STRUCTURAL AND FUNCTIONAL STUDY OF
BOVINE HERPESVIRUS 1 GLYCOPROTEIN B IN
THE INTERACTION WITH MADIN DARBY
BOVINE KIDNEY CELLS

A Thesis
Submitted to the Faculty of Graduate Studies and Research
in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Veterinary Microbiology
University of Saskatchewan

by
Yuanhao Li
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of the requirements for

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ABSTRACT

Entry of herpesviruses is mediated by the interactions between viral glycoproteins and cellular receptors. Among these glycoproteins, gB plays an important role. In this study, my major focus was to study gB’s functions in the virus entry process and the structural requirements for gB to conduct its functions. The virus model in my study is bovine herpesvirus 1 (BHV-1), a member of the alphaherpesviruses.

BHV-1 gB is a type I integral membrane protein with a potential transmembrane anchor at the C-terminal region. A cleavage site in the middle divides this molecule into two subunits, gBb and gBc. In this study, a truncated gB, gBt (residues 1 to 763), and N-terminal subunit, gBb (residues 1 to 505), were first expressed under the control of the bovine heat-shock protein 70A (hsp70A) gene promoter in stably transfected Madin Darby bovine kidney (MDBK) cells. Both forms of gB were secreted into the medium with apparent molecular weights as anticipated, and they were reactive to all gB-specific monoclonal antibodies used in this study. Affinity-purified gBt and gBb were able to elicit antibody responses in mice to an extent comparable to those induced by authentic gB. These results suggest that gBt and gBb retain
the structural and antigenic properties of authentic gB. Furthermore, the intracellular processing of gBt and gBb was similar to that of authentic gB in virus-infected cells. Finally, gBt was proteolytically cleaved after conversion of the high mannose-containing precursor to the mature form. These truncated gBs that were prepared served as reagents for the core of my studies.

In order to study the transport of recombinant forms of gB, a truncated form of gBc (gBct), containing residues 506 to 763, was expressed in the same system, which resulted in the production of a protein retained in the endoplasmic reticulum (ER). Thus, our results suggest that gBb is required for the transport of gB from the ER to the Golgi.

To gain further insight into the mechanism of the attachment process of BHV-1, affinity-purified authentic gB, gC and gD from BHV-1-infected cells and membrane anchor-truncated, soluble gB, gC and gD from stably transfected cell lines were examined for their cell binding properties on MDBK cells. All glycoproteins tested exhibited saturable binding to MDBK cells. Addition of exogenous heparin or treatment of cells with heparinase to remove cellular heparan sulfate (HS) prevented both gC and gB from binding to cells, but had no effect on gD binding. Assessment of competition between gB, gC and gD for cell binding revealed that gC was able to inhibit gB binding, whereas other combinations showed no effect. Cell-bound gC could be dissociated from cells by
heparin or heparinase treatment. The response of cell bound gB to heparin and heparinase treatment differed between the authentic and soluble forms; while soluble gB was susceptible to such treatment, a significant portion of cell-bound authentic gB was resistant to the treatment. Binding affinity analysis showed that soluble gB and both forms of gC and gD each had single binding kinetics with comparable dissociation constants (Kd), ranging from $1.5 \times 10^{-7}$ M to $5.1 \times 10^{-7}$ M, whereas authentic gB exhibited dual binding kinetics with Kd1 = $5.2 \times 10^{-7}$ M and Kd2 = $4.1 \times 10^{-9}$ M. These results demonstrate that BHV-1 gC binds only to cellular HS, gD binds to a non-HS component, and gB initially binds to HS, which is followed by a secondary high-affinity binding to a non-HS receptor. Furthermore, we found that while authentic gB was able to inhibit viral plaque formation, soluble gB, which retains the HS-binding property, but lacks the high affinity binding property, was defective in this respect. These results suggest that the interaction between gB and its high-affinity receptor may play a critical role in virus entry.

In order to confirm that BHV-1 gB can bind to heparin-like structures, conventional heparin affinity chromatography was also used. It was found that under our experimental conditions, BHV-1 gB did bind to immobilized heparin. Recombinant gBt and gBb were also shown to bind heparin-Sepharose as well as HS on MDBK cells, whereas gBct could not bind heparin-Sepharose. Thus, it was suggested that at least
one heparin-binding domain is localized in gBb, which agrees with the presence of clusters of prolines and basic residues, thought to be essential for heparin binding.

Since gBt has been shown to have no high-affinity binding site, we compared it with the authentic gB in order to study the structural requirements for gB's high-affinity binding activity. First, the oligomerization status of authentic gB and different truncated forms of gB were compared. Using chemical cross-linking and sucrose gradient centrifugation, it was found that BHV-1 gB was able to form dimers. gEb was unable to oligomerize, whereas the two other forms, gBt and gBtM (residues 1 to 807), were efficiently dimerized, indicating that the region between residues 506 to 763 was required for gB oligomerization. The affinity-purified gB and truncated products were also shown to consistently form oligomers. However, purified gBt and gBtM did not block the high-affinity cellular receptor, suggesting that oligomerization was not the reason for the loss of the high-affinity binding activity on these truncated forms of gB. Further characterization showed that epitope I, which spans a N-terminal juxtamembrane region and is recognized by monoclonal antibody 1B10, was lost from gBt and gBtM, indicating that both truncated forms of gB are conformationally changed. Therefore, the structure around this particular region may be required for the existence of the high-affinity binding site of gB.
The expression of gBt and gBtM has shown that gBt was efficiently secreted, whereas gBtM, which includes the first two segments of transmembrane region at its carboxy terminus, was unstably retained on the cell surface. Another form of truncated gB, gBtMA (residues 1 to 829), which contains all three membrane spanning segments of the transmembrane region, was also expressed. Recombinant gBtMA was primarily retained intracellularly with some unstable surface anchorage. Another truncated gB, gBtDAF, which contains the residues 1 to 763 of gB (gBt) and a human decay-accelerating factor (DAF) carboxy tail, was expressed as a control. The DAF fragment provided a signal for the addition of a glycosyl phosphatidylinositol-based membrane anchor, which could target the gBt chimeric protein to the cell membrane. Immunofluorescence staining and pulse-chase kinetic studies support the suggestion that gBtM, gBtMA, and gBtDAF may be retained on nuclear and cellular membranes via different segments of the transmembrane region or DAF fragment respectively. These gB products showed different functions in MDBK cells and therefore allowed me to develop a hypothesis of their cellular interactions. In cells expressing gBt or gBtM, no cell fusion was observed, whereas cells expressing gBtMA clearly showed fusion. In gBtDAF cells, overexpression and cellular accumulation of recombinant gB products did not cause fusion, which supports our contention that the fusion phenomenon in gBtMA cells is caused by the fusogenic activity of the expressed gBtMA. With
the help of sequence analysis, our results indicate that segment 2 of the transmembrane anchor region might be a fusogenic domain rather than a membrane anchor segment for BHV-1 gB, whereas the real anchor is segment 3.

In conclusion, BHV-1 gB can bind to HS and another non-HS receptor on MDBK cells. We assume that high-affinity binding to the non-HS receptor is important for BHV-1 infectivity. BHV-1 gB forms dimers in infected cells and in virions, and its dimerization domain may be located between residues 506 to 763. The cytoplasmic domain of BHV-1 gB is important for the existence of the high-affinity binding site. Without the cytoplasmic domain, the truncated gB derivatives exhibit conformational changes and loss of the high-affinity binding site. By comparing the expression of different gB derivatives in MDBK cells, it was found that in the putative transmembrane region, segment 3 is the real membrane anchor, whereas segment 2 is the fusogenic domain.
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ABBREVIATIONS USED IN THIS WORK

aa    amino acid
Asn   asparagine
α-TIF α gene trans-inducing factor
BHV-1 bovine herpesvirus 1
BHV-2 bovine herpesvirus 2
BSA   bovine serum albumin
CPE   cytopathic effect
cpm   counts per minute
C-terminal carboxy-terminal
Cys   cysteine
DNA   deoxyribonucleic acid
EBV   Epstein-Barr virus
EHV-1 equine herpesvirus 1
EHV-2 equine herpesvirus 2
ER    endoplasmic reticulum
FBS   fetal bovine serum
GAG   glycosaminoglycan
Gly   glycine
HA    haemagglutinin
HCMV  human cytomegalovirus
HHV-6 human herpesvirus 6

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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HS</td>
<td>heparan sulfate</td>
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<td>HSPG</td>
<td>heparan sulfate proteoglycan</td>
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<td>kilobase</td>
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<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MCP</td>
<td>major capsid protein</td>
</tr>
<tr>
<td>MDBK cells</td>
<td>Madin Darby bovine kidney cells</td>
</tr>
<tr>
<td>MEM</td>
<td>modified Eagle’s medium</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>met-free MEM</td>
<td>methionine-free minimal essential medium</td>
</tr>
<tr>
<td>moi</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>m.w.</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque-forming units</td>
</tr>
<tr>
<td>PI</td>
<td>post-infection</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>PRV</td>
<td>pseudorabies virus</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RIIPA</td>
<td>radio immunoprecipitation assay</td>
</tr>
<tr>
<td>roe</td>
<td>rate of entry</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>syn</td>
<td>syncytia-forming</td>
</tr>
<tr>
<td>ts</td>
<td>temperature sensitive</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>UL</td>
<td>unique long</td>
</tr>
<tr>
<td>US</td>
<td>unique short</td>
</tr>
<tr>
<td>vhs</td>
<td>virion host shut-off protein</td>
</tr>
<tr>
<td>VZV</td>
<td>varicella-zoster virus</td>
</tr>
</tbody>
</table>
1.0 LITERATURE REVIEW

1.1 HERPESVIRUSES

Herpesviruses are large, highly complex enveloped DNA viruses, which have been isolated from a wide range of animal species, human beings, amphibians, reptiles, fish, and birds (Ardans 1990; Roizman 1993; Roizman and Baines 1991). Virus infection is initiated in epithelial cells of the skin or the mucosal membrane. Following a primary infection, the virus normally establishes latency with the genome persisting in some unknown association with host cells.

1.1.1 Classification of herpesviruses and their clinical problems

The family Herpesviridae consists of three subfamilies, named alpha-, beta-, and gamma-herpesvirinae, which are distinguished from each other based upon biological properties including host range, duration of reproductive cycle, cytopathology, latent infection, genome structure, and sequence homologies (Roizman 1993).

The alphaherpesviruses (herpes simplex virus group)
have a variable host range, are generally highly cytopathic in cell culture, have a relatively short replicative cycle (<24 h), spread rapidly in culture, and frequently cause latent infections in ganglia. There are two genera, *simplexvirus* and *varicellovirus* in this subfamily. Examples include herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), bovine herpesvirus-1 (BHV-1), bovine herpesvirus-2 (BHV-2), equine herpesvirus-1 (EHV-1), and pseudorabies virus (PRV).

Betaherpesviruses have a restricted host range and a long replicative cycle, with infected cells often becoming enlarged (cytomegaly). Latency can be established in numerous tissues including secretory glands, lymphoreticular cells, kidneys and other tissues. There are two genera, *cytomegalovirus* and *muromegalovirus* in this subfamily. Examples include human cytomegalovirus (HCMV), human herpesvirus 6 and 7 (HHV-6, HHV-7), and equine herpesvirus-2 (EHV-2).

Gammaherpesviruses (lymphoproliferative virus group) replicate in lymphoblastoid cells and may cause lytic infections in certain types of epithelial and fibroblastic cells. Their host range is narrow. Latency is frequently established in lymphoid tissue. There are two genera, *lymphocryptovirus* and *rhadinovirus* in this subfamily. Examples include Epstein-Barr virus (EBV) and Marek’s disease virus.
Another major way of classifying herpesviruses is based on their viral genomes. The sequence arrangements in herpesvirus DNAs can divide herpesviruses into six groups designated by the letters A, B, C, D, E, and F (Roizman 1993). In group A, exemplified by channel catfish herpesvirus, a large fragment from one terminus is directly repeated at the other terminus. In group B, exemplified by herpes saimiri virus, the terminal sequence is repeated numerous times at both termini. In group C, exemplified by EBV, the terminal repeat is small, but there are other repeated sequences located in several places along the genome. In group D, exemplified by VZV, the sequence from one terminus is repeated in an inverted orientation internally. This allows the unique short region (US) to invert relative to the unique long (UL) sequence to form two isomeric forms of the genome. In group E, exemplified by HSV and HCMV, sequences from both termini are repeated in an inverted orientation and linked internally. This allows both UL and US to invert relative to the other, resulting in four equimolar populations of isomer. In group F, exemplified by tupaia herpesvirus, the sequences at the two termini are not identical and are not repeated directly or in an inverted orientation.

Currently, from over 100 herpesviruses, seven have been isolated from humans including HSV-1, HSV-2, HCMV, VZV, EBV, HHV-6, and HHV-7, five from horses, four from cattle,
two from pigs, three from chickens.

In human herpesviruses, a high infection rate in the population is very common. Viruses can spread very easily among people, especially in children. A high rate (70%-95%) of HSV infection has been found worldwide even though there is significant country to country variation (Roizman 1993). Although primary HSV infections are mostly asymptomatic, recurrent cutaneous lesions usually occur. Severe clinical HSV problems include keratoconjunctivitis, skin infections, neonatal infection, encephalitis, and disseminated disease.

HCMV infects more than 50%-80% of the population at an early age. Congenital infection occurs in about 0.2%-2.2%, which may cause cytomegalic inclusion disease and central nervous system defects in newborns. Hepatitis, interstitial pneumonitis, and infectious mononucleosis-like disease can be found in young children. It is also a major problem in immunosuppressed individuals, and patients with acquired immunodeficiency syndrome.

EBV causes infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, and lymphoma in immunodeficient individuals. VZV causes the primary chickenpox (or varicella) infection in children. The virus may go to the nervous system to enter a latent state. Upon reactivation, VZV travels down the sensory nerve to cause a vesicular rash known as shingles or herpes zoster.

Latent infections with herpesviruses are normally
asymptomatic. The latently infected cells can evade the host’s immune system. Under certain conditions like trauma, stress, infection, immunosuppression, etc., latent herpesvirus infections can be reactivated to enter a productive infection. In some cases, this may generate severe clinical problems.

In domestic animals, herpesviruses may cause abortion, respiratory disease, fever, emaciation, and occasional neurologic disease, etc. (Ardans 1990). Every year, significant economic losses are caused by herpesvirus infections in different groups of animals, especially livestock animals.

1.1.2 Structure of the virus

Herpesviruses all have similar structural properties. Generally, they are known as large enveloped viruses with a double-stranded DNA genome enclosed in an icosahedral capsid, which is surrounded by the tegument and an envelope. The general chemical and physical properties of herpesviruses are summarized in Table 1-1.

The virion has four main structural components: envelope, tegument, capsid, and core.

(a) Envelope

The envelope is typically triple-layered as seen with the aid of electron microscopy. This envelope is derived from the inner nuclear membrane of the host cell, and has small
spikes on its surface. The spikes are viral glycoproteins which form approximately 8 to 24 nm long projections protruding out of the virion (Stannard et al., 1987), and are involved in various interactions with host cells during virus entry and spread. There are more spikes and different types of glycoproteins on the herpesvirus envelope than on the surfaces of many other enveloped viruses, implying a more complicated mechanism for virus infection.

<table>
<thead>
<tr>
<th>Table 1-1. Chemical and Physical properties of herpesviruses</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (nm)</td>
<td>120-300 (virion); 100-110 (capsid)</td>
</tr>
<tr>
<td>Symmetry of capsid</td>
<td>Icosahedral (20 sided)</td>
</tr>
<tr>
<td>Triangulation number</td>
<td>T=16</td>
</tr>
<tr>
<td>Number of capsomers</td>
<td>162 (150 hexameric and 12 pentameric)</td>
</tr>
<tr>
<td>DNA</td>
<td>Double-stranded, linear, 31-75% G+C, 80-150 MDa (120-230 kbp)</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Linked to envelope proteins</td>
</tr>
<tr>
<td>Lipid</td>
<td>Located in envelope</td>
</tr>
<tr>
<td>Virion mass</td>
<td>Approximately 1450 MDa (HSV-1)</td>
</tr>
<tr>
<td>Density in CsCl</td>
<td>1.20-1.29 g/cm³ (virion)</td>
</tr>
</tbody>
</table>

Hay et al., 1987.
Roizman and Baines 1991.
(b) Tegument

The structural layer between the capsid and the envelope is called the tegument (Roizman and Furlong 1974). The size of this layer is variable between different herpesviruses. The fibrous matrix comprising the tegument contains different types of proteins, thought to play a role in transcription during productive infection.

