. The localization of *U_L9*, *U_L10* (gM), and *U_L11* genes is shown. In the constructed plasmid, the gM gene is deleted and GFP is inserted in that position with the insertion of *BamHI* and *KpnI* sites. The GFP was flanked with a 730bp 5′ gM flanking region and 676 bp 3′ gM flanking region along with a mutated start codon for *U_L10*.

Table 5.2. Primer list for construction of ΔgM BoHV-1 and ΔgM Rev BoHV-1 recombinant viruses.

Primer Name	Primer Sequence
Primer 1	3′GGAGGTACCGTATTACCGCCATGCATTAG5′
Primer 2	3′GAGGGATCCTGCCGATTTCGGCCTATTGG5′
Primer 3	3′GCGGAATTCGCGCTGCATCTCGTCACTTTCATCG5′
Primer 4	3′GAAGGTACCCGCCAACCATACCGCTAAGGAGACC5′
Primer 5	3′GAGAAGCTTCGTAAAGCTGCGCCGACAGGAG5′
Primer 6	3′CTGCGCGGAGCCCGCGATGACGGCAAC5′
Primer 7	3′GAAGGATCCCGCCAACCATACCGCTAAGGAGACC5′
Primer 8	3′GGCGTTGCCGTCATCGCGGGCTCCGCGCAG5′

For construction of ΔgM Rev BoHV-1, the *U_L10* gene with the flanking homologous regions was amplified from the WT BoHV-1 genome using primers 3 and 5, and the amplified product was transfected with the ΔgM BoHV-1 genome for homologous recombination in FBT cells. Plaques that did not express GFP were plaque purified to prepare ΔgM Rev BoHV-1 stocks.

5.5.8 Viral Growth Kinetics

MDBK cells were seeded in 35 mm dishes and infected at 80–90% confluency with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1 at a MOI of 1. Cells and culture media were collected at 0, 5, 10, 15, 20, 25, 30, and 35 h post infection. Cells were put through 2 freeze–thaw cycles to release the virus. The titers of the virus released from the cells (intracellular) and the virus from the culture media (extracellular) were determined in MDBK cells in a 24-well plate at different time points.

5.5.9 Confocal Microscopy

Cells were seeded in 2-well Permanox Lab-Tek chamber slides (Nunc, Thermo Fisher) and infected at 80–90% confluency with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1 at various MOIs. Cells were fixed at different times post infection with 4% paraformaldehyde (Sigma-Aldrich Canada Ltd.), washed with phosphate buffered saline (10 mM Na2HPO4, 1.8 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS)), and then blocked overnight with 1% goat serum. The cells were permeabilized the following day with 0.1% Triton X-100. Subsequently, they were incubated for 2–3 h at room temperature (22 ± 1 °C) with respective primary antibodies prepared in 1% goat serum, followed by Alexa 488-conjugated goat anti-rabbit or goat anti-mouse IgG, Alexa 633-conjugated goat anti-rabbit or goat anti-mouse IgG, or both Alexa 488- and Alexa 633-conjugated IgG (Invitrogen, Thermo Fisher), for 1–2 h at room temperature. Finally, the cells were treated with Prolong Gold with DAPI (Invitrogen, Thermo Fisher). A Leica SP8

confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to analyze the cells.

5.5.10 Transmission Electron Microscopy (TEM)

Monolayers of 80–90% confluent MDBK cells were infected with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1 at a MOI of 5. When all the cells showed a rounded morphology, the monolayers were washed with PBS (Gibco, Life Technologies) and fixed with 2% glutaraldehyde (Electron microscopy Sciences, Hatfield, PA, USA) and 0.1M sodium cacodylate (NaCac) (Canemco, Gore, QC, Canada), pH 7.3, for 3–5 h at 4 °C. The fixative along with the cells were collected and the remaining cells were gently scraped off, and the suspension was centrifuged at 300× g for 5 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 0.1 M NaCac. The fixed cells were then centrifuged, and the pellet was mixed with warm 1% low-melting agarose. The cells were centrifuged again and placed in the refrigerator to allow the agarose to solidify. Subsequently, the agarose was removed from the centrifuge tubes and the excess agarose was removed from the cell pellets. The pellets were then placed in 0.1M NaCac at 4° overnight. Samples were fixed in freshly prepared osmium tetraoxide (Fisher scientific) (1% OsO4 in 0.1M NaCac), for 1 h at room temperature. Samples were rinsed with water, dehydrated, and stained with ethanol and uranyl acetate (UrAc) (Greenfield Global, Brampton, ON, Canada) at room temperature as follows: 50% ethanol, 5–10 min; UrAc in 70% ethanol, 1 h; 70% ethanol, 5–10 min; 95% ethanol, 5–10 min; and three times with 100% ethanol, 5–10 min. This was followed by three rinses in propylene oxide. Subsequently, infiltration in Epon/Araldite was performed as follows: 1 part Epon/Araldite (Electron Microscopy Sciences); 2 parts propylene oxide (Thermo fisher) for 30 min: 2 parts Epon/Araldite: 1 part propylene oxide for 1–2 h; and pure Epon/Araldite overnight. Samples were then oriented in molds and fresh Epon/Araldite was added. The samples were polymerized at 60 °C for 24 h. Blocks were sectioned to around 90 nm with a diamond

knife and were placed on copper grids for observation under the Hitachi HT7700 (Tokyo, Japan) transmission electron microscope.

5.6 Results

5.6.1 Glycoprotein M (pU_L10) Interacts with VP8 (pU_L47)

Previously, we showed that VP8 (pU_L47) localizes to the Golgi during viral infection but not after transfection, suggesting that a viral component is involved in its translocation to the Golgi. To identify the component(s) responsible for the translocation of VP8 (pU_L47) from the cytoplasm to the Golgi, a potential interaction of VP8 (pU_L47) with one of the BoHV-1 glycoproteins was investigated. MDBK cells were infected with WT BoHV-1 at a MOI of 5, and the cells were collected at 18 h post infection. Cell lysates were incubated with VP8-specific monoclonal antibodies overnight at 4°C, followed by Protein G Sepharose beads and elution with SDS loading dye. The cell lysates were analyzed by Western blotting and probing with antibodies specific to glycoproteins B (pU_L27), C (pU_L44), D (pU_S6), E (pU_S8), M (pU_L10), N ($pU_L49.5$), and L (pU_S1). Only glycoprotein M (pU_L10) was found to co-precipitate with VP8 (pU_L47) (Figure 5.2), implying that gM (pU_L10) is the most probable candidate aiding in the translocation of VP8 (pU_L47) from the cytoplasm to the Golgi.

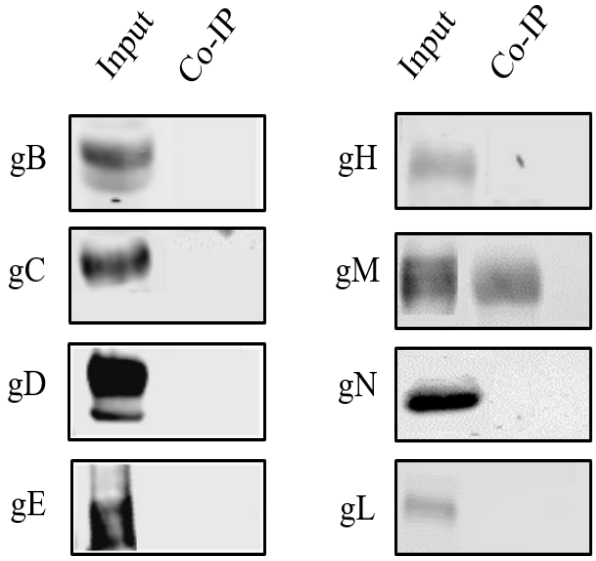


Figure 5.2. Identification of the glycoprotein interacting with VP8 (pU_L47). Lysates from MDBK cells infected with WT BoHV-1 were incubated with rabbit VP8-specific antibody followed by Protein Sepharose G beads. After elution by SDS loading dye, the samples were subjected to SDS-polyacrylamide gel electrophoresis on an 8% (gB (pU_L27), gC (pU_L44), gD (pU_S6), gE (pU_S8), gH (pU_L22), gM (pU_L10)) or 15% (gN ($pU_L49.5$), gL (pU_S1)) gel and transferred to 0.45 µm nylon membranes. Glycoproteins were detected with antibodies specific for gB (pU_L27), gC (pU_L44), gD (pU_S6), gE (pU_S8), gH (pU_L22), gM (pU_L10), gN ($pU_L49.5$), or gL (pU_S1), followed by IRDye 680RD goat anti-mouse IgG and IRDye 800RD goat anti-rabbit IgG. Glycoprotein M (pU_L10) was found to precipitate with VP8 (pU_L47). A fraction of the whole cell lysate was used as input control. Glycoproteins B (pU_L27), gC (pU_L44), gD (pU_S6), gE (pU_S8), gH (pU_L22), gM (pU_L10), gN ($pU_L49.5$), and gL (pU_S1) were all present in the infected cell lysate.

To confirm the interaction between VP8 (pU_L47) and gM (pU_L10), MDBK cells were infected with WT BoHV-1 at a MOI of 5, and the cells were collected at 18 h post infection. Subsequently, the cells were lysed and incubated with antibodies specific for either gM (pU_L10) or VP8 (pU_L47), followed by binding to Protein G Sepharose beads and elution in SDS loading dye at 95 °C. The eluates were analyzed by Western blotting, and antibodies specific for VP8 (pU_L47) and gM (pU_L10) were used for detection. This demonstrated that gM (pU_L10) precipitates together with VP8 (pU_L47) and vice versa (Figure 5.3), further supporting that there is an interaction between VP8 (pU_L47) and gM

(pU_L10). After incubation of the lysates with only Protein G Sepharose beads without antibodies, no gM (pU_L10) or VP8 (pU_L47) was found to be present.

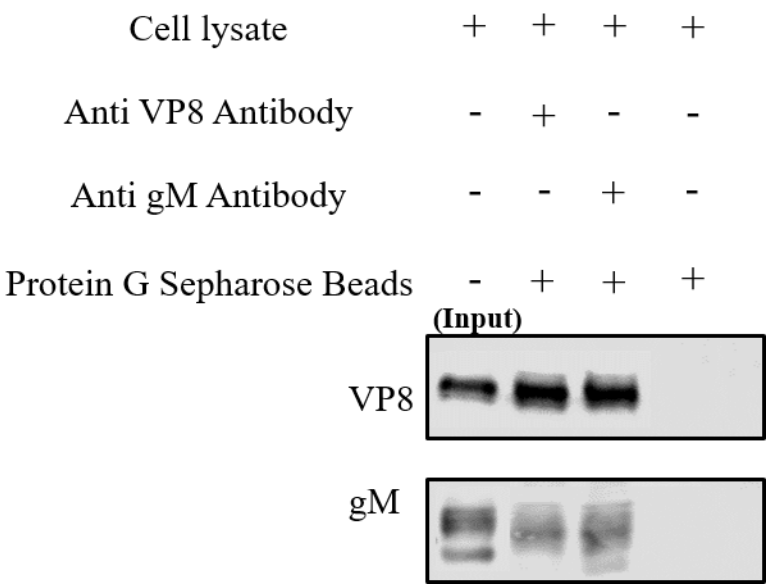


Figure 5.3. VP8 (pU_L47) and gM (pU_L10) interact in WT BoHV-1-infected cells. Cell lysates from MDBK cells infected with WT BoHV-1 were incubated with VP8-specific or gM-specific antibodies, followed by Protein Sepharose G beads or with Protein G Sepharose without antibodies. After elution by SDS loading dye, the samples were subjected to SDS-polyacrylamide gel electrophoresis on an 8% gel and transferred to 0.45 µm nylon membranes. A fraction of the whole cell lysates was used as input control. VP8 (pU_L47) and gM (pU_L10) were detected with monoclonal anti-VP8 and rabbit anti-gM antibodies, followed by IRDye 680RD goat anti-mouse IgG and IRDye 800RD goat anti-rabbit IgG, respectively.

To confirm that the interaction between VP8 (pU_L47) and gM (pU_L10) is independent of other viral factors, ΔNLS VP8-FLAG and gM-HA plasmids were co-transfected into EBTr cells and incubated either with anti-FLAG or anti-HA resins before being eluted in SDS loading dye at 95 °C. Because VP8 (pU_L47) moves into the nucleus in transfected cells and cannot move to the cytoplasm in the absence of pU_S3, ΔNLS VP8-FLAG was used, which expresses VP8 (pU_L47) without NLS, such that it stays cytoplasmic. The eluates were then subjected to Western blotting; antibodies specific for FLAG and HA tags were used to detect the ΔNLS VP8-FLAG and gM-HA proteins,

respectively. As shown in Figure 5.4, gM-HA protein was detected in the eluate after incubation of the co-transfection lysate with anti-FLAG resin, while VP8-FLAG protein was detected in the eluate after incubation of the co-transfection lysate with anti-HA resin. No VP8 was found after incubation with anti-HA beads in absence of gM-HA, and no gM was found after incubation with anti-FLAG beads in absence of VP8-FLAG. This experiment confirms that VP8 (pU_L47) and gM (pU_L10) interact with each other and that this interaction is independent of other viral factors.

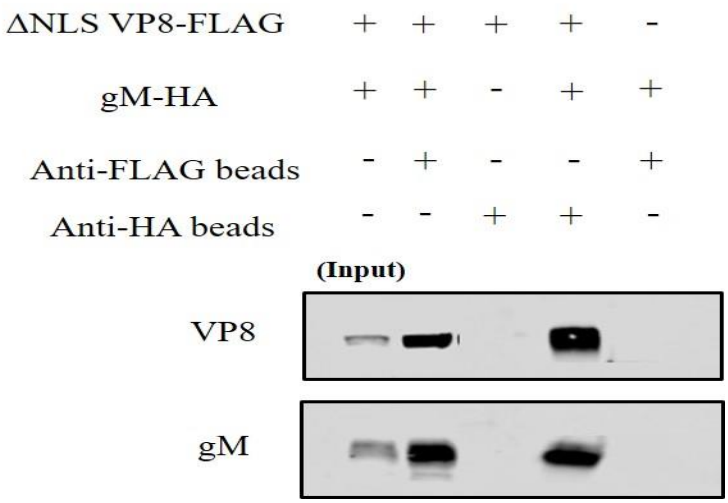


Figure 5.4. VP8-FLAG and gM-HA interact with each other in co-transfected cells. EBTr cells were co-transfected with ΔNLS VP8-FLAG and pgM-HA, or with ΔNLS VP8-FLAG or pgM-HA, and the lysates were incubated with anti-FLAG or anti-HA beads. After elution by SDS loading dye, the samples were subjected to SDS-polyacrylamide gel electrophoresis on an 8% gel and transferred to 0.45 µm nylon membranes. A fraction of the whole cell lysates was used as input control. VP8-FLAG and gM-HA were detected with monoclonal anti-FLAG and anti-HA antibodies, respectively, followed by IRDye 680RD goat anti-mouse IgG.

5.6.2 Glycoprotein M (pU_L10) and VP8 (pU_L47) Co-localize at the Golgi apparatus

In order for gM (pU_L10) to be the glycoprotein responsible for translocation of VP8 (pU_L47) to the Golgi, VP8 (pU_L47) and gM (pU_L10) must co-localize at the Golgi. To investigate this, MDBK cells were infected with WT BoHV-1 at a MOI of 5, fixed with paraformaldehyde at 7 h post infection, and analyzed by confocal microscopy. Panel A of

Figure 5.5 displays VP8 (pU_L47) and Golgi, and the merged image shows the localization of VP8 (pU_L47) in the Golgi at 7 h post infection. Panel B depicts the localization of gM (pU_L10) in the Golgi at 7 h post infection. The merged image in Panel C indicates VP8 (pU_L47) co-localizing with gM (pU_L10) at 7 h post infection. From this three-way study, it can be confirmed that VP8 (pU_L47) and gM (pU_L10) co-localize in the Golgi at 7 h post infection, which suggests that gM (pU_L10) supports the translocation of VP8 (pU_L47) to the Golgi after its phosphorylation by pU_S3 and export from the nucleus to the cytoplasm.

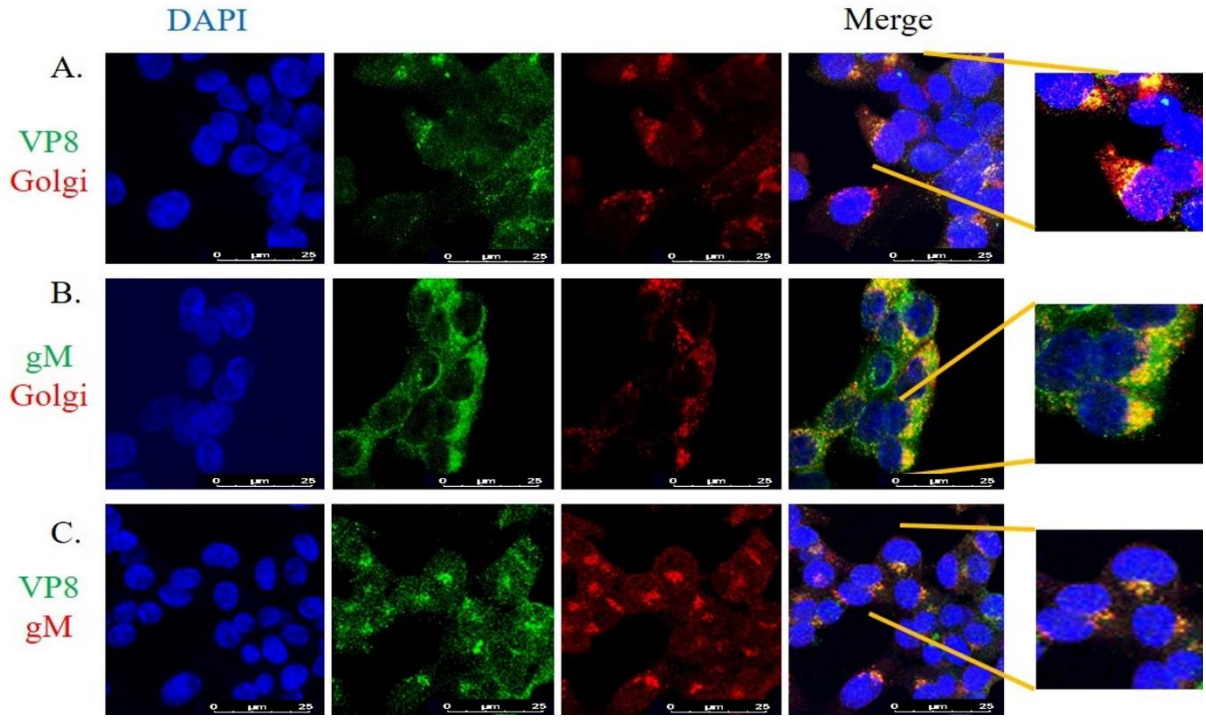


Figure 5.5. Co-localization of VP8 (pU_L47) and gM (pU_L10) at the Golgi in cells infected with WT BoHV-1. MDBK cells infected with WT BoHV-1 were fixed and incubated with antibodies specific for VP8 (pU_L47), gM (pU_L10), or Golgi. Alexa 488-conjugated goat anti-rabbit or goat anti-mouse IgG and Alexa 633-conjugated goat anti-rabbit or goat anti-mouse IgG were used to detect the primary antibodies. (A) VP8 (green) and Golgi (red), and localization of VP8 (pU_L47) in the Golgi in the merged image (yellow). (B) Glycoprotein M (green) and Golgi (red), and localization of gM (pU_L10) in the Golgi in the merged image (yellow). (C) VP8 (green) and gM (red), and co-localization of VP8 (pU_L47) and gM (pU_L10) as shown in the merged image (yellow). The nuclei were stained with Prolong gold DAPI. Cells in the boxes are magnified 5×. The cells were examined with a Leica SP8 confocal microscope. 1 cm represents 5µm in the scale.

To confirm the involvement of gM (pU_L10) in the translocation of VP8 (pU_L47) to the Golgi, three sets of EBTr cells were transfected with ΔNLS VP8-FLAG or gM-HA plasmids individually, or co-transfected with gM-HA and ΔNLS VP8-FLAG plasmids. Transfected cells were fixed at 24 h post transfection. Antibodies specific for VP8 (pU_L47), gM (pU_L10), and Golgi were used to identify ΔNLS VP8-FLAG, gM-HA, and the Golgi apparatus, respectively. In Figure 5.6, panel A shows the distribution of VP8 (pU_L47) in cells transfected with ΔNLS VP8-FLAG plasmid; in the absence of gM (pU_L10), localization of VP8 (pU_L47) was predominantly cytoplasmic. Panel B shows the localization of gM (pU_L10) in the Golgi in cells transfected with gM-HA plasmid. In the cells co-transfected with ΔNLS VP8-FLAG and gM-HA plasmids, the merged images show localization of ΔNLS VP8-FLAG (Panel C) and gM-HA (Panel D) in the Golgi. In panel E, co-localization of ΔNLS VP8-FLAG and gM-HA is shown. These results confirm that the presence of gM-HA is sufficient to mediate the translocation of VP8 (pU_L47) to the Golgi.