(c) Capsid

The capsid structure is similar in all herpesviruses. It is an icosahedral structure, with 150 hexavalent capsomers (hexons) and 12 pentavalent capsomers (pentons).

(d) The core

From electron microscopic observation, the herpesvirus DNA is packed into a centrally located electron-dense spherical mass with an overall diameter of about 30-75 nm (Booy et al., 1991; Furlong et al., 1972; Nazerian 1974).

From studies of the virion structure, it is clear that the envelope is the outermost layer to surround the viral particle. The glycoprotein projections on the envelope extend 8-24 nm from the viral particle. Therefore, these protein spikes must be the first group of viral components encountered by host cells.

1.1.3 Viral replication processes

The life cycle of herpesviruses is illustrated in
Fig. 1-1. Herpesviruses use the membrane glycoproteins for attachment and penetration to follow an entry pathway which has been proposed according to the most extensively studied prototype, HSV-1 (Fuller and Lee 1992). Attachment is initially mediated by the binding of glycoprotein C and/or B to cellular heparan sulfate proteoglycan (HS, HSPG) structures. After initial attachment, the subsequent interactions between viral glycoproteins and cellular receptors enable virions to establish stable attachment to the cell surface, which is followed by penetration, a step where the virus envelope fuses with the cell membrane to release the capsid into the cytoplasm. The de-enveloped viral capsid is then transported to the nuclear membrane and the linear DNA molecule is released into the nucleus through nuclear pores. In a productive infection, transcription, replication of viral DNA, and assembly of new capsids takes place in the nucleus. The viral DNA may go into latency in the host genome and be reactivated later.

In productive infection, upon entry of the virus into the cell, viral proteins, such as the tegument proteins are released. These proteins control the transcription and translation of viral genes in the cell. Viral DNA is transcribed by host RNA polymerase II with the participation of viral factors at all stages of infection. The viral proteins are produced in a coordinately regulated and sequentially ordered cascade manner. Their genes are grouped
Fig. 1-1. Life cycle of herpesvirus. The numbers indicate the sequential activities. Arrows indicate the intracellular movements and products.
into three categories as $\alpha$ (encoding immediate early proteins), $\beta$ (encoding early proteins), and $\gamma$ (encoding late proteins). Briefly, the transcription of $\alpha$ genes by cellular enzymes is induced by the $\alpha$ gene trans-inducing factor ($\alpha$-TIF). The synthesized $\alpha$ gene products are necessary for the transcription of $\beta$ genes. With the presence of $\alpha$ and $\beta$ gene products, a subsequent round of $\gamma$ gene transcription and translation is started. Meanwhile, viral DNA is replicated by a rolling cycle mechanism that yields head-to-tail concatamers of unit length viral DNA, which is cleaved and packaged into the preformed capsids. Such capsids attach to the underside of the nuclear membrane patches containing viral proteins and are enveloped.

1.1.4 Bovine herpesvirus 1

Bovine herpesvirus 1 was first isolated by Madin and coworkers in 1956 as the viral pathogen responsible for infectious bovine rhinotracheitis (Madin et al., 1956). This is an acute, contagious bovine disease, which causes fever, depression, drop in milk production and emaciation (Wyler et al., 1989). A secondary bacterial infection, Pasteurella haemolytica, may follow the primary viral infection and cause bronchopneumonia. This bovine respiratory disease complex is referred to as Shipping Fever (Babiuk et al., 1988; Tikoo et al., 1995; Yates, 1982). Additional clinical problems associated with BHV-1 include vulvovaginitis, conjunctivitis,
encephalitis, and generalized systemic infections. In pregnant cows and young calves, abortions and severe respiratory tract infections have been reported. Due to the loss of animals, abortions, decreased milk production, and loss of weight, BHV-1 causes serious economic losses all over the world.

BHV-1 is a member of the Alphaherpesvirinae subfamily. Its genome belongs to group D, resembling that of PRV, EHV-1, EHV-3, and VZV, therefore, BHV-1 is subclassified in the genus Varicellovirus (VZV-like viruses) rather than with BHV-2 in the genus Simplexvirus (Brown 1989). By cross-neutralization studies, there is only one serotype found in BHV-1 (Nyaga and McKercher 1979). However, based on genome analysis and viral polypeptide patterns, BHV-1 can be subclassified into five subtypes (Wyler et al., 1989). The primary host of BHV-1 is cattle; other animals like rabbits, pigs, sheep, goats, and wild ruminants are susceptible (Wyler et al., 1989). The virus has a broad host range in cell culture and it can cause cytopathic effects in cell monolayers.

The BHV-1 genome consists of approximately 135 to 140 kilobase pairs (kbp) (Mayfield et al., 1983). It is composed of a unique long segment UL (104 kbp), and a unique short segment US (11 kbp) flanked by inverted repeat regions (2x12 kbp) (Mayfield et al., 1983). The inverted repeat regions allow the unique short segment to exist in two orientations,
a characteristic of the group D type of viral genome. Fifty four transcripts have been identified in a productive BHV-1 infection (Wirth et al., 1989). At least 33 structural proteins and at least 15 non-structural proteins have been reported to be produced during productive infection (Bolton et al., 1983; Misra et al., 1981). Based on [3H]-glucosamine labeling, there are 11 protein bands present on reducing SDS-PAGE gels, which are identified as glycoproteins (Misra et al., 1981). Some of the glycoproteins have been well characterized and some have been identified from their open reading frames (ORF) (Fitzpatrick et al., 1989; Khadr et al., 1996; Khattar et al., 1995; Leung-Tack et al., 1994; Liang et al., 1996; Meyer et al., 1991; Misra et al., 1988; Tikoo et al., 1990). These glycoproteins can mediate viral interactions with cellular receptors to initiate BHV-1 infection (Liang et al., 1991; Okazaki et al., 1987). They are also important for the immune responses in host animals, therefore, they are candidates for vaccine development (Babiuk et al., 1987; van Drunen Littel-van den Hurk et al., 1993).

**1.2 PROTEINS OF HERPESVIRUSES**

In some herpesviruses, the genomes have been completely sequenced, which enables the identification and prediction of potential ORFs (Baer et al., 1984; Chee et al.,
1990; Davison and Scott 1986; Hatfull et al., 1988; McGeoch et al., 1985, 1988). Protein products for many of the ORFs have been identified, whereas some ORFs remain to be studied. In HSV-1, there are more than 70 proteins present in the viral particle, which can be generally defined as glycoproteins, tegument proteins and capsid proteins (Haarr and Skulstad 1994; Ward and Roizman 1994).

1.2.1 Glycoproteins

Viral glycoproteins are the major structural components of the envelope in herpesviruses. The lipid components of the envelope in herpesviruses are derived from the nuclear membrane of host cells. On the envelope, virus-coded glycoproteins make up the short, densely packed spikes present on the surface of virions (Stannard et al., 1987). Herpesviruses specify a number of different glycosylated proteins, but not all glycoproteins become components of the envelope (Roizman and Baines 1991). The herpesvirus glycoproteins are involved in a variety of protein-protein interactions between the virus and the host cells. These glycoproteins are also important targets of the host immune response (Babiuk et al., 1987).

As the outside projections of the virion, the membrane glycoproteins have roles in virus-host cell interactions and they are involved in virus entry including attachment and penetration. For virus attachment, gC, gB, and
gD have been found to bind HS or non-HS components to establish the initial and stable binding of virions to permissive cells (Fuller and Lee 1992; Johnson et al., 1984; Johnson and Ligas 1988; Johnson et al., 1990; Kuhn et al., 1990; Liang et al., 1991). A group of glycoproteins including gB, gD, gH, gK, and gL have been found to be involved in viral penetration. Some glycoproteins are involved in cell-to-cell spread of the virus and immune evasion or other viral activities during the infection. In Table 1-2, the identified glycoproteins of selected herpesviruses are summarized. These glycoproteins are grouped in families using the glycoprotein nomenclature adopted in 1994.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Homologues</th>
<th>Necessary</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>gB</td>
<td>gII(VZV, PRV), gI (BHV-1), gp14(EHV-1), UL55(HCMV), BALF4 (gp110, EBV)</td>
<td>Yes</td>
<td>Involved in attachment and fusion, induces neutralizing antibodies</td>
</tr>
<tr>
<td>gC</td>
<td>gpV(VZV), gIII( PRV, BHV-1), gp13(EHV-1)</td>
<td>No</td>
<td>Binds cellular HSPG for attachment, induces neutralizing antibodies. Binding of C3b component of the complement pathway for immune evasion.</td>
</tr>
<tr>
<td>gD</td>
<td>gp50(PRV), gIV(BHV-1)</td>
<td>Yes</td>
<td>Involved in attachment and penetration, induces neutralizing antibodies</td>
</tr>
</tbody>
</table>

Table 1-2. Herpesvirus glycoproteins and their functions
<table>
<thead>
<tr>
<th>Designation</th>
<th>Homologues with other in HSV-1 name</th>
<th>Necessary for virus infectivity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>gE</td>
<td>gpI(VZV), gI(PRV), gp17/18 (EHV-1)</td>
<td>No</td>
<td>Forms complex with gI for transport to plasma membrane and constitutes a high-affinity Fc receptor, induces neutralizing antibodies which inhibit fusion</td>
</tr>
<tr>
<td>gG</td>
<td>gX(PRV)</td>
<td>No</td>
<td>Involved in virion entry, egress and cell-cell spread</td>
</tr>
<tr>
<td>gH</td>
<td>gpIII(VZV), gp85(EBV), p86(HCMV),</td>
<td>Yes</td>
<td>Plays a role in fusion, affects viral entry, egress and cell-cell spread, induces neutralizing antibodies</td>
</tr>
<tr>
<td>gI</td>
<td>gpIV(VZV), gp63(PRV),</td>
<td>No</td>
<td>Forms complex with gE, the complex contributes to intracellular transport and to membrane fusion, also constitutes a high-affinity Fc receptor</td>
</tr>
<tr>
<td>gJ</td>
<td></td>
<td>No</td>
<td>Predicted glycoprotein based on sequence analysis</td>
</tr>
<tr>
<td>gK</td>
<td></td>
<td>Yes</td>
<td>Plays a role in entry, egress and cell-cell spread</td>
</tr>
<tr>
<td>gL</td>
<td>gp25(EBV)</td>
<td>Yes</td>
<td>Complexed with gH, plays role in fusion</td>
</tr>
<tr>
<td>gM</td>
<td></td>
<td>No</td>
<td>Cell-to-cell spread</td>
</tr>
</tbody>
</table>

a Balan et al., 1994  
b Haarr and Skulstad 1994  
c McGeoch et al., 1993  
d Roizman 1993  
e Spear 1993  
f Ward and Roizman 1994
In addition to the glycoproteins listed in Table 1-2, other potential membrane proteins are under investigation. Previously, the HSV-1 genome was reported to have the potential of encoding 17 integral membrane proteins (McGeoch et al., 1985, 1988). Based on sequence prediction and preliminary studies, products of UL20, UL24, UL34, UL43, UL45, UL49.5 are believed to be membrane proteins (Haarr and Skulstad 1994; Roizman and Sears 1993; Ward and Roizman 1994).

Viral glycoproteins are synthesized on membrane-bound polyribosomes of the rough endoplasmic reticulum (RER). During their synthesis, the N-terminal signal peptide, a fragment with primarily hydrophobic residues, directs the translocation of the polypeptide chain across the membrane of the RER. This signal fragment can be cleaved by cellular signal peptidase (Zwizinski et al., 1980). In the lumen of the RER, high-mannose oligosaccharides are transferred to asparagine in the Asn-X-Ser/Thr sequence, the potential glycosylation site, of newly synthesized polypeptides (Hirschberg 1987). After the proteins are transported to the Golgi, the high-mannose oligosaccharides are further processed to form hybrid or complex type oligosaccharides. All oligosaccharides are modified by cellular enzymes, which reside in the RER or the Golgi (Hirschberg 1987). The carbohydrate structures of the viral glycoproteins play roles in maintaining correct folding and directing intracellular
transport. They help to maintain the physicochemical properties and to modulate the antigenicity of the glycoprotein. They are also important in protecting the molecule against proteolytic degradation (Bruck et al., 1982; Olden et al., 1985; Von Figura and Hasilik 1986).

1.2.2 Tegument proteins

The herpesvirus tegument is an amorphous structure, accounting for approximately 50% of the volume of the virion. A large number of proteins, particularly phosphoproteins, can be found in relative abundance in the tegument. These proteins are neither completely released by nonionic detergent treatment nor associated with the nucleocapsids (Haarr and Skulstad 1994; van Drunen Littel-van den Hurk et al., 1995). They are structural components and they play a role in regulating viral infection. The major tegument protein in HSV-1 is α-TIF, also known as VP16, Vmw65, ICP25, and the gene product of UL48 (Campbell et al., 1984; McGeoch et al., 1988). This protein is required for virion assembly and it transactivates α gene expression by interacting with a complex of other factors to activate the α gene promoters (Ace et al., 1988; Batterson and Roizman 1983; Campbell et al., 1984). Another important tegument protein is the virion host shut-off (vhs) protein, a 58 kDa phosphorylated protein encoded by UL41 (Fenwick and Walkwer 1978; Kwong et al., 1988; McGeoch et al., 1988; Nishioka and Silverstein 1978;
Smibert et al., 1992). This protein causes degradation of cellular mRNA and shuts off host cell protein synthesis early after infection (Kwong et al., 1988; Read et al. 1993). An UL13 encoded protein kinase is also found in the tegument, and it is responsible for phosphorylating several virion proteins (Coulter et al., 1993; Cunningham et al., 1992; Overton et al., 1992; Smith and Smith 1989). Other tegument proteins are identified as VP1/2 (UL36), VP11/12 (UL46), VP13/14 (UL47), and VP22 (UL49) (Elliott and Meredith 1992; McLean et al., 1990; McNabb and Courtney 1991, 1992; Zhang and McKnight 1993). During virus replication, products of VP13/14 and VP22 have been found to associate with the nuclear matrix and bind DNA, although their functions are not very clear (Blair and Honess 1983; Pinard et al., 1987). A recent study in HSV-1 identified an essential protein encoded by UL25, likely a tegument protein, which is involved in viral penetration and capsid assembly (Ali et al., 1996). Furthermore, the gene products of US9, UL11, and two transcriptional regulatory proteins, ICP4 and ICP0, are also associated with the tegument and thus, may be considered tegument proteins (Frame et al., 1986; Maclean et al., 1989; Yao and Courtney 1989, 1992).

In HCMV, the major tegument constituents are pp150, the product of UL32, and pp65, the product of UL83 (Jahn et al., 1987, 1987a; Landini et al., 1987; Ruger et al., 1987). One minor tegument protein, pp71, which shares amino acid
sequence similarity with pp65 and is the gene product of UL82, has been identified as a \textit{trans}-activator of gene expression (Liu and Stinski 1992; Nowak et al., 1984; Ruger et al., 1987). Other phosphoproteins, pp28, pp67 are also tegument constituents (Mocarski 1993).

In BHV-1, VP8 was found to be one of the most abundant tegument proteins with homology to HSV-1 VP13/14 (product of UL47) (Carpenter and Misra 1991; Misra et al., 1981; van Drunen Littel-van den Hurk et al., 1995). It may participate in modulating a gene expression (van Drunen Littel-van den Hurk et al., 1995). Another product of the UL49 homologue has been identified as a tegument protein which is dispensable for virus growth \textit{in vitro} (Liang et al., 1995a).

1.2.3 Capsid proteins

In thin sections of infected cell nuclei, three capsid forms can be found: A, an empty capsid shell with no internal structure; B, an intermediate form without viral DNA but containing a proteinaceous core inside the capsid; C, a full form which contains the viral genome. All these forms are produced during viral maturation. The capsid and internal proteins can be obtained by treatment of purified virions with detergents and/or lipid solvents. It has been found that the HSV-1 B capsid is composed of seven proteins, they are assembled into the icosahedral structure via a mechanism
which is unclear (Rixon 1993; Thomsen et al., 1995).

In HSV-1, VP5 is identified as the major capsid protein (MCP) which is the predominant structural subunit of both the hexons and pentons (Costa et al., 1984; Desai et al., 1993; Newcomb and Brown 1989). There are six copies of VP5 in hexons and five copies in pentons (Newcomb et al., 1993; Trus et al., 1992). A homologous protein encoded by UL86 in HCMV has also been found to be the MCP (Chee et al., 1989). A smaller, less abundant protein, VP19C, encoded by UL38 in HSV-1 or UL46 in HCMV has been recognized as a minor capsid protein. This protein is a DNA-binding protein that may anchor viral DNA to the capsid (Braun et al., 1984; Chee et al., 1990; Irmiere et al., 1985; Pertuiset et al., 1989; Rixon et al., 1990). In HSV-1, a 40 kDa protein (p40, ICP35, VP22a), the product of UL26.5, has been found to associate with the virion and aid in capsid maturation (Braun et al., 1984a). Its homologue in HCMV is called assembly protein, which is a nonstructural phosphoprotein, involved in virus assembly (Gibson et al., 1990; Robson and Gibson 1989). In HSV-1, other proteins like VP23, VP24, and VP26, which are products of UL18, UL26, and UL35 respectively, have been reported to be capsid proteins (Davison and Scott 1986; McNabb and Courtney 1992; Newcomb and Brown 1991; Rixon et al., 1990). In general, VP5 and three other proteins, VP19C, VP23, and VP26, make up the outer shell. The core of the B capsid is composed of three proteins, VP21, VP24, and VP22a.
1.3 HERPESVIRUSES AND HOST CELL INTERACTION

Herpesvirus entry into permissive cells is a complex multistep process involving sequential interactions between several viral glycoproteins and distinct cellular membrane constituents. In addition to the complex interactions involved in entry, different subfamilies of the herpesviruses may have different entry mechanisms. In this thesis, entry of alphaherpesviruses will be described. Infection by alphaherpesviruses appears to be initiated by the attachment of the virion, via gC and/or gB, to HSPG moieties on the cell surface. Subsequent steps include attachment of other viral glycoproteins to unidentified cell-surface receptors. The interactions of viral glycoproteins with membrane components leads to stable attachment followed by penetration of the nucleocapsid into the cell. Entry occurs by direct fusion of the viral envelope with the cell membrane (Cooper 1994; Fuller and Lee 1992). Although a large number of cell surface components have been identified as potential virus receptors, their exact roles in attachment and facilitating viral entry, especially in herpesviruses, is not clear (Heywood 1994; Lentz 1990).

1.3.1 Heparan sulfate as a primary receptor for herpesviruses

The surface of mammalian cells is coated with
proteoglycans, which are proteins containing carbohydrates called glycosaminoglycans (GAG). These carbohydrates on proteoglycans are polymers of disaccharide repeats, which are mostly highly sulfated and negatively charged. The most prominent GAG found in proteoglycans are heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate (Couchman and Woods 1993; Jackson et al., 1991; Kjellén and Lindahl 1991).

HSPG on cell surfaces has been demonstrated to be the initial receptor involved in the interaction with some herpesviruses (Compton et al., 1993; Kart and Gehrz 1992; Mettenleiter et al., 1990; Okazaki et al., 1991; Sawitzky et al., 1990, 1990a; Vanderplasschen et al., 1993; WuDunn and Spear, 1989). There are a few lines of evidence to support this. (1) Herpesviruses directly bind to heparin, as demonstrated by inhibition of virus attachment to cells by soluble heparin, and binding of virus or viral glycoproteins to immobilized heparin (Kari and Gehrz 1992; Lycke et al., 1991; Mettenleiter et al., 1990; Okazaki et al., 1991; Shieh et al., 1992; WuDunn and Spear 1989). (2) Agents that bind to heparan sulfate can block virus adsorption (Campadelli-Fiume et al., 1990; Compton et al., 1993; Herold and Spear 1994; Langeland et al., 1988). (3) Enzymatic removal of cell-surface heparan sulfate reduces binding and infectivity of subsequently added virus, whereas digestion of other cell-surface GAG has no effect (Compton et al., 1993; Mettenleiter
et al., 1990; Okazaki et al., 1991; Vanderplasschen et al., 1993; WuDunn and Spear, 1989). (4) Mutant cell lines which are defective in heparan sulfate synthesis (but not other GAG) generally exhibit reduced binding of herpesvirus (Gruenheid et al., 1993; Vanderplasschen et al., 1993) and are resistant to herpesvirus infection (Shieh et al., 1992).

Since heparan sulfates are negatively charged, the initial step of herpesvirus entry may include an electrostatic interaction. Studies suggest that gC homologues, in different herpesviruses, play the dominant role in mediating this initial binding (Herold et al., 1991; Liang et al., 1991a, 1993; Mettenleiter et al., 1990; Okazaki et al., 1991; Sawitzky et al., 1990, 1990a). gC has been shown to form the longest projections on the surface of virions and contain multiple heparin-binding domains (Flynn and Ryan 1996; Liang et al., 1993; Stannard et al., 1987). However, gC is not essential for infectivity since gC-deficient mutants are still able to infect cells (Herold et al., 1991, 1994; Liang et al., 1991, 1993; Robbins et al., 1986). Furthermore, the HS-binding domain of gC can be replaced by other heterologous heparin-binding domains without dramatically changing viral infectivity (Flynn and Ryan 1995). All these results suggest that during the early stage of infection, gC homologues help to bring virion and cell together by binding to cellular HS moieties, but this is not the only way to initiate virus binding on the cell
surface. In some herpesviruses, another glycoprotein, gB, can also bind HS (Byrne et al., 1995; Herold et al., 1991; Kari and Gehrz 1992; Vanderplasschen et al., 1993), therefore, it may also initiate virus infection by binding with cellular HS. In HSV-2, the heparin-binding activity of gB has been suggested to play the dominant role in virus binding to permissive cells (Gerber et al., 1995).

Previous studies have shown that HSV-1 gC and gB may interact with distinct structures of the heterogeneous chains of heparan sulfate (Herold and Spear 1994; Herold et al., 1995). During adsorption to polarized epithelial cells, wild type (wt) HSV-1 can attach to both the apical and the basal surface, whereas gC-negative mutants only attach to the basal surface (Sears et al., 1991). This may indicate that there are different binding moieties for gC or gB on the surface of polarized epithelial cells. The difference in gC and gB binding provides further evidence that there are different mechanisms involved in the initial attachment of herpesviruses to cells. Although the concept of HS-dependent attachment by herpesviruses is popular, HS-independent attachment is also found (Karger et al., 1995; Mettenleiter et al., 1990; Subramanian et al., 1994). This diversity suggests the existence of various mechanisms for herpesvirus attachment.
1.3.2 Non-HS structures for viral attachment

The interaction of alphaherpesvirus glycoproteins with non-HS receptors on cells was first recognized using HSV-1 as a model (Johnson and Ligas 1988; Johnson et al., 1990; Kuhn et al., 1990). It was found that incubation of cells with soluble forms of gD could block entry but not attachment of wt virus (Johnson et al., 1990). These results suggested that cells susceptible to HSV-1 infection have a limited number of gD-specific receptors, which are distinct from heparan sulfate and unaffected by the addition of exogenous heparin (Johnson and Ligas 1988; Johnson et al., 1990; Lee and Fuller 1993). Using soluble forms of HSV-1 gD, Brunetti et al. (1994) suggested that mannose 6-phosphate may be the cellular receptor for gD. In another alphaherpesvirus, BHV-1, an uncharacterized 60 kDa cellular protein was suggested to be the receptor for BHV-1 gD (Thaker et al., 1994).

In HCMV, an uncharacterized 34 kDa membrane protein, probably annexin-II, was found to mediate binding of the virus to cells (Adlish et al., 1990; Nowlin et al., 1991; Taylor and Cooper 1990; Wright et al., 1994). Annexin-II is a member of the lipocortin family of proteins with known Ca\textsuperscript{2+}-dependent phospholipid binding and vesicle aggregation capabilities. Additional cellular components were also found to be involved in HCMV entry, probably at the fusion step (Keay et al., 1989; Keay and Baldwin 1991).
Attachment of EBV is mediated by the interaction of the major viral glycoprotein gp350/220 and the CD21 molecule on the surface of B lymphocytes (Tanner et al., 1987; Wells et al., 1982). Monoclonal antibodies to the CD21 glycoprotein can block viral attachment (Fingeroth et al., 1984; Weis et al., 1984). Furthermore, purified CD21 binds EBV, and CD21 expressed on heterologous cells confers to these cells the ability to adsorb EBV (Ahearn et al., 1988; Li et al., 1992; Motz et al., 1987; Nemerow et al., 1985, 1986; Siaw et al., 1986). These lines of evidence indicate that the type 2 complement receptor (CR2 or CD21) is a specific cellular receptor recognized by EBV. Human CR2 is a member of the gene family of regulators involved in complement activation, with primary specificity for the d region of C3. This glycoprotein has 15 or 16 imperfect 60 amino acid repeats, a transmembrane domain, and a short carboxy-terminal cytoplasmic domain (Moore et al., 1987; Weis et al., 1988). The EBV binding activity is associated with the two amino-terminal repeat domains (Carel et al., 1990; Molina et al., 1991; Moore et al., 1991). During entry of EBV, the CR2 molecule acts as the receptor for viral gp350/220 and as a signal-transducing molecule.

1.3.3 Virus entry and cell fusion

In eukaryotic cells, membranes play a dominant role in both structure and function. The cell is enclosed by a
membrane, has a membrane-delineated nucleus and many membranous organelles. Membrane fusion reactions are involved in many cellular activities, such as endocytosis/exocytosis, sperm-egg fertilization, organelle formation, and infection by enveloped viruses. To initiate a productive infection, the viral genome generally migrates into the nucleus of the host cell by penetrating the membrane system. Therefore, entry of enveloped viruses requires fusion of the viral envelope with membranes of the host cell. Fusion can occur in a pH-dependent or pH-independent manner (White 1990). In pH-dependent fusion, virion binding is followed by receptor-mediated endocytosis to internalize the virion and subsequent fusion of the viral envelope with membranes of the intracellular vesicle. The fusion process is triggered by an intracellular endosomal pH change and conformational changes in the viral fusion proteins (White 1990). The best characterized model is haemagglutinin (HA) of influenza virus (Bentz et al., 1993). Members of orthomyxoviruses, togaviruses, rhabdoviruses, and bunyaviruses require low pH to fuse (Stegmann et al., 1989; White 1990).

pH-independent fusion is involved in the entry of paramyxoviruses, coronaviruses, retroviruses and herpesviruses (Compton et al., 1992; Miller and Hutt-Fletcher 1992; Stegmann et al., 1989; White 1990). Electron microscopy studies demonstrated that entry is mediated by fusion of the viral envelope with the plasma membrane of the cell at

27
neutral pH (Fuller and Spear 1987; Fuller et al., 1989; Fuller and Lee 1992; Wittels and Spear 1990). Obviously, the glycoproteins on the viral envelope play a role in the fusion process. Support for this idea is forthcoming from experiments involving virosomes or lipid vesicles containing HSV-1 glycoproteins which can fuse with cell membranes (Johnson et al., 1984). However, the entire fusion mechanism remains unclear.

To date, many viral fusion proteins have been identified in different families of enveloped DNA or RNA viruses. These proteins are class I glycoproteins present in the viral envelope. In addition to causing fusion, these glycoproteins may also be involved in binding the virus to cellular receptors (Haywood 1994). In the biosynthetic process, some fusion proteins are first made as larger precursors, then cleaved into two polypeptide chains. The two pieces remain associated by disulfide bonds or by non-covalent interactions. Generally, the C-terminal portion anchors the protein to the viral envelope via a hydrophobic transmembrane domain. In most C-terminal polypeptides of fusion proteins, a fusion domain can be found, which is also relatively hydrophobic.

Fusion domains are generally conserved within, but not between, virus families. They are distinct from signal sequence or transmembrane domains as they have different hydrophobicity values and are also rich in alanine and
glycine (White 1990). Predictive analysis shows that fusion domains may form amphipathic helical structures with more hydrophobic amino acids falling on one side of the helix. The proteins containing fusion domains are normally oligomerized where each monomer contacts the others by the hydrophobic faces, thereby burying the fusion domains within the proteins. Upon interaction with cell membranes, the conformation of the protein may change so as to expose the hydrophobic fusion domains, promoting the mixing of lipid components from two opposite bilayers. In influenza virus, the HA represents a prototypic fusion protein. It is a trimer structure that projects from the viral envelope like a rod. Each HA monomer has disulfide-bonded HA1 and HA2 subunits which are products of posttranslational proteolytic cleavage from the HA precursor. Cleavage is required for fusion activity and hence for viral infectivity. The fusion domain is located at the N-terminal end of the HA2 subunit. This domain is highly conserved in HA from different virus strains. In its native structure, the hydrophobic fusion domain in each HA monomer is hidden at the interface between the subunits of the trimer. During infection, an acid-induced conformational change dissociates the HA1 domains in the trimer and also shifts the position of the HA2 to expose the fusion domains so that they can interact with the target membrane. The interaction of the fusion domains with the target cell membrane results in the insertion of the
hydrophobic segment into the lipid bilayer hydrophobic core. Therefore, the fusion domains interact with lipid components of both the viral and the target membranes, forming a fusion pore in the centre of the HA aggregate. This process leads to fusion of the viral and cellular membranes (White 1992). For viruses with more complex genomes, such as herpesviruses, the fusion mechanism still remains elusive, and may actually require the coordinated participation of several distinct glycoproteins (Zhu and Courtney 1988).

In vitro studies have found that in HSV-1, gB, gD, gH, gK, and gL are essential for virus replication (Cai et al.; 1988; Desai et al., 1988; Forrester et al., 1992; Hutchinson and Johnson 1995; Johnson and Ligas 1988; Little et al., 1981; Roop et al., 1993). Virus mutants lacking any one of these glycoproteins can still absorb to cells, but fail to penetrate or to fuse with the cell membrane (Cai et al., 1988; Desai et al., 1988; Forrester et al., 1992; Fuller and Lee 1992; Ligas and Johnson 1988; Peeters et al., 1992). Monoclonal antibodies (mAbs) to each of these glycoproteins fail to block attachment of wt virions to cells, although they do greatly reduce viral infectivity (Dubuisson et al., 1992; Fuller and Spear, 1985, 1987; Fuller et al., 1989; Highlander et al., 1987, 1988; Rodriguez et al., 1993). Therefore, it was suggested that these mAbs may block virus penetration after the initial attachment step. The failure of the attached virions to fuse with cells can be further
demonstrated by the application of a chemical fusogen, polyethylene glycol, which helps to deliver the attached virions across the membrane junction (Fuller and Spear, 1987; Fuller et al., 1989). Taken together, these results suggested that these glycoproteins are involved in virus penetration. Furthermore, these results demonstrate that fusion induced by herpesviruses is a complex process. Previously, syncytial mutations have been mapped to different genes in HSV-1, including UL27 (gB), UL53 (gK), UL20 and UL24 (Baines et al., 1991; Bond and Person 1984; Bzik et al., 1984a; Hutchinson et al., 1992; Jacobson et al., 1989; Pogue-Geile et al., 1984; Ruyechan et al., 1979; Sanders et al., 1982). Recently, the product of UL45 was found to be also important for viral fusion. Without the gene product of UL45, HSV-1 syncytial mutants can not form syncytia (Haanes et al., 1994). In another report, the product of UL25 has been found to be essential for HSV-1 entry (Ali et al., 1996). The multicomponent fusion process has also been reported in other herpesviruses. For example, in PRV, the homologues of gB, gD, and gH have all been implicated in cell fusion (Eliot et al., 1990; Zuckermann et al., 1989).

Since herpesvirus induced cell fusion is a complex process, involving several components, different viral proteins must rely on a cooperative mechanism to conduct fusion activity. It has been suggested that in HSV-1, gB, gD, and gH/gL must be present on the same cellular membrane in
order to act in cis to cause cell fusion (Davis-Poynter et al., 1994). In contrast, HSV-1 gK has been shown to be retained in the perinuclear and nuclear membranes but not on the cell surface (Hutchinson et al., 1995). Therefore, instead of playing a direct role in fusion, gK may play a role in regulating fusion of infected cells. The suggested function of gK provides strong evidence that fusion induced by herpesvirus is a complex process, in which some proteins have a modulatory function. Since the fusion involves a cascade of interdependent processes, any change in one of the viral components involved in this process may cause changes in the fusogenic property of the virus.