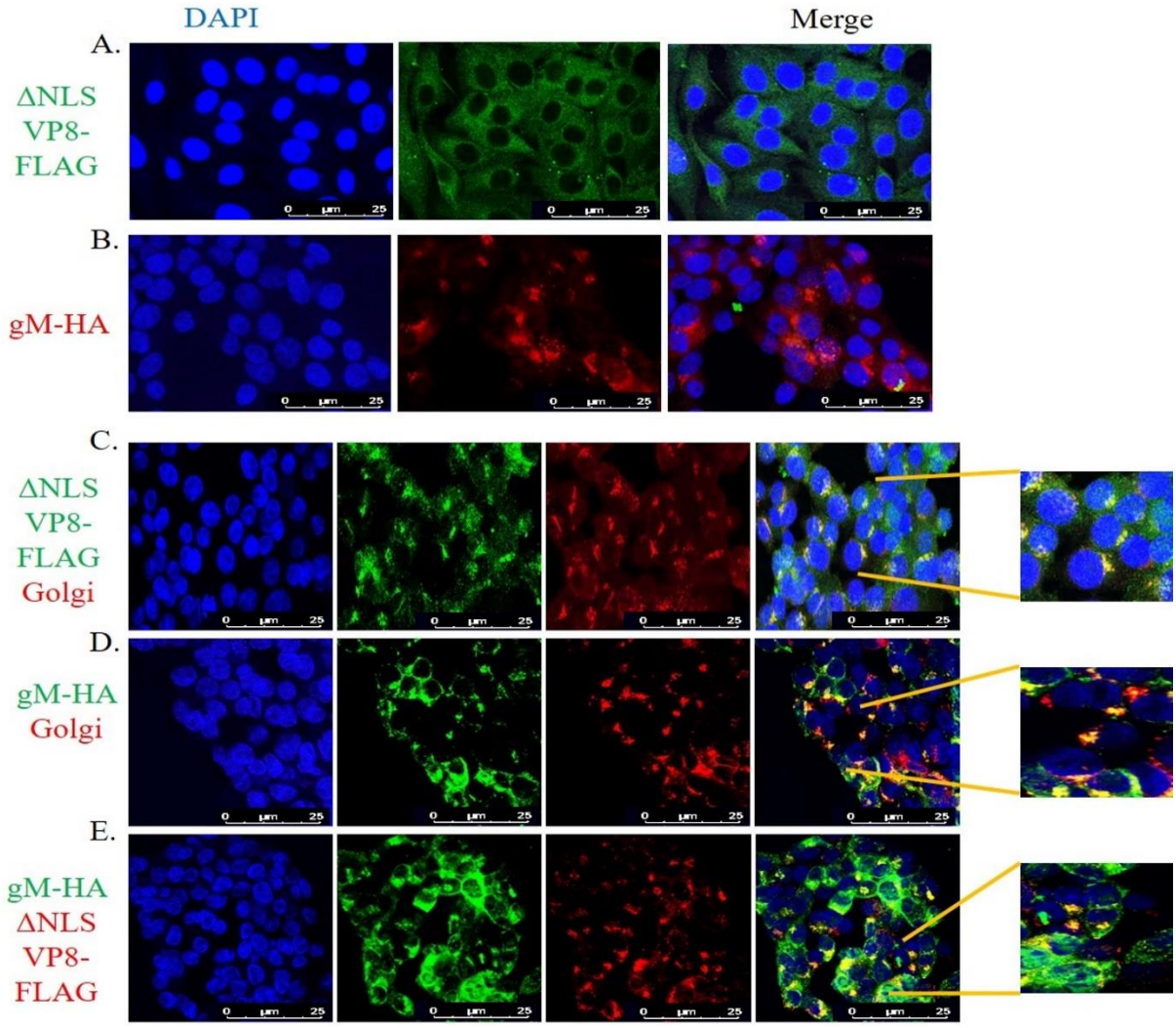
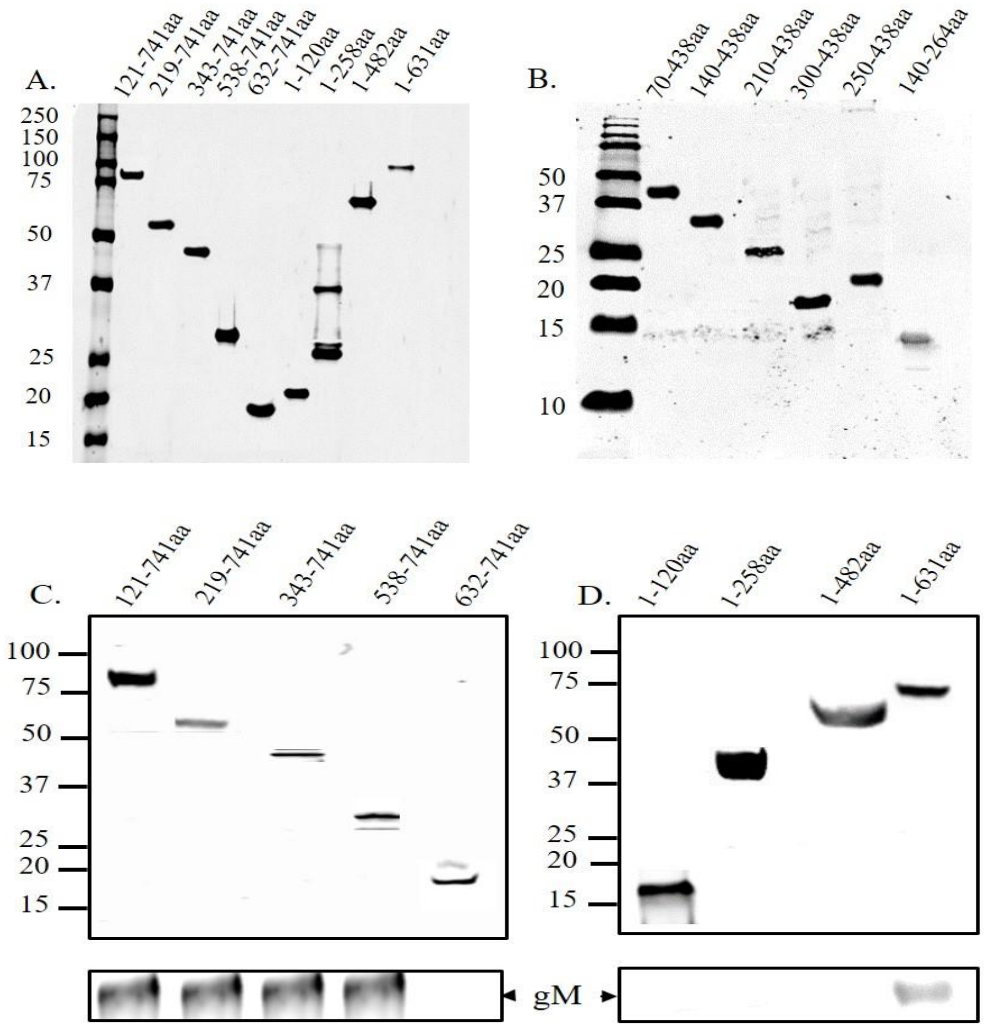


Figure 5.6. Localization of ΔNLS VP8-FLAG and gM-HA protein in cells transfected with pΔNLS VP8-FLAG and pgM-HA individually (A,B) or in combination (C–E). EBTr cells transfected with ΔNLS VP8-FLAG and/or gM-HA were fixed and incubated with antibodies specific for VP8 (pU_L47), gM (pU_L10), or Golgi. Alexa 488-conjugated goat anti-rabbit or goat anti-mouse IgG and Alexa 633-conjugated goat anti-rabbit or goat anti-mouse IgG were used to detect the primary antibodies. (A) Distribution of ΔNLS VP8-FLAG (green) in the cytoplasm. (B) Localization of gM-HA (red) in the Golgi. (C) ΔNLS VP8-FLAG (green), Golgi (red), and localization of VP8 in the Golgi in the merged image (yellow). (D) gM-HA (green), Golgi (red), and localization of gM in the Golgi in the merged image (yellow). (E) gM-HA (green) and ΔNLS VP8-FLAG (red), and co-localization of VP8 (pU_L47) and gM (pU_L10) as shown in the merged image (yellow). The nuclei were stained with Prolong gold DAPI. Cells in the boxes are magnified 5×. The cells were examined with a Leica SP8 confocal microscope. 1 cm represents 5μm in the scale.

5.6.3 A Domain between Amino Acids 538–632 in VP8 (pU_L47) Interacts with a Domain between Amino Acids 210–300 in gM (pU_L10)

To identify the interaction domains of VP8 (pU_L47) and gM (pU_L10), several C-terminal and N-terminal deletions were made in the VP8 (pU_L47) and gM (pU_L10) proteins, and the truncated versions were cloned into a plasmid with FLAG and HA tag, respectively. EBTr cells were co-transfected with the VP8 (pU_L47)-deletion mutants and full-length pgM-HA or gM-HA deletion mutants and full-length pVP8-FLAG and collected at 24 h post transfection. The expression of the truncated VP8 (pU_L47) and gM (pU_L10) proteins is shown in Figure 5.7A,B, respectively. Lysates from cells co-transfected with VP8 (pU_L47) deletion mutants and full-length gM-HA were incubated with anti-FLAG resin, and those from cells co-transfected with gM-HA deletion mutants and full-length VP8 FLAG were incubated with anti-HA resin. The lysates were then eluted in SDS loading dye at 95 °C. When the eluates were subjected to Western blotting, with HA- and FLAG-specific antibodies for detection, VP8 (pU_L47) was found to interact with amino acids 210–300 of gM (Figure 5.7C,D), while gM (pU_L10) interacted with amino acids 538–632 of VP8 (Figure 5.7E). Figure 5.8A, B show a schematic of the interaction between the respective truncated proteins and full-length proteins, and the interaction domains on VP8 (pU_L47) and gM (pU_L10), respectively. Prediction of the secondary structure of VP8 (pU_L47) and gM (pU_L47) using PsiPred (http://bioinf.cs.ucl.ac.uk/psipred, accessed on 18 May 2022) shows that the region of amino acids identified to be responsible for the interaction between VP8 (pU_L47) (538–632) (Figure 5.8C) and gM (pU_L47) (210–300) (Figure 5.8D) contain helix–coil–helix structures, which are known to be characteristic of functional domains (Dawson, Sillitoe et al. 2017).


A.
121-741aa
219-741aa
343-741aa
538-741aa
632-741aa
1-120aa
1-258aa
1-482aa
1-631aa
250
150
100
75
50
37
25
20
15
B.
70-438aa
140-438aa
210-438aa
300-438aa
250-438aa
140-264aa
50
37
25
20
15
10
C.
121-741aa
219-741aa
343-741aa
538-741aa
632-741aa
100
75
50
37
25
20
15
D.
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1-631aa
100
75
50
37
25
20
15
gM

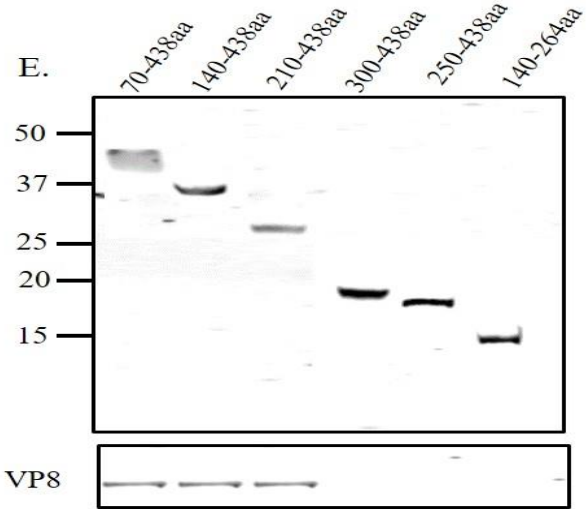
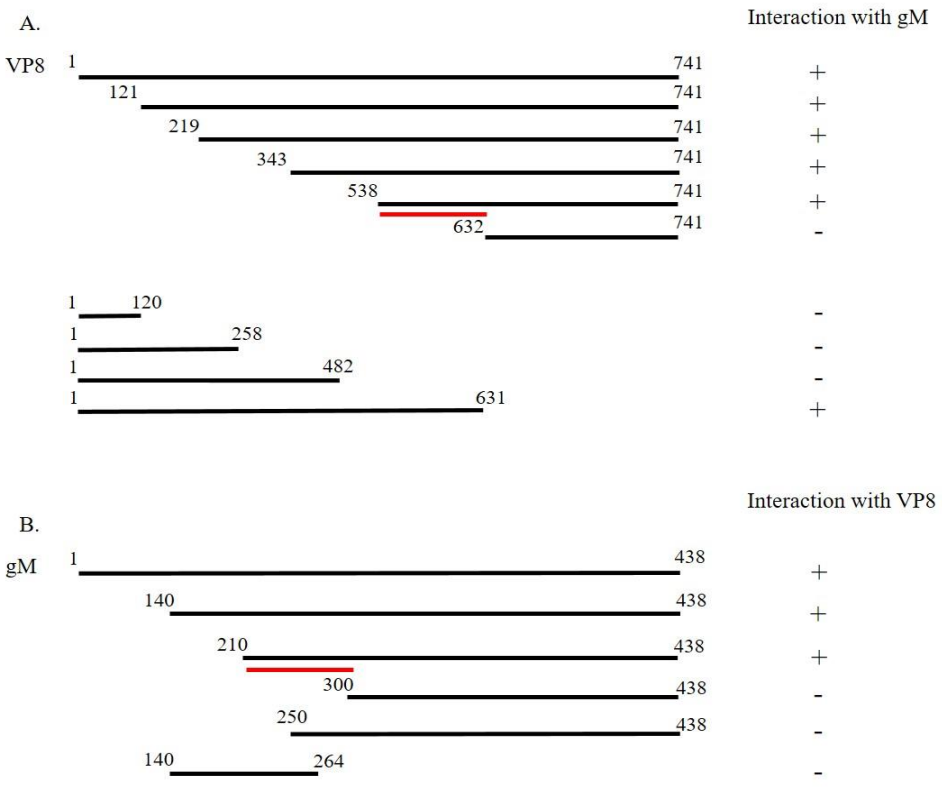


Figure 5.7. Identification of the interacting domains of VP8 (pU_L47) and gM (pU_L47). EBTr cells were co-transfected with FLAG-tagged truncated VP8 versions and pgM-HA or with HA-tagged truncated gM versions and pFLAG-VP8, and the lysates were either directly analyzed by SDS-PAGE (A,B) or incubated with anti-FLAG or anti-HA beads (C–E). After elution by SDS loading dye, the samples were subjected to SDS-polyacrylamide gel electrophoresis on a 4–15% gel and transferred to 0.45 µm nylon membranes. (A) Expression of truncated versions of VP8 protein. (B) Expression of truncated versions of gM protein. (C) Immunoprecipitation of VP8 protein versions by full-length gM-HA shows interaction of gM-HA with all N-terminal deletions except the deletion lacking amino acids 1–631. (D) Immunoprecipitation of C-terminal deletions by full-length gM-HA shows an interaction of gM-HA with VP8 protein consisting of 1–631 amino acids, but not with the other VP8 versions. (E) Immunoprecipitation of truncated gM-HA proteins with full-length VP8-FLAG shows an interaction of VP8-FLAG in the presence of the amino acids 70–438, 140–438, and 210–438 of gM-HA. Molecular weight markers (kDa) are shown in the left margins.


A.
Interaction with gM
VP8
1
741
+
121
741
+
219
741
+
343
741
+
538
741
+
632
741
-
1
120
-
1
258
-
1
482
-
1
631
+
B.
Interaction with VP8
gM
1
438
+
140
438
+
210
438
+
300
438
-
250
438
-
140
264
-

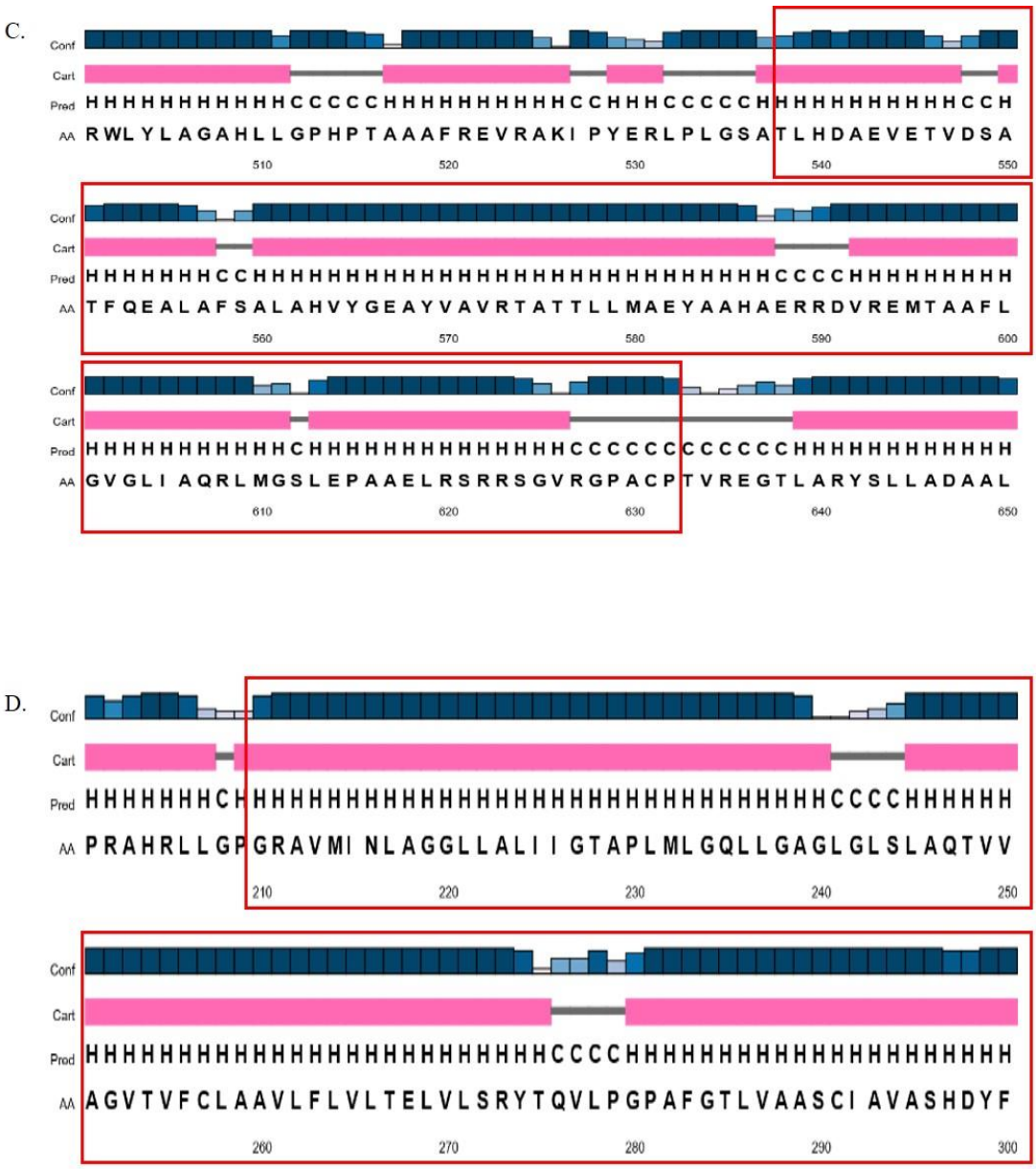


Figure 5.8. Representation of the domains of VP8 (pU_L47) and gM (pU_L10) that interact with each other. (A) Schematic representation of interaction between truncated versions of VP8-FLAG with full-length gM-HA. (B) Schematic representation of interaction between truncated versions of gM-HA with full-length VP8-FLAG. (C) Predicted secondary structure of the domain of VP8-FLAG (538–632) involved in the interaction with gM-HA. (D) Predicted secondary structure of the domain of gM-HA (210–300) involved in the interaction with VP8-FLAG.

5.6.4 The ΔgM BoHV-1 Titer Is Significantly Reduced and Its Egress Is Affected

To test the requirement of gM (pU_L10) for the translocation of VP8 (pU_L47) to the Golgi, ΔgM BoHV-1 and ΔgM Rev BoHV-1 were constructed. To first compare its replication efficiency to WT BoHV-1, the ΔgM BoHV-1 recombinant virus was tested in a one-step growth curve. This showed that the growth rate of ΔgM BoHV-1 was slower than that of WT BoHV-1. The intracellular and extracellular virus in ΔgM BoHV-1-infected cells showed a ~100-fold reduction in the titer when compared to that of WT BoHV-1 (Figure 5.9). No virus was detected intracellularly at 5 h post infection in ΔgM BoHV-1-infected cells (Figure 5.9A), and in the extracellular media up to 10 h post infection (Figure 5.9B), indicating a delay in viral assembly and particle egress from the cell.

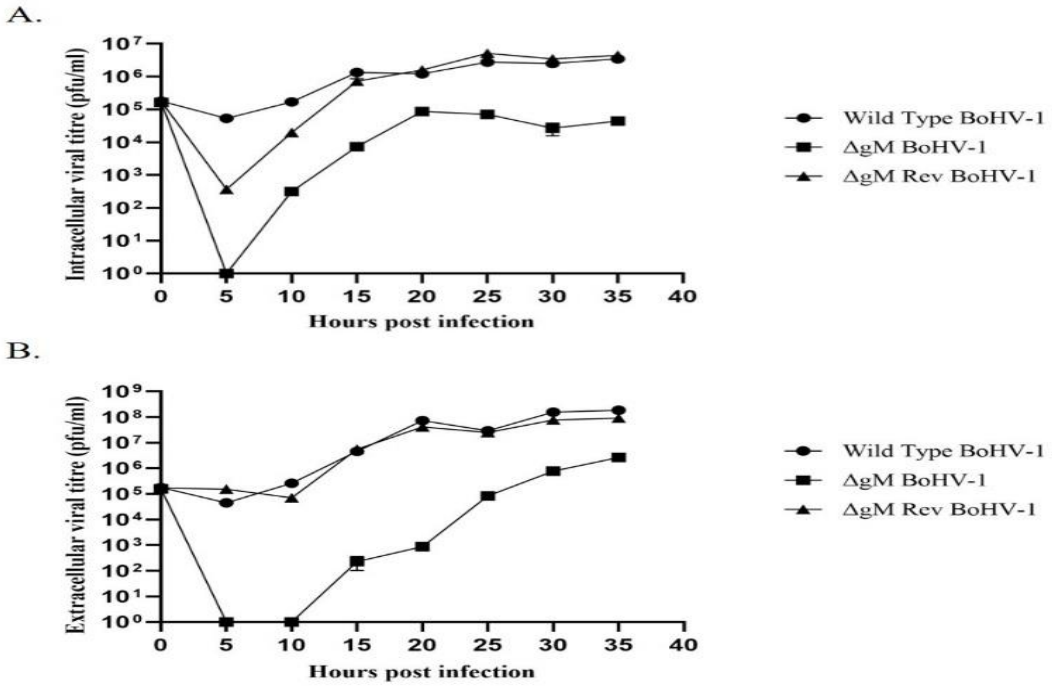
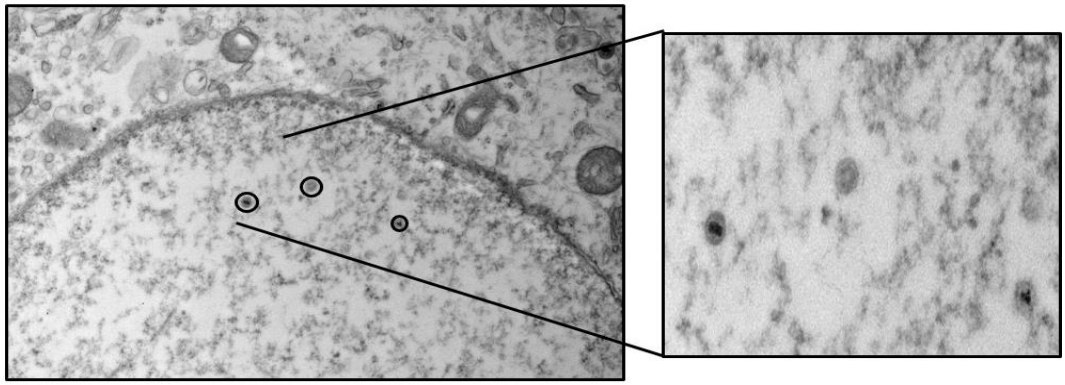


Figure 5.9. One-step growth curve of WT BoHV-1, ΔgM BoHV-1, and ΔgM Rev BoHV-1. MDBK cells were infected with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1 at a MOI of1, and cells and supernatant media were collected at different times after infection. (A) Viral titer of the intracellular virus. (B) Viral titer of the extracellular virus. The error bars indicate standard deviation. Time 0 hours post infection indicates input virus.

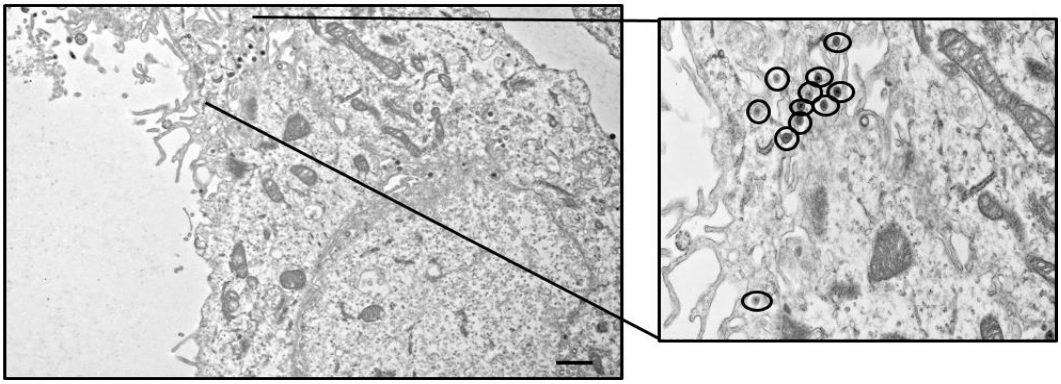
5.6.5 ΔgM BoHV-1 Shows Delayed Egress Compared to WT BoHV-1 and ΔgM Rev BoHV-1

As the one-step growth kinetics of ΔgM BoHV-1, WT BoHV-1, and ΔgM Rev BoHV-1 appeared to show a delay in egress of the virus in ΔgM BoHV-1-infected cells, TEM was performed for confirmation. MDBK cells were infected with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1 at MOI 5 and examined at 7 h post infection (Figure 5.10A–F). The mean values of the number of virus particles observed inside the nucleus and outside the cells in cells infected with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1 are shown in Figure 5.10 G,H, respectively. In the cells infected with WT BoHV-1, 2.14 ± 0.69 viral particles were observed inside the nucleus (Figure 5.10 A,G) and 12.14 ± 1.71 at the plasma membrane, egressing the cells (Figure 5.10 B,H). The ΔgM BoHV-1-infected cells showed 11.42 ± 0.79 virus particles inside the nucleus (Figure 5.10 C,G), and none were found at the plasma membrane (Figure 5.10 D,H). In ΔgM Rev BoHV-1-infected cells, the cells had 2.57 ± 1.07 virus particles inside the nucleus (Figure 5.10 E,G) in contrast to 11.14 ± 1.57 virus particles outside the plasma membrane (Figure 5.10 F,H), which is similar to a WT BoHV-1 infection. These observations showing more virus particles inside the nucleus than at the plasma membrane in ΔgM BoHV-1-infected cells when compared to the WT BoHV-1 or ΔgM Rev BoHV-1 infected cells further support the delay in egress and maturation of ΔgM BoHV-1 in infected cells.