Among the fusion-related proteins, there are questions regarding which protein(s) are involved directly as fusogenic proteins, and which proteins regulate fusion. Mutations in the gB sequence can cause different degrees of fusion, ranging from syncytial or defective fusion to where gB null mutants totally lose the cell fusion ability (Baghian et al., 1993; Cai et al., 1987, 1988; Gage et al., 1993; Navarro et al., 1992; Weise et al., 1987). Furthermore, it appears that gB alone can induce spontaneous fusion in mammalian cells expressing this glycoprotein. This has been demonstrated using different herpesviruses including HSV-1, HCMV, and BHV-1 (Ali et al., 1987; Cai et al., 1988; Fitzpatrick et al., 1988, 1990; Tugizov et al., 1994). Therefore, gB may play a direct role as a fusogenic protein.
A large fraction of syncytial HSV-1 mutants contain mutations in the gene encoding gK (Bond and Person 1984; DebRoy et al., 1985; Hutchinson et al., 1992; Pogue-Geile et al., 1984; Read et al., 1980; Ruyechan et al., 1979). However, mutations in gK may change the fusion regulating profile and result in syncytial mutants. In mutants without UL20 or UL24 genes, it was suggested that fusion deregulation is affected and the mutants show a syncytial phenotype (Baines et al., 1991; Jacobson et al., 1989; MacLean et al., 1991; Sanders et al., 1982). From studies where individual glycoproteins were expressed in mammalian cells, gD alone also caused fusion (Campadelli-Fiume et al., 1988; Tikoo et al., 1990). Recently, the VZV gH/gL complex has also been reported to cause fusion in transfected cells (Duus et al., 1995). However, their possible roles as fusogenic proteins need further study.

Another important unanswered question is whether these proteins play a similar role in virus entry and in direct cell-to-cell spread. Virus penetration, which involves fusion of the viral envelope with the cell membrane, and the cell-to-cell spread of virus, which requires membrane fusion of infected cells with uninfected cells, appear to be different steps in herpesvirus infection. During recurrent herpesvirus infections, direct cell-to-cell spread of the virus is critical since neutralizing antibodies prevent extracellular spread (York and Johnson 1993). Further
evidence that the fusion mechanisms in virus-cell vs cell-to-cell spread may be different was provided by experiments which showed that both gB and gD homologues in PRV were essential for viral penetration, but only gB was required for cell-to-cell spread of the virus (Peeters et al., 1992). In HSV-1, gE and gI are not required for virus-cell infection, however, they can facilitate cell-to-cell spread of virus in cultured cells or in vivo (Balan et al., 1994; Dingwell et al., 1994; 1995; Zsak et al., 1992). Antibodies to gE can inhibit cell fusion caused by syncytial strains (Chatterjee et al., 1989) and deletion of gE and gI also changes the fusion properties of the syncytial mutants (Balan et al., 1994). All these results indicated that viral proteins may have different functions in virus penetration or cell-cell fusion.

1.4 GLYCOPROTEIN B IN HERPESVIRUSES

Homologues of glycoprotein B have been detected in all herpesviruses studied to date (Borchers et al., 1991; Buckmaster et al., 1988; Bzik et al., 1984, 1986; Chou and Marousek, 1992; Cranage et al., 1986; Eberle and Black 1991; Ellinger et al., 1993; Griffin 1991; Guo 1990; Hammerschmidt et al., 1988; Keller et al., 1986; Limbach et al., 1994; Maeda et al., 1992; Maes et al., 1988; Meredith et al., 1989; Misra et al., 1988; Pellet et al., 1985, 1985a; Poulsen et
al., 1991; Pumphrey and Gray 1994; Riggo et al., 1989; Robbins et al., 1987; Ross et al., 1989; Stewart et al., 1994; Whalley et al., 1989; Whitbeck et al., 1988; Zamb 1987). This family of glycoproteins shares similar structural and functional characteristics and is important for the infectivity of herpesviruses. In this thesis, gB homologues from some selected herpesviruses are discussed.

1.4.1 Sequence conservation

Among the herpesvirus glycoproteins, gB homologues exhibit the highest conservation in their primary structure (Table 1-3). They all have hydrophobic amino-terminal signal sequences, carboxy-terminal membrane anchor sequences and cytoplasmic tails. However, the signal sequences in different gBs are not well conserved. A number of gB homologues including BHV-1, PRV, EHV-1 have unusual long signal sequences (Meredith et al., 1988; Misra et al., 1988; Riggo et al., 1989; Robbins et al., 1987; Whalley et al., 1989; Whitbeck et al., 1988; Zamb 1987). The function of this long signal sequence is not clear. The ectodomain region of all gBs is well conserved, especially the 10 cysteine residues and many N-linked glycosylation sites. The conservation patterns of cysteine residues and N-linked glycosylation sites among different gB homologues suggest that the secondary and tertiary structures of the proteins are also similar. This conservation has been suggested to be due to
the important function of gB in viral infectivity. Around the middle of the gB molecule, there is a poorly conserved region, which contains the cleavage site in some herpesvirus gBs.

Herpesvirus gB is a type I integral membrane protein. It has been shown that gB uses the transmembrane region to anchor the protein and leaves the carboxy terminal region inside the cytoplasm of the infected cells (Basgoz et al., 1992). In all herpesviruses, the transmembrane anchor region shows extremely high conservation (Fig. 1-2). This region has been previously thought to span the membrane three times (Pellet et al., 1985a).

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* Calculated by PALIGN program (Version 1.05) in PC/GENE software (Release 6.70).
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Conservation

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Conservation

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Conservation

** ** . . . . . . .

Fig. 1-2. Conservation of transmembrane region of herpesviruses. "**" and "." indicate perfect and good conservation respectively. "=" indicate the potential boundary for individual membrane spanning segment.

Recently, the long transmembrane region in herpesviruses has drawn much attention. In the sequence alignment of the transmembrane region of herpesviruses, it
was found that the polar residues (serine) and glycine are well conserved, and such conservation pattern is not required for a membrane anchor. Therefore, whether anchorage is the only function of this region remains to be determined.

The cytoplasmic tail region in herpesvirus gBs is poorly conserved. However, in alphaherpesviruses, two conserved helices are present, which are important in gB's fusion activity (Gage et al., 1993).

In addition to sequence homology, many gB products also share antigenic cross-reactivity (Balachandran et al., 1987; Hammerschmidt et al., 1988; Snowden et al., 1985), suggesting that they have similar structures.

1.4.2 Protein structure

Although herpesvirus gBs show high levels of homology, the protein structures are not exactly the same. For example, in the primary structure of gBs of PRV, BHV-1, EHV-1, and EHV-4, an unusually long signal sequence can be found (Meredith et al., 1988; Misra et al., 1988; Riggo et al., 1989; Robbins et al., 1987; Whalley et al., 1989; Whitbeck et al., 1988; Zamb 1987). As shown in Table 1-4, with exception of gB of HSV-1, HSV-2, BHV-2, and EBV, the gB homologues have a proteolytic cleavage site, resulting in cleavage of gB during processing (Cranage et al., 1986; Keller et al., 1986; Meredith et al., 1988; Misra et al., 1988; Pellet et al., 1985; Riggo et al., 1989; Robbins et
al., 1987; Whalley et al., 1989; Whitbeck et al., 1988; Zamb 1987). Cleavage of gB occurs in the Golgi by cellular protease (Vey et al., 1995; Whealy et al., 1990). This conserved hydrophilic cleavage site appears to have been lost in the gBs of HSV-1, HSV-2, and BHV-2. In EBV gB, although the cleavage site is present, the molecule is not processed through the Golgi apparatus, therefore, it remains uncleaved (Gong and Kieff 1990).

In those viruses where cleavage occurs, the resulting fragments remain linked to each other by intermolecular disulphide bonds. While the majority of mature gB is cleaved, small amounts always remain uncleaved (van Drunen Littel-vanden Hurk et al., 1986; 1990; Whealy et al., 1990). The cleavage of gB has been shown not to be essential for gB’s function in viral infectivity, however, it may affect virus spread from cell to cell (Blewett and Misra 1991; Kopp et al., 1994).

Oligomerization of gB homologues has been observed in several herpesviruses (Britt and Vugler 1992; Claesson-Welsh and Spear 1986; Sarmiento and Spear 1979; Whealy et al., 1990). It was suggested that proper oligomerization is required for the function of gB, as well as for productive viral infection (Chapsal and Pereira 1988; Haffey and Spear 1980). In HSV-1 gB, the cytoplasmic and transmembrane regions are dispensable for oligomerization (Ali 1990; Pereira et al., 1989; Qadri et al., 1991), however, two regions in the
ectodomain have been found to be involved in oligomer formation of gB. The upstream domain is located between residues 93 to 282 (Highlander et al., 1991). The other domain is localized between residues 596 to 711 (Ali 1990; Highlander et al., 1991; Navarro et al., 1993). By comparing the results reported by different groups, it appears that the downstream domain is critical for oligomerization and that the upstream site only plays a minor role. This conclusion is based on the observation that deletion of the downstream site (residues 616 to 711) totally abolishes gB oligomerization (Cai et al., 1988a). Moreover, a virus mutant, which has a deletion of the downstream site in gB can not form infectious virus and oligomerized gB (Desai et al., 1994). A recent study provided strong evidence that a 28-amino-acid domain, residues 626 to 653, is responsible for gB oligomerization (Laquerre et al., 1996). When this stretch was introduced into the carboxy-terminal region of gB, an unoligomerized polypeptide, oligomerization occurred.

Table 1-4. Structural properties of herpesvirus gB

<table>
<thead>
<tr>
<th>Herpesviruses</th>
<th>Subfamily</th>
<th>Molecular mass (kDa)</th>
<th>Proteolytic cleavage</th>
<th>Oligomerization</th>
<th>Dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>α</td>
<td>133</td>
<td>No</td>
<td>No</td>
<td>Dimer</td>
</tr>
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<td>α</td>
<td>135</td>
<td>No</td>
<td>No</td>
<td>Dimer</td>
</tr>
<tr>
<td>VZV</td>
<td>α</td>
<td>98</td>
<td>Yes</td>
<td>Dimer</td>
<td></td>
</tr>
<tr>
<td>BHV-1</td>
<td>α</td>
<td>130</td>
<td>Yes</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
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<td>α</td>
<td>120</td>
<td>Yes</td>
<td>Dimer</td>
<td></td>
</tr>
<tr>
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<td>130</td>
<td>No</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
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<td>143</td>
<td>Yes</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
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<td>150</td>
<td>Yes</td>
<td>Dimer</td>
<td></td>
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<tr>
<td>EBV</td>
<td>γ</td>
<td>110</td>
<td>No</td>
<td>unknown</td>
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40
The high-mannose precursor of gB can form oligomers in the ER during gB maturation (Ali 1990; Whealy et al., 1990). After oligomerization, gB is transported into the Golgi to be further glycosylated and/or cleaved. For HSV-1 gB transportation, the dimerization and the cytoplasmic region are not required (Navarro et al., 1993). Residues 441 to 475 have been shown to contain signals sufficient for processing and transport of gB through the exocytic pathway (Cai et al., 1987; Navarro et al., 1993). However, the carboxy terminus of gB may contain sequences that facilitate transport of gB to the cell surface (Navarro et al., 1993; Pachl et al., 1987). Alternatively, truncation of the carboxy terminus of the protein may alter the higher structure of gB in such a way that efficient transport is blocked (Pachl et al., 1987).

1.4.3 Functional studies

There are several lines of evidence to indicate that herpesvirus gB plays a role in membrane fusion. First, gB-specific mAbs that have post-adsorption neutralizing activities can block virus penetration. Second, mutants with defective gB’s may bind to permissive cells but fail to penetrate. Third, mutations in the cytoplasmic domain and ectodomain of gB can change the fusogenic phenotypes of the virus. Fourth, expression of gB in transfected cells may cause cell fusion.

Neutralizing mAbs directed at gB have been widely
used to define the regions involved in gB's fusion activity (Britt and Vugler 1988; Dubuisson et al., 1992; Navarro et al., 1992; Peeters et al., 1992; Pereira et al., 1990; Rauh and Mettenleiter 1991). In HSV-1 gB, domain D1 (residues 1-50), domain D2 (residues 1-457), and domain D3 (residues 600-690) are major targets of complement-independent neutralizing mAbs (Navarro et al., 1992; Pereira et al., 1990). These domains may function in virus penetration, membrane fusion, and cell-to-cell spread. MAbs against these domains do not block attachment of the wt virus, however, the attached virions fail to fuse with the cell membrane. In HCMV, mAbs that recognize epitopes on gB between amino acid (aa) 411 and 476, antigenic domains D1 and D2, preclude fusion of the virion envelope with the plasma membrane and prevent cell-to-cell spread (Navarro et al., 1993). Similar findings have been reported with other herpesviruses, including PRV and BHV-1 (Dubuisson et al., 1992; Peeters et al., 1992; Rauh and Mettenleiter 1991).

In HSV-1, many temperature-sensitive (ts) mutants, which can not produce correct forms of gB under non-permissive temperature, have been found. The defect in processing the gB precursor to the mature gB form affects virus infectivity (Bzik et al., 1984; DeLuca et al., 1984; Haffey and Spear 1980; Navarro et al., 1991; Schaffer et al., 1978). The mutant viruses can bind to cells but they do not penetrate cells. Further infection by these mutants can be
promoted by the addition of PEG, suggesting that gB functions in viral fusion. Syncytia-causing mutations have been located in gB, which also suggest gB's function in causing fusion (DeLuca et al., 1982; Goodman and Engel 1990; Holland et al., 1983; Little et al., 1981; Pogue-Geile and Spear 1987). Mutations in different regions of gB have been found to induce two different types of fusion alterations: a change in the rate of entry (roe) and generation of syncytial mutations (syn). Mutations at aa 437 or aa 189 can reduce or completely inhibit fusion activity (Cai et al., 1988; 1988a). Mutations at aa 277 and aa 373 affect the conformation of gB and generate ts mutants tsJ20 and tsJ12 respectively (Bzik et al., 1984a; DeLuca et al., 1984; Little and Schaffer 1981; Sarmiento and Spear 1979). A serine to proline substitution at aa 357 is responsible for the mutant ts1-8, which has low plaquing efficiencies at non-permissive temperature (Arko et al., 1991). An alanine to valine substitution at aa 552 of gB can slow the viral penetration rate (Bzik et al., 1984a; DeLuca et al., 1982). Therefore, all the mutations which occur in the ectodomain of gB may decrease fusion activity. Similarly, mutations in the carboxy terminal region have been known to cause syncytia formation (Bzik et al., 1984a; Cai et al., 1988; Engel et al., 1993; Ruyechan et al., 1979). A leucine to histidine substitution at residue 787, an alanine to valine substitution at residue 825, an arginine to histidine substitution at residue 828, a valine to alanine
substitution at residue 855, and an arginine to histidine substitution at residue at 857 are responsible for the syncytial phenotype (Balan et al., 1994; Bzik et al., 1984a; Engel et al., 1993). Other single amino acid substitution mutants have been found that generate syncytial phenotypes, which are mostly associated with two hydrophilic, $\alpha$-helical regions of the gB cytoplasmic domain (Gage et al., 1993). By linker insertion at residue 816 and 817, it is also found that this mutation causes a syncytial phenotype (Cai et al., 1988). Fig. 1-3 illustrates the identified locations which affect the fusion property of the HSV-1 gB.

<table>
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<tr>
<th>100</th>
<th>200</th>
<th>300</th>
<th>400</th>
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<td>TMA</td>
<td>↑↑</td>
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</tbody>
</table>

Fig. 1-3. Genome of HSV-1 gB and the mutations which affect fusogenic property. S and TMA indicate signal and transmembrane anchor region respectively. Single arrows represent mutations which cause decreased fusion (decreased rate of entry), and double arrows indicate mutations which cause syn mutants.
A very useful way of studying gB is to express gB in transfected mammalian cells. In HSV-1, HCMV, and BHV-1, expression of gB has been shown to cause cell fusion (Ali et al., 1987; Cai et al., 1987; Fitzpatrick et al., 1988, 1990; Tugizov et al., 1994). These data suggest that expression of gB can mediate syncytia formation independent of any other viral proteins. This provides strong evidence that gB may play a direct role in fusion.

The expression of gB in mammalian cells has been adapted to study the functional domains on gB without constructing gB mutant viruses (Navarro et al., 1991, 1993; Tugizov et al., 1994). By using a cell line which expresses truncated HCMV gB, with the deletion between residues 411 to 447, it was found that the recombinant gB still caused cell fusion (Tugizov et al., 1995). This result indicates that the potential fusion domain of HCMV gB is located outside of this region. However, mAbs which recognized epitopes in this region (between residues 411 and 476) precluded fusion of the virion envelope with the plasma membrane and cell-to-cell spread (Navarro et al., 1993), suggesting the existence of fusion-related epitopes in this region. Therefore, the fusion caused by the truncated gB, which has no fusion-related epitopes (aa 411-447), suggests that these mAbs may interfere with the conformation of gB that accompanies cell-cell fusion (Tugizov et al., 1995). This conclusion helps to explain the fusion mechanism by distinguishing the fusion domain and the
conformation of the fusion protein.

At this moment, although the mechanism of fusion is not clear, it is believed that the ability of gB to mediate fusion is influenced by a number of viral components. All these viral proteins are involved as fusion proteins or as modulators that regulate fusion. In normal herpesvirus infections, no cell fusion is observed, suggesting that fusion is an optimized process. However, any change of a fusion component may influence the fusion activity. This has been shown as syncytia-inducing mutations can be located in several locations along the viral genome, as well as in the carboxy-terminus of gB (Bond and Person 1984; Bzik et al., 1984; DebRoy et al., 1985; DeLuca et al., 1982; Little and Schaffer 1981; Pogue-Geile and Spear 1987). The proteins encoded by these genes are components of the fusion complex. Mutations in these genes can cause malfunction of the fusion protein or fusion modulator and generate syncytia. Since different proteins are involved in the modulation of fusion, it is most likely that the modulating proteins can affect the conformation of the fusion protein, probably gB, to establish a proper configuration for fusion.

The function of gB has been found to have strict structural requirements. Although in HSV-1, truncation of gB after residue 863 has no apparent effect on the function of gB (Huff et al., 1988), chain termination after residue 851 results in a mutant protein which fails to complement a gB-
negative virus (Cai et al., 1987, 1988) or function in cell-cell fusion (Cai et al., 1988). The defect in oligomerization can also affect its function and cause the mutant virus to lose infectivity. Recent structural and functional studies in HSV-1 gB have identified the membrane anchor and oligomerization domains (Gilbert et al., 1994; Laquerre et al., 1996; Rasile et al., 1993). Other structural domains required for the functions of gB, especially fusion, have not been reported.

Although gB homologues have the highest degree of conservation among herpesviruses, their role in viral infectivity has not been shown to be universal. The gB homologue in EBV, gp110, is not a major component of the virion, whereas in infected cells, gp110 can only be found perinuclearly but not on the cell surface (Gong et al., 1987). Antibodies against gp110 fail to neutralize EBV infectivity (Gong and Kieff 1990). Therefore, the function of EBV gB may not be the same as its counterparts in other herpesviruses. However, a recent study demonstrated that in EBV, gB is essential for virus entry or assembly (Herrold et al., 1996). These studies further support the contention that gB homologues play a very important role in herpesvirus infection.
2.0 EXPERIMENTAL DESIGN AND OBJECTIVES

Virus infection is initiated through specific interaction(s) between viral attachment proteins and their receptors on the surface of permissive cells. In herpesviruses, glycoproteins on the viral envelope mediate virus entry into host cells. Among them, gB is an important viral glycoprotein which plays an essential role in virus attachment and penetration. This glycoprotein and its counterparts in other herpesviruses share the highest degree of conservation among all glycoprotein families, suggesting that they may have similar functions in viral infectivity.

The early events in the entry of alphaherpesviruses into host cells have been shown to be initiated by the interaction between viral gC and heparan sulfates on permissive cells. In HSV, gB is also a heparin-binding glycoprotein. However, previous studies suggested that BHV-1 gB does not bind heparin. There is evidence that in BHV-1, the attachment is mediated by different viral glycoproteins, including three major viral glycoproteins, gB, gC, and gD. In order to get an insight into their roles as viral attachment proteins, a new strategy was applied by using purified
glycoproteins in a binding assay toMDBKcells. Both
authentic and truncated forms of gB, gC, and gD were tested
in this study. The relationship between viral proteins and
acellular HS structures was characterized by using soluble
heparin and heparinase to identify the glycoprotein that
binds HS. For further confirmation of our observations from
the protein-binding study, conventional heparin affinity
chromatography was used.

In order to study the function of gB in the virus
entry process and the structural requirements for this
function, different forms of gB were expressed in transfected
mammalian cells. This allowed us to study the function of gB
without using mutant viruses. In this thesis, different
aspects of the recombinant gBs were investigated including
structure, antigenicity, and maturation.

To characterize the structure and function of BHV-1
gB, my specific objectives were to investigate: a) the
ability of gB to bind to permissive cells and to HS; b) the
post-translational modifications of gB and sequences which
are involved in oligomerization and transport of gB; c) the
structural requirements for gB to bind to cells; d) the
fusion and membrane anchor domains of gB.
3.0 PRODUCTION AND CHARACTERIZATION OF BOVINE HERPESVIRUS 1 GLYCOPROTEIN B ECTODomain DERIVATIVES IN AN hsp70A GENE PROMOTER-BASED EXPRESSION SYSTEM

3.1 INTRODUCTION

Bovine herpesvirus-1 (BHV-1) is a member of the Alphaherpesvirinae subfamily and an economically important pathogen of cattle (Gibbs and Rweyemamu, 1977). The genome of BHV-1 encodes more than 33 structural proteins, some of which are membrane associated glycoproteins (Bolton et al., 1983; Misra et al., 1981). At present, three major glycoproteins, gB, gC and gD, have been well characterized (Collins et al., 1984; Marshall et al., 1986; van Drunen Littel-van den Hurk et al., 1984; van Drunen Littel-van den Hurk and Babiuk 1986). The corresponding genes have also been mapped and sequenced (Fitzpatrick et al., 1989; Misra et al., 1988; Whitbeck et al., 1988; Tikoo et al., 1990; Zamb 1987).

BHV-1 gB consists of 933 amino acids with features characteristic of type I integral membrane proteins, which include a putative signal peptide sequence located between residues 50 to 66, the transmembrane domain between residues 759 to 828, and a 104-amino-acid cytoplasmic tail (van Drunen
Littel-van den Hurk et al., 1992; Whitbeck et al., 1988; Zamb 1987). Like the gB homologues of most other herpesviruses, including pseudorabies virus (PRV) (Lukacs et al., 1985), varicella-zoster virus (VZV) (Montalvo and Grose 1987), human cytomegalovirus (HCMV) (Britt and Vugler 1989) and equine herpesvirus (EHV) (Meredith et al., 1989; Whalley et al., 1989), mature BHV-1 gB exists predominantly as a covalently linked heterodimeric complex of N-terminal subunit gBb and C-terminal subunit gBc. The two subunits are derived by proteolytic cleavage from a common primary translation product, while a small fraction of gB remains in an uncleaved form (Lukacs et al., 1985; Meredith et al., 1989; van Drunen Littel-van den Hurk et al., 1989).

In BHV-1, glycoprotein B is one of the essential viral glycoproteins (Whitbeck et al., 1988; Wyler et al., 1989). Previous studies have shown that gB plays an important role in virus entry, including initial virus attachment and subsequent penetration involving membrane fusion (Dubuisson et al., 1992; Fitzpatrick et al., 1988, 1990; Liang et al., 1991; van Drunen Littel-van den Hurk et al., 1992). It is now well established that attachment of BHV-1 is initiated via an interaction between viral gC and cellular heparin-like components. However, since gC is nonessential (Liang et al., 1991), other viral attachment protein(s) must exist. Previously, it has been demonstrated that in addition to gC, purified gB as well as gD, when added during virus
adsorption, could effectively block infections of both wild-type and gC-negative BHV-1 (Liang et al., 1991). Therefore, both gB and gD may also participate in the process of BHV-1 attachment. The evidence for gB’s involvement in the penetration step is based on the observations that gB-specific antibodies possess postadsorption neutralization activity (Dubuisson et al., 1992) and that expression of gB in transfected cells can induce syncytium formation and membrane fusion (Fitzpatrick et al., 1988, 1990; van Drunen Littel-van den Hurk et al., 1992). These suggested functions of BHV-1 gB are also consistent with what has been described for the homologous protein of HSV-1. BHV-1 gB also represents one of the primary viral antigens recognized by host defense mechanisms in naturally infected animals (Collins et al., 1984; Marshall et al., 1986). Furthermore, immunization of cattle with purified gB could elicit significant protection from a BHV-1 challenge (Babiuk et al., 1987; Gao et al., 1994; Israel et al., 1992; van Drunen Littel-van den Hurk et al., 1990). Because of its potent immunogenicity, it has been suggested that gB may be an important candidate for developing a BHV-1 subunit vaccine.

In order to further study the biological function of gB as well as to explore its potential use as a BHV-1 vaccine, we sought to establish bovine cell lines expressing gB and its subunits. Because expression of full-length gB has been shown to cause membrane fusion and to be toxic to
transfected cells (Fitzpatrick et al., 1988, 1990), we constructed a gB gene which lacks the sequence coding for the putative transmembrane and cytoplasmic domains (gBt). For gBb, the subunit representing the amino terminal fragment of the proteolytic products, the gene was constructed to code a protein which terminates at the proteolytic site. To express these forms of gB, we chose a heat-inducible, bovine heat-shock protein 70A (hsp70A) gene promoter-based expression system (Kowalski et al., 1993). Results of this study demonstrate that both gBt and gBb can be expressed at high levels in this novel expression system. The resultant gB products appear to be fully processed and secreted, and retain antigenic properties similar to their counterpart of the virus. Further investigation of the exocytic pathway of recombinant forms of gB suggests that the gBb subunit is well exposed and provides the transport signal for intracellular movement of gB, since another recombinant gB, the truncated C-terminal subunit gBct, is retained in the endoplasmic reticulum where it is synthesized.

3.2 MATERIALS AND METHODS

3.2.1 Virus, cells and reagents.

The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratories, Ames Iowa, and was propagated in Madin-Darby bovine kidney (MDBK) cells grown in
minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO). Lipofectin and G418 were from GIBCO; Tran [\textsuperscript{35}S]-label, which includes radiolabeled methionine and cysteine (Met/Cys), was from ICN (Mississauga, Ontario).

3.2.2 Construction of transfer vectors and transfected cell lines.

Plasmid p3KHSPG4HU (Kowalski et al., 1993), which contains a truncated BHV-1 gD gene under the control of the bovine hsp70 gene promoter and the aminoglycoside phosphotransferase gene under the control of the SV40 early promoter, was used as the parental plasmid for constructing transfer vectors. The restriction enzyme sites within the gB gene, which are relevant to the transfer vector construction are presented in Fig. 3-1. To generate the gBb gene transfer vector, p3KHSPGBb, two sets of oligonucleotides of both strands were synthesized. The first set of oligonucleotides contained the gB coding sequence for amino acid residues 1 to 21, which was flanked by a NcoI site containing the translation initiation codon ATG at the 5' end and a Sphi site at the 3' end. The second set of oligonucleotides, which were flanked with Sphi and SalI sites, contained the coding sequence for amino acid residues 487 to 505, followed by a translation stop codon TGA. These oligonucleotides were joined together at the Sphi site and cloned into NcoI and
SalI sites of plasmid polink/NcoI (Liang et al., 1992). The resultant plasmid contains 5' and 3' coding sequences of the gBb, separated by the SphI site (The SphI site is not from the original gB sequence and used only to contribute to the plasmid construction). To generate a full-length gBb gene, a 1.4 kilobase-pair (kbp) TthlllI-XhoI gB fragment was isolated from plasmid psd106 (Mayfield et al., 1983) and cloned at the corresponding sites of the above plasmid, resulting in plasmid polink/gBb. The final construct, p3KHSPGgB, was generated by replacing the NcoI-XhoI gD fragment in the p3KHSPG4HU with the NcoI-SalI gBb fragment from polink/gBb.

To construct the gBt transfer vector, we took advantage of a unique PvuII site naturally situated at gB codon 743. A set of oligonucleotides corresponding to the coding sequence for gB residues 743–763, followed by a translation stop codon TGA and a SalI recognition sequence were synthesized. It is important to note that the codons for Isoleucine 752 and Asparagine 753 were modified to accommodate a ClaI site for the purpose of facilitating screening of the plasmid to be generated. The synthetic PvuII-SalI 3' gB fragment was ligated with a 2 kbp SalI-XhoI 5' gB fragment from p3KHSPGgB (the SalI site is located within the hsp70 promoter sequence) and a 0.8 kbp XhoI-PvuII gB fragment (middle portion of gB) from psd106 to generate a SalI-SalI gBt gene fragment. This fragment was then cloned into SalI and XhoI sites of p3KHSPG4HU to replace the gD
gene. The resulting plasmid was named p3KHSPGBt.

In order to express the truncated gBC part, a heterologous signal sequence from BHV-1 gC (Fitzpatrick et al., 1989) was used in the construction of transfer vector p3KHSPGBct. The synthesized oligonucleotides introduce the gC signal fragment into the transfer vector and the gB gene via the NcoI and XmaI sites respectively.

To establish recombinant gB expressing cell lines, approximately 4 x 10⁶ MDBK cells were transfected with 5 μg of a transfer vector using Lipofectin (Felgner et al., 1987; Kowalski et al., 1993). After transfection, the cells were grown in the presence of 666 μg of G418 per ml medium. The G418-resistant cells were screened for gB production by an immune dot blot assay using gB-specific monoclonal antibodies (mAbs). The positive cells were isolated and subjected to single cell cloning. One of the gBt-positive clones, gBt03, one of the gBb-positive clones, gBb03, and one of gBct-positive clones, gBct15, were used in all subsequent studies.

3.2.3 Enzyme-linked immunosorbent assay (ELISA).

For quantitation of gB produced by the cell lines, microtiter plates were coated with 200 μl per well of the test samples or affinity-purified BHV-1 gB standard, in carbonate-bicarbonate coating buffer, pH 9.6, and incubated at 4°C overnight. Following incubation, the plates were washed three times with phosphate-buffered saline (PBS)
containing 0.025% Tween-20 (PBS-Tween), and incubated with 3% gelatin in PBS-Tween (blocking solution) for 1 h at 24°C. One hundred μl of gB-specific mAb at a 1:1000 dilution in blocking solution was added to each well, and the plates were incubated at 37°C for 2 h and then washed as above. Two hundred μl of mouse IgG specific horseradish peroxidase-conjugated goat antibody (BioRad, Missisauga, Ontario), diluted at 1:2000 in blocking solution, was added to each well, followed by incubation at 37°C for an additional 2 h. This was followed by three washes and addition of 150 μl of 1 mg/ml ABTS [2,2′-amino-di-(3-ethylbenzthiazoline sulfonate)] (Boehringer-Mannheim, Doval, Quebec) per well. After 1 h at 24°C, the plates were read at a wavelength of 405 nm using an ELISA plate reader. In each plate, serial dilutions of affinity-purified gB with known concentrations were incubated as standard. gB concentrations in the test samples were calculated based on the standard curve.

To determine gB-specific antibody titers in mouse serum, plates were coated with 0.1 μg purified gB per ml, which was followed by the same procedures described above, except that test samples, instead of gB mAbs, were added to the gB-coated plates.

3.2.4 Immunoprecipitation assays.

Subconfluent gB expressing cells were subjected to heat-shock for 6 h at 43°C or left at 37°C. Subsequently, the
cells were starved for methionine in methionine-free medium (GIBCO) supplemented with 2% dialysed FBS for 30 minutes and labeled with 50 μCi of [\textsuperscript{35}S]-methionine per ml of methionine-free medium containing 2% dialysed FBS for 18 h. After labeling, the cells and supernatant were harvested separately. Immunoprecipitation assays were carried out as previously described using a mixture of gB-specific mAbs (van Drenen Littel-van den Hurk et al., 1992). Antibody-precipitated samples were separated on SDS-8.5% polyacrylamide gels.