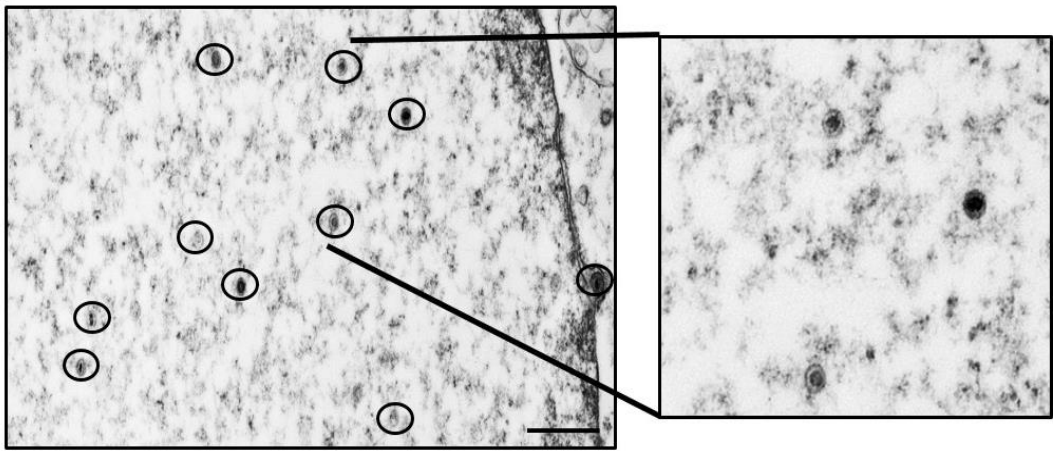
A.



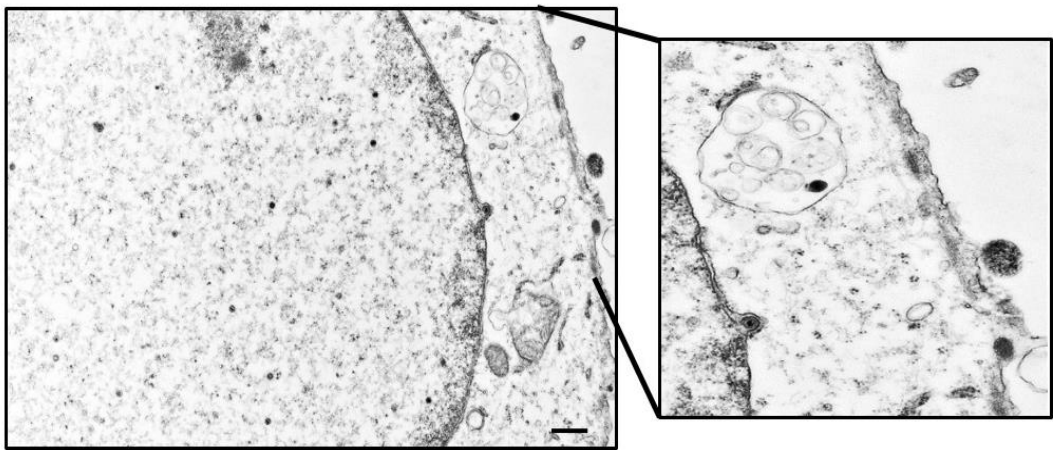
B.



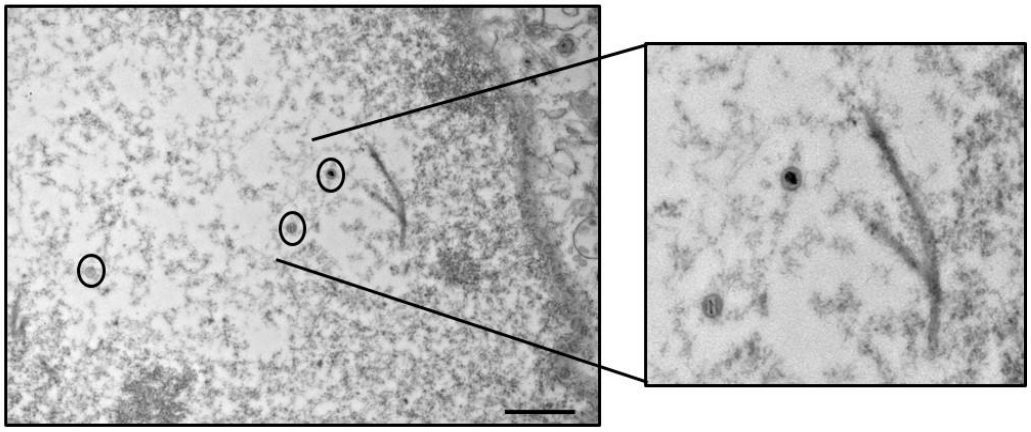
C.



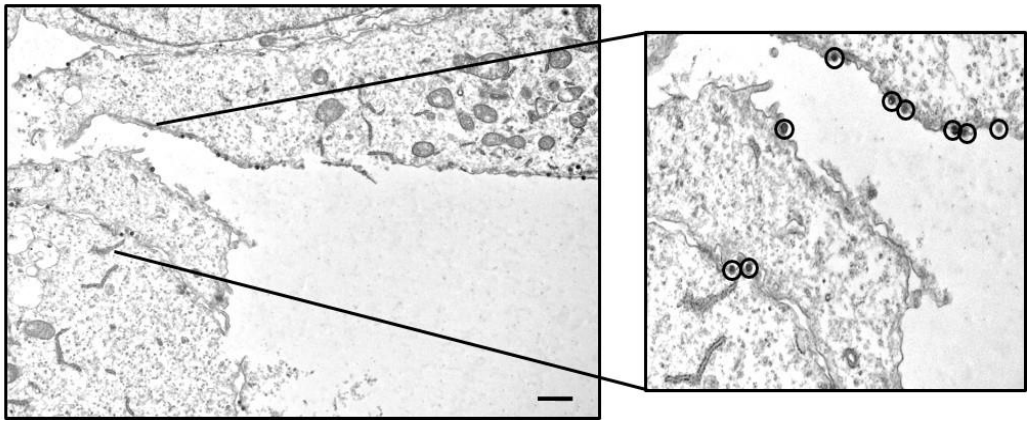
D.



E.



F.



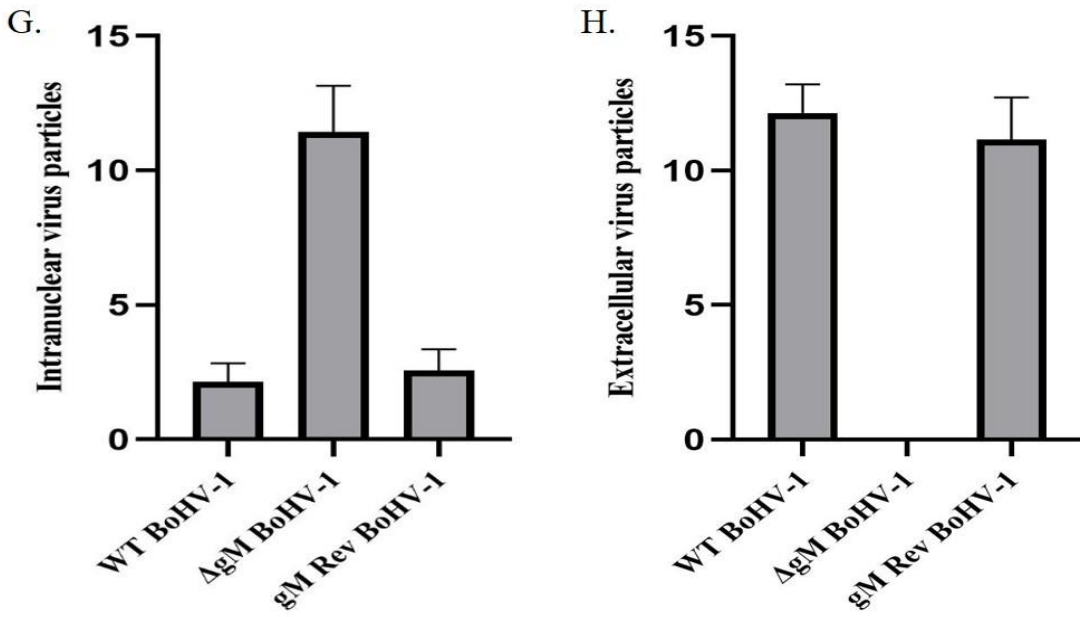


Figure 5.10. Transmission electron microscopy of cells infected with WT-BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1, and processed at 7 h post infection. (A) A 20K magnified image of the nucleus of the cells infected with WT BoHV-1. A part of the nucleus is magnified 50K. The circles show the virus particles inside the nucleus. (B) A 6K magnified image of the cell membrane of the cells infected with WT BoHV-1. A part of the plasma membrane is magnified 20K. The circles show the virus particles outside the membrane and in between cells. (C) A 20K magnified image of the nucleus of the cells infected with ΔgM BoHV-1. A part of the nucleus is magnified 50K. The circles show the virus particles inside the nucleus. (D) A 6K magnified image of the cell membrane of the cells infected with ΔgM BoHV-1. A part of the cell membrane is magnified 20K. The circles show the virus particles egressing the nucleus; no virus was present on or outside the cell membrane. (E) A 20K magnified image of the nucleus of the cells infected with ΔgM Rev BoHV-1. A part of the nucleus is magnified 50K. The circles show the virus particles inside the nucleus. (F) A 6K magnified image of the cell membrane of the cells infected with ΔgM Rev BoHV-1. A part of the cell membrane is magnified 20K. The circles show the virus particles outside the cell membrane. The bars represent 0.2 µm. Mean values of the number of virus particles (G) inside the nucleus and (H) outside the cells in MDBK cells infected with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1, observed by TEM. Cells were infected at an MOI of 5 and fixed and processed 7 h post infection. The mean number of virus particles inside the nucleus and outside the cells ± SD of seven cells per sample is shown.

5.6.6 VP8 (pU_L47) Does Not Localize to the Golgi in the Absence of gM (pU_L10)

MDBK cells were infected with ΔgM BoHV-1 or ΔgM Rev BoHV-1 at a MOI of 5 to check the localization of VP8 (pU_L47) to the Golgi at 7 h post infection. As shown in Figure 5.11 A, VP8 (pU_L47) was spread throughout the cytoplasm; accordingly, the merged image was devoid of any yellow, confirming that VP8 (pU_L47) does not localize to the Golgi when MDBK cells are infected with ΔgM BoHV-1. However, when MDBK cells were infected with ΔgM Rev BoHV-1 (Figure 5.11 B), the ability of VP8 (pU_L47) to localize into the Golgi was restored, as shown by the yellow Golgi in the merged image. The restoration of the ability of VP8 (pU_L47) to localize to the Golgi in the presence of gM (pU_L10) confirms the indispensable nature of gM (pU_L10) in the localization of VP8 (pU_L47) to the Golgi, and possibly indirectly in the packaging of VP8 (pU_L47).

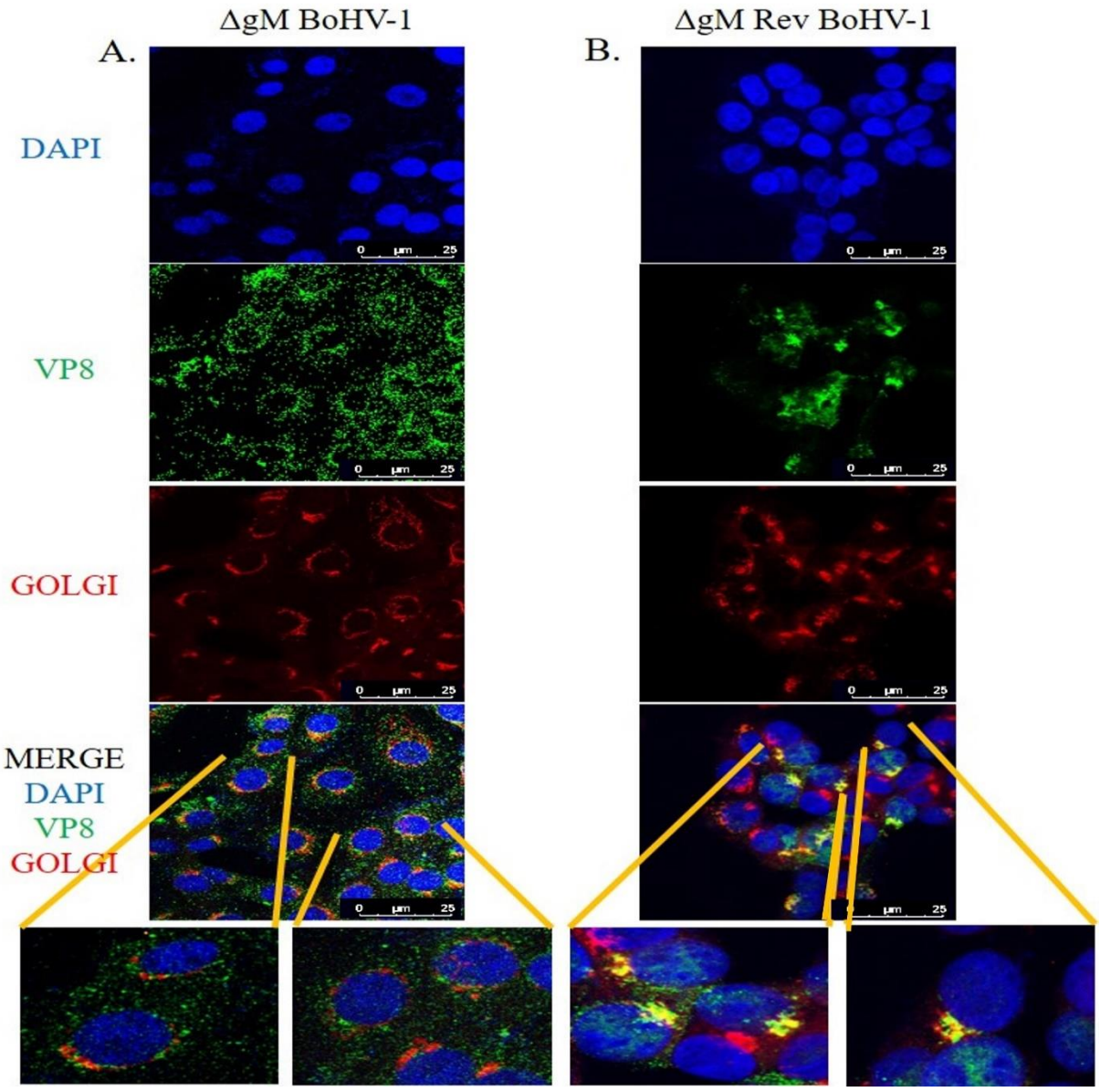


Figure 5.11. Localization of VP8 (pU_L47) in cells infected with ΔgM BoHV-1 or ΔgM Rev BoHV-1. (A) Cells infected with ΔgM BoHV-1. VP8 (pU_L47) is shown in green, and Golgi is shown in red. The merged image does not show any yellow which indicates that VP8 (pU_L47) does not localize in the Golgi in the absence of gM (pU_L10). (B) Cells infected with ΔgM Rev BoHV-1. VP8 (pU_L47) is shown in green, and Golgi is shown in red. The yellow in the merged image shows the localization of VP8 (pU_L47) in the Golgi. 1 cm represents 5µm in the scale.

5.6.7 The Amount of VP8 (pU_L47) in Mature Virus Is Considerably Reduced in gM-Deleted Virus

To test the effect of gM (pU_L10) on the final packaging of VP8 (pU_L47), the amount of VP8 (pU_L47) in the mature virus isolated from MDBK cells infected with ΔgM BoHV-1 was determined. MDBK cells were infected with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1 at a MOI of 5, and supernatants were collected at 18 h post-infection. Virus was purified on a sucrose gradient as described previously (Sucharita, Zhang et al. 2021), and the purified virus was subjected to Western blotting, with VP8-specific antibodies for detection (Figure 5.12). VP5 (pU_L19) (capsid protein) was identified and used as a loading control on the gel. Based on densitometry analysis, the amount of VP8 (pU_L47) in the mature ΔgM BoHV-1 was 33.8%, compared to 100% in the virus from cells infected with WT BoHV-1. In ΔgM Rev BoHV-1-infected MDBK cells, the amount of VP8 (pU_L47) in the mature virus was found to be 89.2%. A considerable reduction in the amount of VP8 (pU_L47) in the mature virus in the absence of gM (pU_L10) and a major restoration in the amount of VP8 in ΔgM rev BoHV-1 confirms the indispensable role of gM (pU_L10) in localization to the Golgi and hence packaging of VP8 (pU_L47) into the virus.

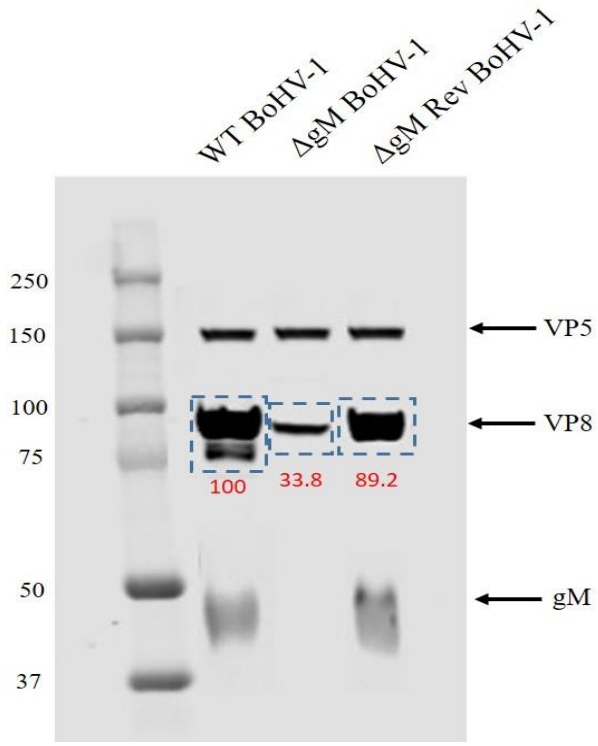


Figure 5.12. Amounts of VP8 (pU_L47) present in virus purified from cells infected with WT BoHV-1, ΔgM BoHV-1, or ΔgM Rev BoHV-1. VP8 (pU_L47) was detected using VP8-specific monoclonal antibody. The amount of VP8 (pU_L47) was reduced to 33.8% in ΔgM BoHV-1 compared to 100% in WT BoHV-1 and 89.2% in ΔgM Rev BoHV-1. Molecular weight markers (kDa) are shown in the left margin.

5.7 Discussion

VP8 (pU_L47) is the most abundant tegument protein of BoHV-1 and localizes to the nucleus early during infection. Following phosphorylation by pU_S3 in the nucleus, it travels to the cytoplasm and finally localizes to the Golgi in BoHV-1-infected cells, where it is packaged into the mature virus (Zhang, Donovan et al. 2019). However, VP8 (pU_L47) does not localize to the Golgi outside the context of infection, indicating the involvement of a viral component in this process. The most predominant viral proteins found in the Golgi apparatus of the host cells infected with BoHV-1, and herpesviruses in general, are the viral glycoproteins. Viral glycoproteins play a variety of roles in the life cycle of a virus, ranging from virus entry into the host cells to virus assembly. In studies performed on HHV, it has

been found that the incorporation of the tegument proteins into the virus depends on the interaction with other proteins, which are either capsid proteins, other tegument proteins, or glycoproteins (Mettenleiter 2006). Various tegument proteins interact with glycoproteins, which aid in their localization to the site of secondary envelopment, where the complete maturation of the virus takes place (Maringer, Stylianou et al. 2012). The site of secondary envelopment and virus maturation is the Golgi, where the majority of the glycoproteins/tegument proteins are packaged into the virions (Owen, Crump et al. 2015, Crump 2018)

Based on these observations, the focus of this study was to identify the glycoprotein(s) involved in the subcellular localization of VP8 (pU_L47) at the Golgi. VP8 (pU_L47) interacts with gM (pU_L10) in BoHV-1-infected MDBK cells (Figure 5.2). Different open reading frames in different herpes viruses encode glycoprotein M. Glycoprotein M is encoded by the *U_L10* gene in HHV-1 (McGeoch, Dalrymple et al. 1988), HHV-2 (Dolan, Jamieson et al. 1998), BoHV-1 (Wu, Zhu et al. 1998), and pseudorabies virus (Brack, Dijkstra et al. 1999). In other alpha herpesviruses, gM (pU_L10) is encoded by ORF50 (varicella zoster virus) (Davison and Scott 1986) or ORF52 (equine herpes virus) (Telford, Watson et al. 1992). Glycoprotein M (pU_L10) exists as a complex with gN ($pU_L49.5$) in all herpesviruses via the formation of disulfide bonds (Wu, Zhu et al. 1998, El Kasmi and Lippé 2015). The gM (pU_L10)/gN ($pU_L49.5$) complex is critical in the packaging of several proteins during the maturation of BoHV-1 (Graul, Kisielnicka et al. 2019). However, VP8 (pU_L47) did not interact with gN ($pU_L49.5$). Glycoprotein M (pU_L10) aids in the packaging of gN ($pU_L49.5$) in HHV-1 (Striebinger, Funk et al. 2016) and pseudorabies virus (Dijkstra, Gerdts et al. 1997); however, in HHV gM (pU_L10), packaging is independent of gN ($pU_L49.5$) (El Kasmi and Lippé 2015). In HHV, gM (pU_L10) also aids in the packaging of several other proteins (Crump, Bruun et al. 2004) for example tegument protein VP22 (pU_L49), as VP22 (pU_L49) packaging is impaired in the absence of gM (pU_L10) (Maringer, Stylianou et al. 2012). Glycoprotein M (pU_L10) promotes the cell-to-cell spread of several herpesviruses, including HHV-1 (Striebinger, Funk et al. 2016),

BoHV-1 (Pannhorst, Wei et al. 2018), varicella zoster virus (Yamagishi, Sadaoka et al. 2008), and equine herpesvirus-4 (Ziegler, Just et al. 2005).

Immunoprecipitation studies with BoHV-1-infected (Figure 5.3) and VP8- and gM-co-transfected (Figure 5.4) cells were performed to further demonstrate an interaction between VP8 (pU_L47) and gM (pU_L10); the results confirmed an interaction between these two proteins. Furthermore, VP8 (pU_L47) and gM (pU_L10) were shown to co-localize to the Golgi at 7 h post infection (Figure 5.5), which indicates a probable role of gM (pU_L10) in the localization of VP8 (pU_L47) to the Golgi. Previous studies demonstrated that in cells transfected with pFLAG-VP8 and pUS3 (viral kinase), VP8 (pU_L47) moves from the nucleus to the cytoplasm but was not localized to the Golgi (Zhang, Donovan et al. 2019). In this study, the translocation of VP8 (pU_L47) to the Golgi did not occur in cells transfected with pFLAG-ΔNLS VP8 alone (Figure 5.6A), which supports the inability of cytoplasmic VP8 (pU_L47) to translocate to the Golgi without involvement of a viral factor. However, in EBTr cells co-transfected with pFLAG-ΔNLS VP8 and pHA-gM, both gM and VP8 (pU_L47) localized to the Golgi (Figure 5.6 C,D). The observation of gM (pU_L10) enabling VP8 (pU_L47) localization at the Golgi in the absence of any other viral factor is significant, suggesting that gM (pU_L10) plays an important role in VP8 (pU_L47) subcellular localization.