3.2.5 Pulse-chase experiment.

Subconfluent gB-expressing cells grown in 6-well plates were incubated at 43°C for 6 h, followed by washing with MEM. Cells were starved for methionine in methionine-free MEM supplemented with 2% dialysed FBS at 37°C for 30 minutes, and labeled by the addition of 200 μCi of [\textsuperscript{35}S]-methionine per ml of MEM at 37°C for 30 minutes. After labeling, supernatants were removed and cells were further incubated in MEM containing 2 mM methionine. At indicated time points, supernatants and cells were collected together, and samples were precipitated with gB specific mAbs and separated on SDS-8.5% PAGE under reducing conditions. For the experiments involving virus infection, normal MDBK cells were infected with BHV-1 at an moi of 5, and the above pulse-chase procedures were conducted at 7 h postinfection.
3.2.6 Affinity-purification of gB proteins and immunization of mice.

Affinity-purification of gB was carried out according to the methods described by van Drunen Littel-van den Hurk and Babiuk (1985). Briefly, a gB-specific immunoadsorbent column was equilibrated with 0.01 M Tris-HCl, 0.5 M NaCl, 0.1% Nonidet P-40, (pH 7.5; column buffer). Culture supernatant from the gB expressing cell lines or BHV-1-infected MDBK cell lysate was applied to the affinity column at a flow rate of 7 ml per hour, which was followed by two sequential washes, once with column buffer and once with column buffer lacking Nonidet P-40. Bound proteins were eluted with 0.05 M diethylamine, pH 11, and the eluant was neutralized, concentrated and dialyzed against 0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH 7.5. The purified proteins were stored at -70°C till use.

To assess the antigenicity of the purified glycoproteins, groups of mice (CD-1 strain) were immunized subcutaneously with 10 µg of protein in complete Freund’s adjuvant, and boosted two weeks later with 10 µg of protein in incomplete Freund’s adjuvant. One week after the boost, serum samples were collected and analysed for anti-BHV-1 antibodies.

3.2.7 Glycosidase digestion of glycoproteins.
The deglycosylation was performed as previously
reported (van Drunen Littel-van den Hurk et al., 1990). Samples were radiolabeled and immunoprecipitated, and the proteins were collected by boiling in 0.8% SDS. For endoglycosidase H (endo H) treatment, the proteins were resuspended in 0.125 M sodium citrate, pH 5.5, and digested with endo H (Boehringer-Mannheim, Laval, Quebec) at 37°C overnight. For N-glycosidase F (PNGase F) treatment, the proteins were resuspended in 0.2 M sodium phosphate buffer, pH 8.6, and digested with PNGase F (Boehringer-Mannheim) at 37°C overnight. After deglycosylation, the samples were precipitated by adding 1 ml cold acetone (-70°C) and pelleted at 15,000 g for 10-15 min at room temperature, then separated by SDS-PAGE.

3.3 RESULTS

3.3.1 Generation of gBt and gBb expressing cell lines.

BHV-1 gB is synthesized as a 933-amino acid precursor, which is subsequently cleaved into two subunits, i.e, the amino terminal fragment gBb and the carboxyl terminal fragment gBc. According to amino acid sequence analysis, the predicted gB transmembrane domain is located between residues 759-828 and the proteolytic cleavage recognition site between residues 505-506. The utility of the cleavage site has been confirmed by amino terminal sequence

determination of gBc showing that the alanine 506 is the amino terminal residue (van Drunen Littel-van den Hurk et al., 1992). On the basis of this information, the gBt and gBb genes were constructed so that they contained the sequences coding for residues 1-763 and for residues 1-505, respectively. Fig. 3-1 depicts the 5' and 3' sequences of authentic gB, gBt and gBb.

To produce gB expressing cell lines, transfer vectors containing the respective gB genes under the control of a hsp70A promoter, which are followed by a neomycin resistance gene, were constructed and used to transfect MDBK cells. The transfected cells were cultured in the presence of G418, a neomycin analog, and resultant G418-resistant cells were screened for gB expression by a gB-specific monoclonal antibody-based immune dot blot analysis. Three cell lines were subsequently established, and named gBt03, gBb03 respectively. These gB expressing cell lines exhibited morphology and growth properties indistinguishable from parental MDBK cells, and the expression of gB products was stable even after several passages in the absence of G418 (result not shown).

3.3.2 gB production in response to heat induction.

For characterization of the molecular properties of gBt and gBb produced by the transfected cells, an immunoprecipitation assay was performed (Fig. 3-2). We
compared heat-activation of protein production in gBt03 and gBb03 cells, and found that both cell lines could produce more gB products following heat-shock treatment (+ HS) than prior to heat-shock (- HS), indicating that gB production is under the control of the hsp70A promoter. Under non-reducing conditions, gBt existed as a single band with a molecular weight of about 115 kDa (Fig. 3–2 B); when reduced, the 74 kDa and 38 kDa bands were dominant, although the 115 kDa band was still visible (Fig. 3–2 A). The 74 kDa band had the same mobility as the gBb subunit present in the BHV-1-infected cell lysate, whereas the 38 kDa band was significantly smaller than the authentic gBc (55 kDa). The difference in apparent molecular weight between the truncated and authentic gBc was estimated to be 17 kDa, which approximates the estimated molecular weight of the truncated portion of gB. Therefore, the reduced molecular weight of gBt could be attributed to the truncation of its transmembrane and cytoplasmic domains. In gBb03 cells, the expressed product had a mobility similar to authentic gBb, where the trace amount of the 46 kDa product observed could be degraded protein. Under the conditions tested, gBt and gBb were readily detected in the culture medium, but little in the cell lysate (Fig. 3–2), indicating they were secreted from the cells.
Fig. 3-2. Immunoprecipitation of gBt and gBb from transfected cell lines. Subconfluent MDBK cells grown in T-25 flasks were heat shocked at 43°C for 4 h and labeled with 35S]-methionine. Cells and culture medium were collected separately and each precipitated with gB-specific monoclonal antibodies. The samples were separated on 8.5% SDS-PAGE gels under reducing conditions (panel A) or under non-reducing conditions (panel B). Also included is gB precipitated from the wt BHV-1 infected MDBK cells (BHV1). C, cellular fraction; M, medium; HS, heat shock; M.W., molecular weight standards (kDa).
3.3.3 Production of recombinant forms of gB under multiple heat-shock inducement.

Since we intended to produce the recombinant gB products in large amounts and to purify them from the media, we characterized the production of gB in the transfected cell lines. The cells were subjected to daily heat-shock treatment by incubation at 43°C for 4 h, whereafter the cells were returned to 37°C for 20 h. This was repeated daily with a medium change prior to the next heat-shock cycle. The collected medium was quantitated for the presence of gB by ELISA, in which the concentrations of gB products in the samples were calculated based on the authentic gB standard (Fig. 3-3). Without heat-shock, both cell lines exhibited a basal level of gB expression; after each heat-shock, there was an approximately 2-5 fold increase in both gBt and gBb production. Of significance, the cells were able to sustain their response to multiple heat shock, even at the tenth cycle of heat-shock treatment there was still at least a two fold induction of gB expression. In this particular experiment, after the tenth cycle, the accumulated gBt and gBb production per million cells was 7.5 µg and 28 µg, respectively. As noticed, the yield of gBt was lower than that of gBb. Since the two cell lines grew equally well, we may speculate that the lower yield of gBt may be caused by lower copy numbers of the gene present in the transfected cells.
Fig. 3-3. Production of gBt and gBb by transfected cell lines in response to heat-shock treatment. Cell lines gBt03 and gBb03 were cultured in MEM with 10% FBS in T-150 flasks until confluent, culture media were then replaced with serum-free MEM, and cells were subjected to heat shock treatment. Each cycle of heat shock treatment consisted of incubating cells at 43°C for 4 h, followed by incubation at 37°C for 20 h. After each heat shock, culture medium was collected, and fresh serum-free MEM was added. The amount of gB present in the culture medium collected after each heat shock was determined by a quantitative ELISA using affinity-purified authentic gB as standard. The results represent averages of duplicate samples. Control, non-heat shocked cells were treated identically.
An advantage of using the gB expressing cell lines is that the recombinant proteins are primarily secreted into the medium, and are therefore easier to purify. After collecting the media and passing them through an affinity column (van Drunen Littel-van den Hurk and Babiuk 1985), the gB products were relatively pure.

3.3.4 Immunogenicity of gBt and gBb.

We previously generated and characterized a panel of gB-specific monoclonal antibodies, which were shown to recognize not only linear but also conformation- and glycosylation-dependent epitopes (van Drunen Littel-van den Hurk and Babiuk 1985). To assess the antigenicity of the gB products, we first examined the reactivity of gBt and gBb with this panel of monoclonal antibodies by ELISA and immunoprecipitation. We found that both gB products reacted with all eight monoclonal antibodies tested, and they all showed a similar degree of reactivity to that exhibited by authentic gB (data not shown).

To further confirm that the recombinant gBs have authentic antigenicity, groups of CD-1 mice were immunized with gBt, gBb or authentic gB. Mice were subcutaneously injected with 10 μg of affinity-purified protein in complete Freund’s adjuvant, and boosted two weeks later with 10 μg of protein in incomplete Freund’s adjuvant. One week after the boost, serum samples were collected and tested for gB-
Fig. 3-4. Serum ELISA and neutralizing antibody titers of mice immunized with gB, gBt and gBb. Three groups of 10 mice each were primed with 10 μg of affinity-purified gB in complete Freund’s adjuvant and boosted two weeks later with 10 μg of the same antigen in incomplete Freund’s adjuvant. Serum antibody titers were determined by ELISA and virus neutralization assays (SN).
specific antibody titers and BHV-1 neutralization titers (Liang et al., 1991; van Drunen Littel-van den Hurk et al., 1994). As shown in Fig. 3-4, the three forms of gB induced comparable immune responses as measured by both methods.

3.3.5 Intracellular processing and export of recombinant gBs.

Previously, it has been shown that truncation in the cytoplasmic region of viral glycoproteins can change the protein’s exocytosis pathway (Doms et al., 1993; Navarro et al., 1993). In this study, the immunoprecipitation result suggested that gBt was able to form heterodimers similar to its authentic counterpart, and that the gBb had a similar molecular weight to authentic gBb, indicating that both gB products produced by the cell lines were properly processed with respect to post-translational modification. In order to further characterize the maturation and transport processes of gBt and gBb, we performed pulse-chase experiments and compared the gB molecules produced by the transfected cell lines with authentic gB in BHV-1-infected cells. In these experiments, pulse-labeling was performed at 7 h postinfection for authentic gB and at 1 h after heat shock for gBt and gBb. In view of the fact that most of the gBb and gBt produced by the cell lines was secreted into the medium, while authentic gB was retained in cells, the samples from cellular and medium fractions were combined for detection of
gB. At time 0, a dominant, 117 kDa band was detected in the BHV-1 infected cells, which represents a high mannose oligosaccharide-containing gB precursor (Fig. 3-5 A) (van Drunen Littel-van den Hurk et al., 1989). This gB precursor was subsequently chased into a 130 kDa fully glycosylated, but uncleaved gB, as well as two cleaved products gBb and gBc. A similar pulse-chase pattern was observed with gBt. With respect to gBb expressed by cell line gBb03, the precursor existed as a 65 kDa band, which was chased into a 74 kDa mature gBb. As non-glycosylated gBb was reported to be 56 kDa (van Drunen Littel-van den Hurk et al., 1989), the observed 65 kDa gBb precursor likely contains the high-mannose oligosaccharides as does the authentic gB precursor. According to densitometry analysis, where the gBb bands, which are present in all three forms of gB, were used to calculate the conversion rates, gBt, gBb and authentic gB in BHV 1-infected cells showed similar rates of conversion from the precursor to the mature form (Fig. 3-5 B).

3.3.6 Expression and deglycosylation analysis of recombinant gBct.

In order to further study the processing and export of recombinant gB products, we also expressed the truncated gBc portion, gBct, containing residues 506 to 763, in the same expression system (Fig. 3-6 A). To construct the transfer vector, a BHV-1 gC signal fragment was added to the
Fig. 3-5. Pulse-chase analysis of intracellular maturation of gB. MDBK cells infected with BHV-1 at 7 h postinfection and gB-expressing cell lines were pulse-labeled with $^{35}$S methionine for 30 min and chased with unlabeled methionine. At indicated time points, both cells and culture medium were collected, precipitated with gB-specific monoclonal antibodies and separated on SDS-8.5% polyacrylamide gels under reducing conditions (Panel A). pgB, pgBt and pgBb are precursors and gB, gBt and gBb are mature products. The density of individual bands was scanned by a Bio-Rad video densitometer (model 620). The percent of gB converted to the mature form at a given time point was calculated on the basis of gBb, a subunit which is present in all three forms of the gB products. Panel B shows the conversion curves of authentic gB, gBt and gBb by plotting the percent of the mature form of each of the gB products against chase time.
N-terminal portion of gBct (Fitzpatrick et al., 1989; Fig. 3-1). To our surprise, this product could not be successfully transported out of the cells (Fig. 3-6 B). The C-terminal subunit of gBt is a fully glycosylated product, which has a molecular weight of 38 kDa (c, Fig. 3-6 B and C). The recombinant gBct has an apparent molecular weight of 36 kDa, and is retained intracellularly (Fig. 3-6 B). In order to compare the polypeptide backbone of gBct to that of gBt, we performed deglycosylation as previously reported (van Drunen Littel-van den Hurk et al., 1990). It was found that the C-terminal subunit of gBt (c) could be reduced to a 34 kDa product by PNGase F digestion (c2 Fig. 3-6 C), which corresponds to the molecular weight of the unglycosylated polypeptide from residues 506 to 763. This subunit is also partially sensitive to endo H digestion (Fig. 3-6 C). Therefore, the C-terminal subunit of gBt contains complex oligosaccharides as well as high-mannose oligosaccharides which are sensitive to PNGase F and endo H respectively. The product c1 obtained by PNGase F treatment probably is an incompletely deglycosylated product. This result is consistent with previous observations (van Drunen Littel-van den Hurk and Babiuk 1986), that gBc possesses N-linked oligosaccharides of the high-mannose type. Both endo H and PNGase F modified the recombinant gBct to migrate at the c2 position, suggesting that it only contains high-mannose oligosaccharides, but no complex oligosaccharides. Our
Fig. 3–6. Expression and deglycosylation of the C-terminal subunit of gBt. Schematic diagram of gBct expression which has a BHV-1 gC signal at the N-terminal position is shown in panel A. Samples from cells (C in panel B) or media (M in panel B) were immunoprecipitated with gBc-specific polyclonal antibody (20) and separated on SDS–8.5% polyacrylamide gels under reducing conditions with a gBt sample as a control (panel B). a, b, c correspond to uncleaved mature gBt and its cleaved products respectively. Endo H (H) and PNGase F (F) treatment of gBt and gBct are shown in panel C. The capital C on top indicates untreated control. c1 and c2 indicate two forms of endoglycosidase digested products from c (panel C). M.W., molecular weight standard (kDa).
results indicate that the recombinant gBct is the same polypeptide as the C-terminal subunit of gBt. However, this product is not fully glycosylated by enzymes in the Golgi, because it can not be properly transported.

3.4 DISCUSSION

In this section, we describe the production and characterization of different forms of the BHV-1 gB ectodomain in stably transfected bovine cell lines by employing a heat-regulated bovine hsp70 gene promoter-based expression system. Results from this study show that high levels of recombinant gB can be produced by this expression system and that the gB products possess structural properties similar to authentic gB.

As with the authentic gB produced in virus infected cells, gBt expressed by the transfected cell line was composed mainly of covalently linked heterodimers. This indicates that gBt is capable of forming inter-chain disulfide bonds and that it is susceptible to intracellular proteolytic cleavage. The large subunit of gBt exhibited a molecular weight similar to the authentic gBb, whereas the small subunit, which contained an 170 amino acid-deletion, showed the expected lower molecular weight. The gBb also showed a molecular weight similar to the authentic gBb (Fig. 3-2). The comparable molecular weights between the
recombinant gB products and authentic gB suggest that the gB molecules expressed by the cell lines are properly glycosylated. In support of this, both gBt and gBb are reactive to five gB-specific monoclonal antibodies which recognize glycosylation-dependent epitopes IVa, IVb and IVc (van Drunen Littel-van den Hurk et al., 1990). In addition, the results from the pulse-chase experiment show that both gBt and gBb have the same maturation rate as the authentic gB in BHV-1-infected cells (Fig. 3-5 B). Taken together, these results suggest that the gB molecules expressed by the hsp70 gene promoter-based system appear to have proper post-translational modifications, including inter-chain disulfide bond formation, proteolytic cleavage and glycosylation.