Domains are the functional units of a protein and play an important role in a protein's function as well as interaction with another protein (Dawson, Sillitoe et al. 2017). The domain between amino acids 538–632 in VP8 (pU_L47) interacted with the domain between amino acids 210–300 in gM (pU_L10) (Figure 5.7). In the secondary structures of these proteins obtained by psiPred software, these regions make up a helix–loop–helix structure, which is a signature structure of a functional domain (Figure 5.8). Furthermore, according to the predicted gM (pU_L10) structure, amino acids 210–300 are located in the cytoplasm; this location agrees with the ability to interact with VP8 (pU_L47). The deletion of the *U_L10* gene from the virus significantly reduced the growth rate as well as the titer of both intracellular and extracellular virus (100-fold) (Figure 5.9), suggesting a delay in

egress of ΔgM BoHV-1, which was confirmed by TEM studies performed on cells infected with WT BoHV-1, ΔgM BoHV-1, and ΔgM Rev BoHV-1 (Figure 5.10). In cells infected with ΔgM BoHV-1, the majority of the virus particles was found to be inside the nucleus, and none were found outside the cell. In contrast, in the cells infected with WT BoHV-1 and ΔgM Rev BoHV-1, the majority of the virus particles was outside the cells and very few were observed inside the nucleus (Figure 5.11). The reduction in the ΔgM BoHV-1 titer and the delay in egress from the ΔgM BoHV-1-infected cells could be correlated to the role of gM (pU_L10) in the packaging of other tegument proteins like VP22 (pU_L49) (Graul, Kisielnicka et al. 2019) or its importance in the cell-to-cell spread of BoHV-1 (Pannhorst, Wei et al. 2018). Furthermore, the localization of VP8 (pU_L47) to the Golgi was hampered, and the amount of VP8 (pU_L47) in the mature virus in cells infected with ΔgM BoHV-1 was reduced to 33.6%, indicating that gM (pU_L10) plays an important role in VP8 (pU_L47) packaging (Figure 5.12). This supports our previous results suggesting that a portion of VP8 (pU_L47) can be incorporated into virions during primary envelopment (Zhang, Brownlie et al. 2016).

Based on this study, it can be concluded that gM (pU_L10) plays an indispensable role in the subcellular localization and packaging of VP8 (pU_L47). The growth rate and titer of ΔgM BoHV-1 are comparable to the growth rate of a BoHV-1 in which VP8 (pU_L47) is not phosphorylated and does not exit the nucleus (Zhang, Brownlie et al. 2016). Given that VP8 (pU_L47) is critical for viral replication, the slow growth rate and reduced titer of ΔgM BoHV-1 could be partially due to the reduced expression of VP8 (pU_L47) and VP8′s inability to be translocated and hence packaged at the Golgi.

5.8 Acknowledgments

Sunil Khattar (University of Maryland, College Park, MD, USA) contributed to the generation of the gM-specific antibody. Sharmin Afroz (AbbVie, Worcester, USA) contributed two of the VP8 (pU_L47) deletion mutants. The Western College of Veterinary,

University of Saskatchewan helped with transmission electron microscopy studies. This article is journal series 996, approved by the Director of VIDO.

5.9 Authors' Contribution

Conceptualization, S.S. and S.v.D.L.-v.d.H.; methodology, S.S. and S.v.D.L.-v.d.H.; software, S.S.; validation, S.S. and S.v.D.L.-v.d.H.; formal analysis S.S.; investigation, S.S.; resources, S.S., S.T. and S.v.D.L.-v.d.H.; data curation, S.S.; writing—original draft preparation, S.S. and S.v.D.L.-v.d.H.; writing—review and editing, S.S., K.Z. and S.v.D.L.-v.d.H.; visualization, S.S., K.Z. and S.v.D.L.-v.d.H.; supervision, S.v.D.L.-v.d.H.; project administration, S.v.D.L.-v.d.H.; funding acquisition, S.v.D.L.-v.d.H. All authors have read and agreed to the published version of the manuscript.

CHAPTER 6

6. GENERAL DISCUSSION AND FUTURE DIRECTION

6.1 General discussion:

During the maturation process of alpha herpesviruses, the incorporation of the structurally arranged amorphous layer of tegument proteins is carried out at different stages (Granzow, Klupp et al. 2001). The first stage of tegument incorporation occurs during the nuclear egress of the virus particles, in the perinuclear space; a few tegument proteins are added during the transport of the primary enveloped virus towards the Golgi, while the final incorporation of the tegument proteins occurs at the Golgi along with the final maturation (Granzow, Klupp et al. 2001, Mettenleiter, Klupp et al. 2006, Mettenleiter, Klupp et al. 2009). For tegument proteins such as VP8 (pU_L47) it can hence be suggested that the incorporation of the protein happens either at the perinuclear region or at the Golgi, or possibly partially at the perinuclear region and partially at the Golgi. A similar pattern of packaging has been confirmed for an HHV-1 tegument protein, VP16 (pU_L48), which is partially packaged during the early envelopment of the virus in the perinuclear space where as both VP13/14 (pU_L47) (VP8 homologue) and VP22 (pU_L49) were absent from the primary enveloped virions (Naldinho-Souto, Browne et al. 2006). pU_L31, pU_L34 and U_S3 also are packaged into the early tegument in HHV-1 and SuHV-1 as they for the basis for the inititiation of tegument incorporation (Granzow, Klupp et al. 2004, Naldinho-Souto, Browne et al. 2006).

Phosphorylation of the nuclear VP8 (pU_L47) by the viral kinase US3 governs the cytoplasmic localisation of VP8 (pU_L47) during the infection cycle of BoHV-1, while non-phosphorylated VP8 (pU_L47) remains nuclear (Zhang, Brownlie et al. 2016). In BoHV-1 encoding non-phosphorylated VP8, approximately 30% of VP8 (pU_L47) is present in the mature virus particles compared to the WT BoHV-1 (Zhang, Afroz et al. 2015). In view of the reduction in the amount of VP8 (pU_L47) in the mature virus in absence of

phosphorylation, a partial packaging of VP8 (pU_L47) during the early tegumentation and a final incorporation at the Golgi was suggested.

Mass spectrometry analysis of the primary enveloped virions extracted from the perinuclear space and the mature virus purified from the extracellular matrix of MDBK cells infected with WT BoHV-1 was carried out to identify the proteins in the virus particles from these fractions. The primary enveloped virions were isolated from the nuclear membrane preparations, the purity of which was confirmed by the absence of nuclear and cellular markers, nucleolin and tubulin, respectively. The purity of the NMP supports the fact that the identified proteins were entirely part of the primary enveloped virus particles. Striking similarities were found in the comprehensive list of the identified proteins in the primary enveloped virus particles and the mature virus fractions in BoHV-1 and HHV-1 (Mocarski Jr 2007, Loret, Guay et al. 2008, Padula, Sydnor et al. 2009). Mass spectrometry identified 11 glycoproteins –gB (pU_L27), gC (pU_L44), gD (pU_S6), gE (pU_S8), gH (pU_L22), gL (pU_S1), gG (pU_S4), gM (pU_L10), gN ($pU_L49.5$), gK (pU_L53) and gI (pU_S7)- in the mature BoHV-1 purified virus. The virus particles from the NMP were devoid of these glycoproteins, which further supports the purity of the primary enveloped virions extracted from the NMP. The presence of the major and minor capsid proteins along with the portal vertex proteins (VP5 (pU_L19), pU_L18 and U_L6) in both fractions confirms the presence of viral capsids in both regions. The majority of the tegument proteins are constituents of the mature virus, which supports the final incorporation of the tegument proteins at the Golgi. Tegument proteins like pU_L2, VP1/2 (pU_L36) and pU_L37 were likely packaged early during assembly as they were found to be components of only the primary enveloped virions in both HHV-1 and SuHV-1-1 (Loret, Guay et al. 2008, Kramer, Greco et al. 2011). In HHV-1, the tegument proteins VP1/2 (pU_L36) and pU_L37 are critically involved in the nuclear egress of the virus and hence are required at the early stages of the infection cycle (Lucchese, Benkirane et al. 2016). In BoHV-1, a few tegument proteins are a part of both primary enveloped virus particles and the mature virus, including pU_L26, pU_L29, pU_L39, pU_L40, VP8 (pU_L47) and VP22 (pU_L49). This indicates early packaging of these proteins

during the early tegumentation process, either partially or completely. This also suggests that these proteins were either retained or lost and regained during the travel of the virus particles to the Golgi where the final maturation takes place. Early partial packaging of critical proteins might also be a possible survival strategy for the virus to escape host defense mechanisms hampering the packaging of important proteins critical for the viral replication or cell-to cell spread.

In the primary enveloped virus fractions of BoHV-1, both VP8 (pU_L47) and VP22 (pU_L49) were identified, which suggests some amount of early packaging of both these proteins. In HHV-1, the tegument protein VP22 (pU_L49) plays a critical role in packaging of other tegument proteins during the maturation process (Pannhorst, Wei et al. 2018, DuRaine, Wisner et al. 2020). A reduced amount of tegument protein VP22 (pU_L49) in cells infected with BoHV-1 ΔUL47 indicated a possible interdependence of VP8 (pU_L47) and VP22 (pU_L49). An interaction between VP8 (pU_L47) and VP22 (pU_L49) was confirmed in the primary enveloped virus particle, as well as in the mature virus, in cells infected with WT BoHV-1 and BoHV-1 YmVP8 through co-immunoprecipitation analysis. The interaction between these two proteins was also found outside the context of infection, which suggests independence from other viral factors. Immunofluorescence microscopy indicated the presence of VP8 (pU_L47) and VP22 (pU_L49) in the perinuclear region at the earlier stages of infection, which supports the possibility of packaging of VP8 (pU_L47) and VP22 (pU_L49) during the early tegumentation process. By analysing cells infected with BoHV-1 ΔUL49 for the presence of VP8 (pU_L47) in the perinuclear region, it was found that in the absence of VP22 (pU_L49), no VP8 (pU_L47) localises in the perinuclear region. This further confirms the requirement of VP22 (pU_L49) for the localisation of VP8 (pU_L47) in the perinuclear region and hence its final packaging.

In cells infected with BoHV-1, after phosphorylation of nuclear VP8 (pU_L47) by pU_S3, VP8 (pU_L47) travels to the cytoplasm and localises to the Golgi where its final packaging takes place. In the absence of infection i.e. when cells were co-transfected with plasmids expressing pU_S3 and VP8 (pU_L47), VP8 (pU_L47) remained cytoplasmic without

being localised to the Golgi (Zhang, Donovan et al. 2019). This indicated the involvement of a viral factor in the translocation of VP8 (pU_L47) to the Golgi. The viral proteins present in the Golgi are predominantly glycoproteins. Viral glycoproteins are critically important for the virus as they have various functions ranging from viral entry into the host to incorporation of other viral components during the viral maturation. Studies on HHV-1 have shown the involvement of viral glycoproteins in the incorporation of other viral proteins, including capsid proteins, tegument proteins and other glycoproteins (Mettenleiter 2006). Glycoproteins also aid in the localisation of the tegument proteins to the site of secondary envelopment (Golgi) for their final incorporation into the virus (Maringer, Stylianou et al. 2012, Owen, Crump et al. 2015, Crump 2018).