The relation between cleavage and glycosylation of BHV-1 gB has not been fully studied. It was postulated, mainly on the basis of relative molecular weights of precursor and cleaved products, that the cleavage took place at the stage of the high mannose-containing precursor (van Drunen Littel-van den Hurk et al., 1986, 1989). According to the results from the pulse-chase experiment (Fig. 3-5 A), the high mannose-containing gB precursor was chased into a higher molecular weight form, gBa, and the cleaved subunits gBb and gBc; while a reduction in the amount of gB precursor was accompanied by a proportional increase of gBb and gBc, the amount of gBa remained constant. The lack of a corresponding increase of gBa suggests it may represent a transient product
between the precursor and the cleavage event. This in turn suggests that proteolytic cleavage occurs after the conversion of the high mannose-containing precursor to the mature form containing complex oligosaccharides, i.e., gBa. This interpretation is consistent with the observations made with PRV (Lukacs et al., 1985; Whealy et al., 1990), VZV (Montalvo and Grose 1987), and EHV-1 (Sullivan et al., 1989). However, in Marek’s disease virus (MDV), gB glycosylation may happen after cleavage (Yoshida et al., 1994).

The processing and transport of viral glycoproteins have been studied extensively in other viruses especially for vesicular stomatitis virus G protein and paramyxovirus hemagglutinin-neuraminidase protein (Doms et al., 1993). Viral glycoproteins are synthesized on the ribosomes attached to the rough endoplasmic reticulum. Then they pass through cellular organelles where they undergo proper posttranslational modifications. In eukaryotic cells, proteins enter the exocytic pathway at the rough endoplasmic reticulum and travel through the Golgi to the cell surface or to other cellular organelles. This process requires the protein to be in the correct conformation. Mutations which block the initial folding and perturb oligomerization can stop the transport to the cell surface (Doms et al., 1993). In our study, both gBt and gBb undergo intracellular processing similar to authentic gB (Fig. 3-5), indicating that their overall conformation is similar to authentic gB.
Since we found that gBt forms dimers similar to authentic gB, but gBb does not (chapter 6.0), the oligomerization of recombinant forms of gB may not be critical for their transport from the ER to the Golgi, therefore, another potential conformational signal may be responsible for transport. As the N-terminal subunit of gBt, gBb, is successfully transported, this raises the question as to which part can provide such a transport signal for BHV-1 gB. Obviously, gBb is the common component in both recombinant products. This was further supported by the expression of gBct, the C-terminal subunit of gBt. This subunit was found to be retained in the ER as a precursor form with high-mannose oligosaccharides, suggesting that the N-terminal subunit of gB, gBb, is required for the transport of the gB ectodomain from the ER to the Golgi where appropriate glycosylation and proteolysis occur. Previous studies using HSV-1 gB also suggested that for the efficient transport through the ER and the Golgi, a fragment which contains residues 441-475 is required, whereas molecular dimerization is not (Cai et al., 1987, 1988; Navarro et al., 1993). From our results, it is possible to draw a three-dimensional picture for recombinant gBt where the gBb subunit must be exposed to provide functional conformation in the intracellular environment, whereas the C-terminal part, gBct, is not. This hypothesis is also supported by the antigenic comparison between the two recombinant forms of gB and the
authentic gB in that they all induce comparable antibody responses in laboratory animals. The observation that gBb, which lacks the small subunit, was as efficacious as authentic gB in inducing an immune response is consistent with observations from a study involving monoclonal antibody epitope mapping (Fitzpatrick et al., 1990a) and further supports the conclusion that the dominant immunogenic epitopes of gB are located within the gBb subunit. This subunit may also be responsible for inducing protective immunity in cattle (Gao et al., 1994; Israel et al., 1992; Leary et al., 1992), suggesting that gBb is the most exposed and immunogenic part in BHV-1 gB.

The hsp70A gene promoter-based expression system differs from most other commonly used expression systems since it does not require the addition of exogenous inducing agents. The induction of foreign gene expression can be simply carried out by raising the temperature. In this study, we extended the characterization of this system with the expression of a highly complex viral glycoprotein, BHV-1 gB. The results show that induction of foreign gene expression by heat treatment in MDBK cells had no adverse effect on various properties of the expressed product such as intracellular processing and antigenicity; high levels of foreign gene expression could be readily achieved by multiple heat shock cycles of the cells. The results of this study demonstrate that both gBt and gBb can be expressed at high levels. The
resultant gB products appear to be fully processed, secreted, and to retain antigenic properties similar to their authentic counterpart. This observation provides the basis for future studies using recombinant forms of gB as subunit vaccine candidates.

In conclusion, the observations made in this study have demonstrated that in BHV-1 gB, gBb is required for the transport of the gB ectodomain from the ER to the Golgi. This study provides us with further information about intracellular processing of BHV-1 gB, and substantiates the utility of the novel, hsp70 gene-promoter based expression system. Furthermore, the availability of gBt and gBb in relatively high quantities should facilitate future studies of this important viral component and its potential use as a BHV-1 vaccine.
4.0 CHARACTERIZATION OF CELL BINDING PROPERTIES OF BOVINE HERPESVIRUS 1 GLYCOPROTEIN B, C, AND D: IDENTIFICATION OF A DUAL CELL BINDING FUNCTION OF gB

4.1 INTRODUCTION

Virus infections are initiated through specific interactions between viral attachment proteins and their receptors on the surfaces of permissive cells. For alphaherpesviruses the initial interaction between viral attachment proteins and cellular receptors is followed by virus penetration involving membrane fusion. These two steps constitute the virus entry process. It is now known that for most cell types, the initial attachment of alphaherpesviruses to permissive cells is mediated by an interaction of their gC molecules with the cellular glycosaminoglycan, heparan sulfate (HS) (for comprehensive reviews, refer to Spear et al., 1992a; Spear 1993). Early studies using antibodies (Dubuisson et al., 1992; Fuller and Spear 1985; Johnson et al., 1990), cell membrane fractionation (Kuhn et al., 1990) and virosomes (Johnson et al., 1984) suggest that gB and gD molecules are also involved in the virus attachment process.
Glycoprotein B of herpes simplex virus type 1 (HSV-1) has been shown to be able to directly bind to HS (Herold et al., 1991). More recent studies showed that for gC negative HSV-1, the interaction between gB and cellular HS plays a dominant role in attachment (Herold et al., 1994). Glycoprotein B of pseudorabies virus (PRV) can also bind heparin-coated beads; however, it does so only in conjunction with gC (Mettenleiter et al., 1990; Sawitzky et al., 1990). For bovine herpesvirus 1 (BHV-1), gC represents the only viral protein identified to date that has the ability to bind to a heparin-like component (Okazaki et al., 1991). HSV-1 gD has been shown to bind to a limited number of specific cellular receptors (Johnson et al., 1990; Johnson and Ligas 1988). Recently, Brunetti et al. showed that HSV-1 gD binds to mannose-6-phosphate receptors (Brunetti et al., 1994), although the relevance of the gD-mannose-6-phosphate receptor interaction in virus infection has yet to be established. Thaker et al. recently showed that BHV-1 gD binds to a 60 kDa cell surface protein (1994). We previously showed that affinity-purified BHV-1 gB, gC and gD could inhibit virus attachment at 4°C and that gC inhibited not only wild type (wt) BHV-1 but also a gC minus mutant to a lesser degree, whereas gB and gD inhibited wt and gC minus viruses to the same extent. These observations led us to propose that BHV-1 attachment is a complex event in that wt virus uses gC to carry out the initial interaction with cellular receptors, which is followed by interactions of gB
and/or gD with their receptors (Liang et al., 1991). Karger and Mettenleiter showed that PRV and BHV-1 attachment to permissive cells can be divided into two stages, an initial heparin inhibition-sensitive stage, and a subsequent heparin-resistant stage (Karger and Mettenleiter 1993). By using isogenic viruses deleted of individual viral proteins, they found that although the wt virus and the gD-negative mutant have similar initial binding, the gD-negative mutant was considerably impaired in heparin-resistant binding, suggesting that the initial attachment of the PRV and BHV-1 to cells via a gC-HS interaction is followed by a gD-mediated HS-independent attachment. A similar attachment mechanism has been revealed for HSV-1 (Fuller and Lee 1992; Lee and Fuller 1993; McClain and Fuller 1994). The data available to date collectively suggest that attachment of alphaherpesviruses to most permissive cells is a complex event, involving at least gC binding to cellular HS and gD binding to other undefined cellular receptors.

In order to further understand the virus attachment mechanism, in the present study we purified both authentic and membrane anchor-truncated forms of BHV-1 gB, gC, and gD and examined each of these glycoproteins for cell binding properties. By employing exogenous heparin as an inhibitor and heparinase treatment to remove cellular HS, we examined whether BHV-1 gB and gD could also bind to cellular HS, and whether binding of any of the glycoproteins to cellular HS
could be coupled to secondary HS-independent binding. In addition, we determined cell binding kinetics and affinity of each of the three glycoproteins. The results from this study reveal for the first time that BHV-1 gB interacts with cells in a two-step fashion, with initial binding to cellular HS followed by secondary high-affinity binding to a non-HS receptor.

4.2 MATERIALS AND METHODS

4.2.1 Virus, cells and reagents.

The BHV-1 Cooper strain was obtained from the National Veterinary Services Laboratories, Ames, Iowa and propagated in Madin-Darby Bovine Kidney (MDBK) cells. MDBK cell lines that express individual secreted gB, gC and gD under the control of a bovine heat shock hsp70 gene promoter were constructed in this institute (Kowalski et al., 1993; Chapter 3.0). All cell lines were maintained in minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO). Heparin of bovine intestinal mucosa (molecular weight, approximately 3000 Da) and heparinase of flavobacterium heparinum were purchased from Sigma (St. Louis, MO); lactoperoxidase was purchased from Boehringer-Mannheim (Doval, Quebec); and Na$^{125}$I was purchased from Amersham (Oakville, Ontario).
4.2.2 Affinity purification of gB, gC and gD.

Affinity purification of gB, gC and gD from BHV-1-infected cells was carried out essentially as previously described (van Drunen Littel-van den Hurk and Babiuk 1985). Briefly, subconfluent MDBK cells were infected with BHV-1 at a multiplicity of infection (moi) of 1; 24 to 48 h postinfection, cells were collected, washed and lysed with 1% Nonidet P-40 (NP-40) and 1% deoxycholic acid, followed by sonication. After centrifugation at 110,000 x g for 1 h to remove the cellular debris, the supernatant was collected and applied onto the immunoabsorbent columns. The columns were sequentially washed with wash buffer (0.1M Tris-HCl, 0.5M NaCl, pH 7.5) containing 0.1% NP-40 and wash buffer without NP-40. In some cases, the columns were further washed once with 1.0 M NaCl in phosphate buffer (pH 7.2). The bound proteins were eluted from the columns with 50 mM diethylamine (pH 11.0), concentrated by ultrafiltration and dialyzed extensively against 0.01 M Tris-HCl-0.15 M NaCl-1 mM EDTA (pH 7.5). The purified glycoproteins were stored at -70°C until use. For purification of secreted glycoproteins, cultures of the individual viral protein-expressing cell lines were subjected to daily heat shock treatment (43°C, 4 h) for 4 to 6 days (Kowalski et al., 1993); culture media were collected, centrifuged to remove cell debris and applied to affinity columns as described above.
4.2.3 Protein iodination.

Protein iodination was carried out by a lactoperoxidase method according to Thorell and Johansson (Thorell and Johansson 1971). About 5 µg of each protein was used for iodination; \(^{125}\text{I}\)-labeled protein was separated from free Na\(^{125}\text{I}\) by chromatography on a Sephadex G-25 column. The labeled proteins were aliquoted and stored at -70°C until use. The specific activities of the labeled proteins ranged between 6 µCi and 20 µCi per µg protein; the trichloroacetic acid precipitable radioactivity was more than 90% of the total radioactivity.

4.2.4 Protein binding assays.

Confluent MDBK cells in 48-well plates were cooled to 4°C. After removing culture medium, cells in each well were incubated with approximately 2 x 10^5 cpm of an \(^{125}\text{I}\)-labeled protein diluted in MEM-FBS at 4°C for 4 h. After incubation, cells were washed three times with MEM containing 10% FBS and lysed with 1% Triton X-100 and 1% sodium dodecyl sulfate (SDS) in phosphate buffered saline (PBS) (lysis buffer). Total cell lysate was collected and counted for radioactivity in a gamma counter. In each plate, wells containing an \(^{125}\text{I}\)-labeled protein together with 100 µg/ml of unlabeled protein were included and used as a control for non-specific background binding. Under the conditions tested, the non-specific binding was always less than 10% of total binding.
Each experiment was performed in triplicate. To assess the effect of heparin and heparinase treatment on glycoprotein binding, four different conditions as follows were employed:

(i) Heparin competition of $^{125}$I-labeled protein binding: Cells were cooled to 4°C and incubated with 50 μl of heparin at a specified concentration per well for 1 h at 4°C prior to the addition of 50 μl of a labeled protein. (ii) Heparinase treatment before addition of labeled proteins: Cells were first treated with 100 μl of different concentrations of heparinase in MEM-FBS containing 0.05% NaN₃ at 37°C for 1 h; after three washes with MEM-FBS, cells were cooled to 4°C and then incubated with 100 μl of a labeled protein per well. (iii) Heparin treatment of cells with bound $^{125}$I-protein: Cells were incubated with a labeled protein for 4 h at 4°C. After incubation, cells were washed three times with MEM-FBS to remove the free $^{125}$I-labeled protein and then incubated with 100 μl of heparin at different concentrations in MEM-FBS per well for another hour at 4°C, followed by washing and lysis as described above. (iv) Heparinase treatment of cells with bound $^{125}$I-protein: The procedure was essentially the same as that described in (iii) except that cells were treated with heparinase at 37°C for 1 h instead of heparin.

4.2.5 Assay for protein binding kinetics.

The assay was carried out according to Lasky et al. (1987). Confluent MDBK cells in 48-well plates were cooled at
4°C for 1 h. After removal of media, cells were incubated with a constant amount of an $^{125}$I-labeled protein (2 x $10^5$ cpm in a 50 µl volume per well) in MEM-FBS in the presence of increasing amounts of an unlabeled protein. After incubation at 4°C for 4 h, the cells were washed three times with MEM-FBS and lysed with 1% Triton X-100 and 1% SDS. Cell associated radioactivity was determined in a gamma counter. Total input, cell bound and free proteins were calculated and subjected to Scatchard analysis (Scatchard 1949).

4.2.6 Viral adsorption inhibition assay.

MDBK cells grown to confluence in 24-well culture plates were cooled at 4°C for 1 h. Viral proteins or heparin were diluted in MEM-FBS to specified concentrations and added to the cells in a volume of 50 µl per well. The plates were incubated for 1 h at 4°C, followed by addition of 50 µl of virus inoculum per well, containing approximately 150 pfu of virus, and incubated at 4°C for another hour. After adsorption, the plates were washed three times with MEM and overlaid with 0.8% agarose in MEM containing 2% FBS. Plaques were counted four days later. Number of viral plaques formed in the absence of an inhibitor was taken as 100% of plaque formation and used as a reference for calculating percentage of viral plaques formed in the presence of an inhibitor. To detect the plaque formation on heparinase treated cells, MDBK cells were incubated with heparinase at 37°C for 1 h before
wash and viral inoculation.

4.3 RESULTS

4.3.1 Production and characterization of membrane-anchor truncated, soluble gB, gC and gD.

Three stable MDBK cell lines that express membrane anchor truncated, soluble BHV-1 gB (gBt), gC (gCt) and gD (gDt), respectively, were established in this institute (Kowalski et al., 1993; Chapter 3.0). The expression of these viral proteins is controlled by a bovine heat shock hsp70 gene promoter, and is highly inducible by subjecting the cells to elevated temperatures, 43°C (Kowalski et al., 1993). These truncated glycoproteins are efficiently secreted. Characterization of the truncated glycoproteins involving immunoprecipitation, reactivity to an extended panels of monoclonal antibodies (mAb) and immunogenicity in cattle or in mice showed that the truncated proteins had properties similar to their authentic counterparts; the truncated gB, like its authentic form, is also cleaved and forms disulfide-linked heterodimers (Chapter 3.0). Figure 4-1 shows the predicted transmembrane domains of gB, gC and gD and the carboxyl termini of the truncated glycoproteins. gBt, gCt and gDt are terminated at amino acid residue 763, 465 and 356, respectively. During construction of the gCt and gDt genes, several exogenous amino acid residues were introduced at
their carboxyl termini; Leu and Asp were added to gCt, and Arg, Val and Ala were added to gDt.