Co-immunoprecipitation studies, employed to screen the glycoproteins interacting with VP8 (pU_L47), indicated an interaction between VP8 (pU_L47) and gM (pU_L10). VP8 (pU_L47) and gM (pU_L10) interacted in cells infected with BoHV-1 and also outside the context of infection, which suggests that the interaction is independent of other viral factors. In HHV-1 and other alpha-herpesviruses gM (pU_L10) exists as a complex with gN ($pU_L49.5$) via formation of disulfide bonds (Wu, Zhu et al. 1998, El Kasmi and Lippé 2015). gM (pU_L10) /gN ($pU_L49.5$) is critical for the packaging of tegument proteins like VP22 (pU_L49) during the maturation of BoHV-1 (Graul, Kisielnicka et al. 2019). gM (pU_L10) has also been found to aid in the packaging of gN ($pU_L49.5$) in HHV-1 (Striebinger, Funk et al. 2016) and in SuHV-1 (Dijkstra, Gerdts et al. 1997). However, in HHV-1 the packaging of gM (pU_L10) is independent of gN ($pU_L49.5$) (Crump, Bruun et al. 2004). Studies on HHV-1 have also shown an involvement of gM (pU_L10) in the packaging of VP22 (pU_L49), as impaired packaging of VP22 (pU_L49) was observed in the absence of gM (pU_L10) in HHV-1 (Maringer, Stylianou et al. 2012).

In cells co-transfected with plasmids expressing gM (pU_L10) and VP8 (pU_L47) without NLS, an interaction between VP8 (pU_L47) and gM (pU_L10) independent of other viral factors was confirmed. Furthermore, co-localisation of gM (pU_L10) and VP8 (pU_L47) was observed in the Golgi at 7 hr post infection with WT BoHV-1. In cells co-transfected

with plasmids expressing gM (pU$_L$10) and VP8 (pU$_L$47) without a NLS a localisation of VP8 (pU$_L$47) to the Golgi was seen at 24 hr post transfection. This suggests an important role of gM (pU$_L$10) in the localisation of the cytoplasmic VP8 (pU$_L$47) to the Golgi for its final packaging without the involvement of any other viral factor.

Domains that form a helix-loop-helix structure are the functional units of a protein (Dawson, Sillitoe et al. 2017). The domains of gM (pU$_L$10) and VP8 (pU$_L$47) that are involved in the interaction between the two proteins were identified. When truncated versions of gM (pU$_L$10) and VP8 (pU$_L$47) proteins were immunoprecipitated with each other it was observed that the domains between amino acids 538-632 of VP8 (pU$_L$47) and amino acids 210-300 of gM (pU$_L$10) were involved in the interaction between these two proteins. To confirm the requirement of gM (pU$_L$10) for the localisation and packaging of VP8 (pU$_L$47) a recombinant BoHV-1 lacking the gM-encoding *U$_L$10* gene was constructed. The one step growth curve showed a reduction of approximately 100-fold in the viral titre in the absence of gM (pU$_L$10) and a delay in nuclear egress. The delay in nuclear egress was confirmed by transmission electron microscopy. Observations of cells infected with WT BoHV-1, ΔgM BoHV-1 and ΔgM Rev BoHV-1 by transmission electron microscopy confirmed the presence of more nuclear virus particles in the absence of gM (pU$_L$10) and more extracellular virus particles in the presence of gM (pU$_L$10) at 7 hr post infection. The reduction in the virus titer and the delay in egress in the absence of gM (pU$_L$10) could be due to the essential role of gM (pU$_L$10) in the incorporation of other viral proteins and its importance in the cell-to-cell spread after the viral infection (Pannhorst, Wei et al. 2018, Graul, Kisielnicka et al. 2019). VP8 (pU$_L$47) was observed to remain cytoplasmic in cells infected with ΔgM BoHV-1 and was not localised to the Golgi; however, the restoration of gM (pU$_L$10) restored the localisation of VP8 (pU$_L$47) to the Golgi. The indispensable role of gM (pU$_L$10) in the packaging of VP8 (pU$_L$47) was further confirmed based on the significant reduction in the amount of VP8 (pU$_L$47) in the mature virus in the absence of gM (pU$_L$10), which was nearly restored with the restoration of gM (pU$_L$10).

Overall, the study shows a novel partial packaging of VP8 (pU_L47) in the perinuclear region during the early tegumentation process. Another tegument protein, VP22 (pU_L47), was confirmed to play an important role in the early packaging of VP8 (pU_L47). After the phosphorylation of nuclear VP8 (pU_L47) by viral kinase pU_S3, VP8 (pU_L47) becomes cytoplasmic. The cytoplasmic VP8 (pU_L47) interacts with gM (pU_L10), suggesting that gM (pU_L10) is responsible for the transport and retention of VP8 (pU_L47) at the Golgi, where its final incorporation into the virus takes place. Schematics indicating stages of VP8 incorporation and its previously known functions (Zhang, Afroz et al. 2015, Afroz, Brownlie et al. 2016, Zhang, Brownlie et al. 2016, Afroz, Garg et al. 2018) is summarised in Fig 6.1.

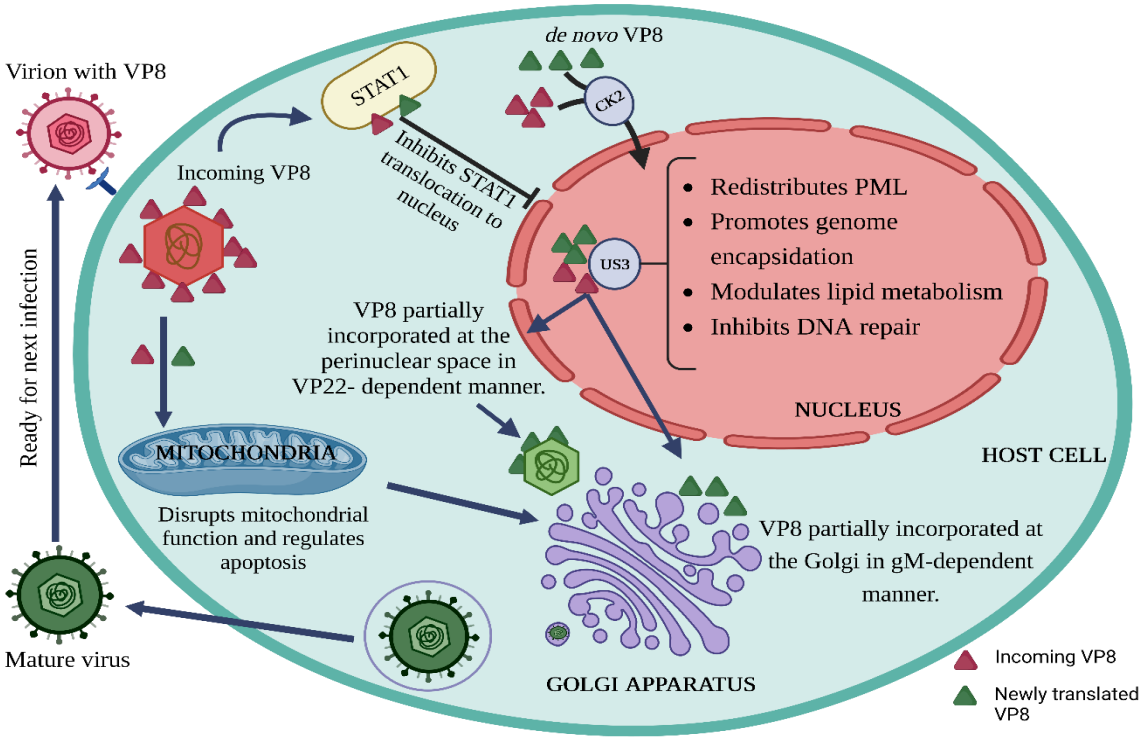


Figure 6.1: Schematic of VP8 (pU_L47) packaging and functions in BoHV-1 infected cells

6.2 Future Directions

This project focuses on the stages of virion incorporation of the major tegument protein of BoHV-1, VP8 (pU_L47). One of the stages of incorporation was the early tegumentation of the virus particle with the involvement of another tegument protein, VP22 (pU_L49). The final incorporation was shown to be at the Golgi with major participation of gM (pU_L10). Despite these discovered novel factors, many stones regarding VP8 (pU_L47) and its packaging into the mature virus still remain unturned.

The phosphorylation of nuclear VP8 (pU_L47) promotes the cytoplasmic localisation of VP8 (pU_L47). VP8 (pU_L47) is also known to contain nuclear export signals for the egress of VP8 (pU_L47); however, egress of non-phosphorylated VP8 (pU_L47) from the nucleus was not observed. This indicates a role of the phosphorylation in either activation of the NES or conformational changes that are responsible for exposing the NES, which is then involved in the export of VP8 (pU_L47) into the cytoplasm. The conformational changes can be studied by purification of the NES of phosphorylated and non-phosphorylated VP8 (pU_L47) and analyse the structure for conformational differences. A possible involvement of host exportin proteins can also be tested by analysis of co-precipitation with VP8 (pU_L47).

According to the results of this study, VP8 (pU_L47) is incorporated at two different stages of tegument assembly, i.e. at the perinuclear region and at the Golgi. However, it is not known whether there is a difference in structure and/or function of VP8 (pU_L47) incorporated in these different locations. This can be evaluated by first identifying the shortest amino acid sequence critical for VP8 (pU_L47) and VP22 (pU_L49) interaction, followed by construction of a recombinant virus with a deleted/mutated interaction domain. Theoretically, the recombinant virus will contain only Golgi-incorporated VP8 (pU_L47). The differences in the function of VP8 (pU_L47) can be investigated by comparing the recombinant virus and WT BoHV-1. Similar experiments can be performed to study the functions of VP8 (pU_L47) incorporated at the perinuclear region by first identifying the shortest amino acid sequence critical for VP8 (pU_L47) and gM (pU_L10) interaction and

subsequently constructing a recombinant virus with the deleted/mutated interaction domain. Theoretically, the recombinant virus will contain VP8 (pU_L47) incorporated at the perinuclear region. The differences in the function of VP8 (pU_L47) can be investigated by comparing the recombinant virus and WT BoHV-1.

HHV-1 VP13/14 (pU_L47) interacts with the nuclear egress complex $pU_L31/34$ to aid in the nuclear egress of the virus (Liu, Kato et al. 2014); however a similar involvement of BoHV-1 VP8 (pU_L47) has not yet been studied. Interaction of VP8 (pU_L47) with the nuclear egress complex can be investigated by testing the precipitation of pU_L31 and pU_L34 with VP8 (pU_L47). Once an interaction between VP8 (pU_L47) and $pU_L31/34$ is established, the necessity of such an interaction for the nuclear egress of BoHV-1 can be tested. This can be carried out by first identifying the interaction domain/specific amino acids of $pU_L31/34$ and later construction of a recombinant virus with the identified domain/amino acids deleted or mutated. The nuclear egress of the virus in cells infected with the constructed recombinant virus can be studied by immunofluorescence.

Similar to HHV-1 VP13/14 (pU_L47), BoHV-1 VP8 is phosphorylated in infected cells but non-phosphorylated in the mature virus (Zhang, Afroz et al. 2015). The factor responsible for the changed phosphorylation status of VP13/14 (pU_L47) in mature virus compared to that in infected cells in HHV-1has been identified to be pUL21 (Benedyk, Muenzner et al. 2021). Based on this, the factor responsible for de-phosphorylation of BoHV-1 VP8 (pU_L47) can be investigated. This can be carried out by conducting mass spectrometric analysis of the proteins co-immunoprecipitating with VP8 (pU_L47). If results coincide with those found for HHV-1, bovine pU_L21 can be characterised for its properties as a viral phosphatase. In case of contrasting results, other potential proteins identified by mass spectrometry can be characterised by interaction and mutation studies. The organelle at which the phosphorylation status of the protein changes can further be investigated by immunofluorescence studies.

To expand the studies involving VP8 (pU_L47) and gM (pU_L10) interaction, a mutant virus with amino acids 210-300 (domain involved in VP8 (pU_L47) interaction) of gM

(pU_L10) deleted can be constructed to further confirm the specific domain (and later amino acid) involved in translocation of VP8 (pU_L47) to the Golgi. Specific amino acids of VP8 (pU_L47) and gM (pU_L10) responsible for the interaction can also be identified by point mutations and the necessity of the interaction can be investigated by construction of recombinant mutant viruses.

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