4.3.2 Effects of heparin or heparinase on the binding of gB, gC and gD to MDBK cells.

To evaluate whether BHV-1 gB, gC and gD were able to bind to cellular HS, we examined the effects of addition of exogenous heparin and treatment of cells with heparinase to remove cellular HS on the binding of affinity-purified, ¹²⁵I-labeled glycoproteins to MDBK cells. Prior to this study, a number of preliminary experiments were performed to optimize the conditions for the binding assay. We found that a binding assay in the presence of normal culture medium, i.e., MEM containing 10% FBS, produced the least non-specific binding and no discernible alteration of cell morphology, and each of the glycoproteins tested reached maximum binding within 4 h of incubation at 4°C (data not shown). These conditions were therefore adopted for the protein binding assays throughout the present study.

As shown in Figure 4-2, authentic (Fig. 4-2A) and soluble (Fig. 4-2B) proteins revealed similar binding properties in response to heparin and heparinase treatment. Heparin or heparinase treatment inhibited both gC and gB binding to cells in a dose-dependent fashion, but had no
gb represents omitted amino acid sequence in the cytoplasmic tail.

Residues added during construction of the mutant molecules, those in authentic 
Whitbeck et al., 1990; underlined amino acids of truncated gc and gb are exogenous 
Fitzpatrick et al., 1989; Tiko et al., 1990, 
highlighted by the enclosed boxes (gb'gc and gb', predicted transmembrane domains of authentic gc and gb are 
\( \text{gb', gc and gb'} \), \( \text{predicted transmembrane domains of authentic gc and gb are} \),

\[
\begin{align*}
\text{gb'gc and gb'} & \quad \text{predicted transmembrane domains of authentic gc and gb are} \\
\text{gb'gc and gb'} & \quad \text{predicted transmembrane domains of authentic gc and gb are}
\end{align*}
\]
effect on gD binding. This establishes that both BHV-1 gC and gB bind to cellular HS, whereas gD binds to a non-HS cellular component. While binding of both gB and gC was susceptible to heparin inhibition, gB and gC nevertheless differed dramatically in their binding capacity. For example, heparin at a concentration of 0.1 μg/ml inhibited more than 50% of both authentic and soluble gB binding; for the same degree of inhibition of gC binding, a 100-fold higher heparin concentration, i.e., 10 μg/ml, was required. It may be noted that although gB and gC showed different susceptibilities to heparin inhibition, their responses to heparinase treatment are similar. This is likely due to the different operation mechanisms involved in these two assays. The heparin treatment is a competition assay, where the effectiveness of inhibition by the exogenous heparin depends on the overall binding affinity or avidity of a labeled protein. In contrast, with heparinase treatment, the inhibition of binding of a labeled protein is caused by removal of cell binding sites. Thus, the observation that gB and gC showed similar responses to heparinase treatment may reflect that heparinase treatment was able to remove the binding sites recognized by gB and by gC proportionally.

The observation that both gB and gC were able to bind to MDBK cells in a HS-dependent manner prompted us to further examine the interrelation between the viral proteins in cell binding. Because authentic and soluble proteins exhibited
Fig. 4-2. Effects of heparin and heparinase treatment on binding of 125I-labeled gB, gC and gD to MDBK cells. For the heparin inhibition assay, confluent MDBK cells grown in 48-well plates were incubated with heparin at the indicated concentrations at 4°C for 1 h, this was followed by addition of approximately 2 x 10^5 cpm of an 125I-labeled authentic glycoprotein (A) (▲, gB; ☐, gC; ◆, gD) or soluble glycoprotein (B) (▲, gBt; ☐, gCt; ◆, gDt) and incubation at 4°C for 4 h. After incubation, cells were washed with MEM-FBS and lysed with 1% Triton X-100-1% SDS. Cell lysates were collected, and radioactivity was counted in a gamma counter. For heparinase treatment, cells were first treated with heparinase at indicated concentrations at 37°C for 1 h. After three washes, the cells were incubated with 125I-labeled glycoprotein for 4 h at 4°C, this was followed by the same procedure described above. The counts per minute bound in the presence of 100 µg of an unlabeled protein per ml was defined as background, which was always less than 10% of total counts per minute bound. The percent of cpm bound was calculated as following: % of bound=([cpm bound in the presence of inhibitor - background cpm]/[cpm bound in the absence of inhibitor - background cpm]) x 100%. Experiments were performed in triplicate, and the data represent means ± standard deviations for triplicate samples.
similar binding properties in response to heparin and heparinase treatment, in this particular experiment only authentic proteins were tested. Cells were incubated with a constant amount of an $^{125}$I-labeled protein in the presence of different concentrations of a second unlabeled, heterologous protein at 4°C for 4 h; the amount of labeled protein bound to cells was determined and compared to the amount of labeled protein bound in the absence of the heterologous protein (Fig. 4-3). gB and gD showed no inhibitory effect on the binding of a second heterologous protein. While gC had no effect on gD binding, it effectively inhibited gB binding. It was noticed that gC at a concentration of 1 µg/ml repeatedly increased the total binding of gB. The reason for this is not clear.

4.3.3 Effects of heparin and heparinase on cell-bound glycoproteins.

The experiments described above showed that gC and gB bind to cellular HS, but they could not exclude the possibility that these viral glycoproteins might bind to additional, non-HS cellular receptors following their initial interaction with HS, i.e., the initial binding of these viral proteins to HS might promote their binding to additional, non-HS receptors. Accordingly, we speculated that should gB and gC be able to engage in a secondary non-HS binding, then after being bound to cells, they would become resistant to
Fig. 4–3. Competition binding with heterologous proteins. Confluent MDBK cells in 48-well plates were incubated with approximately 2 x 10³ cpm of ¹²⁵I-labeled gB (●), gC (○), or gD (◊) in the presence of an increasing amounts of a second unlabeled, heterologous glycoprotein at 4°C for 4 h. After incubation, cells were washed and lysed. Cell lysates were collected, and radioactivity was counted. The percentage of counts per minute bound was calculated as described for Figure 4-2. Experiments were performed in triplicate, and the data represent means ± standard deviations for triplicate samples.
either heparin or heparinase treatment. Therefore, cells were first incubated with an \(^{125}\text{I}\)-labeled glycoprotein at 4\(^\circ\)C for 4 h; after removal of free, unbound protein, the cells were treated with exogenous heparin or heparinase (Fig. 4-4). As expected, bound gD was completely resistant to heparin and heparinase treatments. In contrast, bound gC could be completely removed by either heparin or heparinase treatment. Of interest is that authentic gB and soluble gB showed different responses to heparin and heparinase treatment. While bound soluble gB could be removed completely by either heparin or heparinase treatment, a significant portion of bound authentic gB became resistant to heparin and heparinase treatments. Under the conditions tested, 40\% of the bound authentic gB became refractory to treatment. The maximum effects of heparin and heparinase on the bound authentic gB were observed at a concentration of 100 \(\mu\)g/ml and 1 U/ml, respectively; increasing the concentration of either heparin or heparinase did not result in a further reduction of bound authentic gB, indicating that the residual bound gB was truly resistant, and not merely reduced in sensitivity, to heparin and heparinase treatments. These results suggest that gC binds solely to cellular HS whereas gB appears to bind first to cellular HS and then to a non-HS cellular receptor.
Fig. 4-4. Effects of heparin and heparinase treatment on bound gB, gC and gD. Confluent MDBK cells grown in 48-well plates were incubated with approximately $2 \times 10^5$ cpm of an $^{125}$I-labeled authentic (A) (●, gB; ○, gC; ◆, gD) or soluble protein (B) (▲, gBt; ◇, gCt; ◈, gDt) at 4°C for 4 h. After three washes, cells were incubated either with heparin at 4°C for 1 h or with heparinase at 37°C for 1 h; this was followed by an additional three washes. Cell lysates were collected, and radioactivity was determined. The percentage of counts per minute bound was calculated as described for Figure 4-2. Experiments were performed in triplicate, and the data represent means ± standard deviations for triplicate samples.
4.3.4 Binding affinity analysis.

To further characterize the binding properties of gB, gC and gD, we determined binding affinities of the individual viral glycoproteins on MDBK cells. Cells were incubated with a constant amount of a labeled protein in the presence of increasing levels of an unlabeled homologous protein; the amounts of total input, cell bound and free labeled proteins were determined and subjected to Scatchard analyses. The binding affinity plots from one representative experiment are shown in Figure 4-5. All the glycoproteins tested exhibited saturable binding curves; namely, binding of a labeled protein was susceptible to the competition by an unlabeled homologous protein. Soluble gB and both forms of gC and gD each showed single binding kinetics; in contrast, authentic gB showed a curve characteristic of dual binding kinetics. The calculated dissociation constant (Kd) and number of cell bindings sites of each of the glycoproteins are summarized in Table 4-1. The values presented in Table 4-1 are the means derived from several independent experiments whereas the Scatchard plots were drawn from one set of data (similar results were shown in all experiments). Soluble gB, and both forms of gC and gD had similar dissociation constants ranging from $1.5 \times 10^{-7}$ to $5.1 \times 10^{-7}$ M. The low-affinity binding of authentic gB had a Kd value of $5.2 \times 10^{-7}$ M, which is similar to that of soluble gB, whereas the high-affinity binding has a Kd of $4.1 \times 10^{-9}$ M, two orders of magnitude higher than that
of soluble gB. Among gB, gC and gD, gC has the largest number of cell binding sites, followed by gD and then gB.

<table>
<thead>
<tr>
<th>Viral proteins</th>
<th>Kd (x 10^-7 M)</th>
<th>Binding sites (x 10^5/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gB</td>
<td>5.2 ± 1.5</td>
<td>6.1 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>0.041 ± 0.034</td>
<td>0.084 ± 0.056</td>
</tr>
<tr>
<td>gBt</td>
<td>2.2 ± 1.1</td>
<td>5.4 ± 3.4</td>
</tr>
<tr>
<td>gC</td>
<td>5.1 ± 1.7</td>
<td>46 ± 27</td>
</tr>
<tr>
<td>gCt</td>
<td>3.3 ± 0.4</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>gD</td>
<td>1.5 ± 0.5</td>
<td>9.8 ± 2.6</td>
</tr>
<tr>
<td>gDt</td>
<td>4.2 ± 0.7</td>
<td>9.5 ± 2.8</td>
</tr>
</tbody>
</table>

'The values of authentic glycoproteins are means ± standard deviations for three experiments, and the values for the soluble glycoproteins are means ± standard deviations for two experiments.

4.3.5 Inhibition of viral plaque formation.

In view of the fact that gB was able to bind to cellular HS and its binding to HS could mediate binding to an additional, non-HS receptor, it was of interest to further evaluate the relevance of these observations at the level of virus infection. We speculated that should the interaction of gB with cellular HS play a role in virus entry, heparin or heparinase treatment to block gB-HS interaction should be able to inhibit gC-negative virus infection. Therefore, the effects of heparin and heparinase treatment on gC negative
Fig. 4-5. Competition binding of gB, gC and gD on MDBK cells. Confluent MDBK cells in 48-well plates were incubated with 2 x 10^3 cpm of an ¹²⁵I-labeled protein in the presence of an increasing amount of an unlabeled protein at 4°C for 4 h. After incubation, cells were washed and lysed. Cell lysates were collected, and radioactivity was counted. On the basis of the specific activity of each of the labeled proteins, the amounts of total input, free, and bound protein were determined and subjected to Scatchard analysis (Scatchard 1949). Shown in the inserts are lines representing the best fits as determined by a linear regression analysis. E-1, 10⁻¹.
Fig. 4-5. Competition binding of gB, gC and gD on MDBK cells (Continued).
virus plaque formation were determined. As shown in Figure 4-6, heparin and heparinase treatments inhibited plaque formation by both wt and gC-negative BHV-1, although heparinase treatment was less effective for the gC negative mutant than for wt virus. Of note, the fact that heparinase treatment was not as effective as heparin treatment in inhibiting viral plaque formation was not due to the potential damage of the cells after heparinase treatment. We have repeated the same experiment by including vesicular stomatitis virus and found that either heparin or heparinase treatment had no effect on vesicular stomatitis virus plaque formation.

We previously showed that purified authentic gB, gC or gD is able to inhibit virus adsorption (Liang et al., 1991). Since soluble gB was found to be defective in the HS-independent binding function, we next examined whether the soluble gB would retain the ability to inhibit viral plaque formation. As a control, both soluble gC and gD were also included in this experiment. The results are shown in Figure 4-7. Consistent with previous results, all three authentic glycoproteins showed various degrees of inhibition of viral plaque formation. Soluble gC showed the same inhibitory effect as authentic gC; soluble gD appears to be slightly less effective than its authentic form. The most dramatic difference observed was that between soluble and authentic gB. For example, authentic gB at a concentration of 100 µg/ml
Fig. 4-6. Effects of heparin and heparinase treatment on viral plaque formation. For the heparin inhibition assay (left), confluent MDBK cells grown in 24-well plates were incubated with heparin at the indicated concentrations at 4°C for 1 h; this was followed by addition of approximately 150 PFU of wt BHV-1 (⚫) or gC-negative mutant virus (○) per well. Cells were incubated at 4°C for an additional 1 h. For heparinase treatment (right), cells were first treated with heparinase at the indicated concentrations at 37°C for 1 h. The cells were subsequently washed, infected with either wt or mutant virus, and incubated at 4°C for 1 h. After adsorption, the viral inoculum was removed, and cells were washed once with MEM and overlaid with 0.8% agarose in MEM containing 2% FBS. Plaques were counted 4 days later. The number of plaques formed in the absence of an inhibitor was defined as 100% plaque formation. Experiments were performed in triplicate and the data represent means ± standard deviations for triplicate samples.
Fig. 4-7. Effect of purified BHV-1 glycoproteins on virus plaque formation. Confluent MDBK cells grown in 24-well plates were cooled at 4°C and then incubated with 50 μl of a glycoprotein at the indicated concentrations per well at 4°C for 1 h. After incubation, 50 μl of diluted BHV-1 (about 150 PFU) was added to each well of cells in the presence of glycoproteins; this was followed by an additional 1 h incubation at 4°C. After incubation, cells were washed three times with MEM and overlaid with 0.8% agarose. Viral plaques were counted 4 days later. The number of plaques formed in the absence of a glycoprotein was defined as 100% plaque formation. The data represent means plus standard deviations for triplicate samples. Solid bar, authentic protein; hatched bar, truncated protein.
inhibited about 90% of plaque formation, whereas soluble gB at 200 μg/ml had essentially no effect on plaque formation. These observations from the viral plaque inhibition assay appear to be consistent with what would be predicted on the basis of the binding studies with purified proteins. Of particular interest was that the loss of the high-affinity binding function of soluble gB was concomitantly associated with its inability to inhibit viral plaque formation. It needs to be pointed out that the virus adsorption inhibition assay was performed at 4°C, a temperature at which virus penetration does not occur. Therefore, the results obtained from this experiment are most likely reflecting the events during virus attachment rather than during virus penetration.

4.4 DISCUSSION

In this section, we have described cell binding properties of three major BHV-1 attachment proteins, gB, gC, and gD, in both authentic and soluble forms and have shown that all three viral proteins possess the characteristics for specific saturable binding to MDBK cells; gC and gD bind exclusively to cellular HS and non-HS components, respectively, whereas gB binds to cells in a two-component fashion, with initial binding occurring to HS and secondary binding to a non-HS cellular receptor.
Interaction of gC and gD with MDBK cells. Although prior to this study it had been well established that BHV-1 gC, like its homologues in other alphaherpesviruses, binds to cellular HS, the issue whether the initial interaction of gC with HS could trigger a further interaction of this molecule with other cellular components had not been directly addressed. It has been well documented that HS has a broad modulatory effect on the functions of a variety of proteins (Jackson et al., 1991), and that binding of bFGF to its secondary high-affinity receptors takes place only after it binds to HS (Yayon et al., 1991). According to amino acid sequence analysis, BHV-1 gC has an R-G-D motif typical of ligands of cellular adhesion glycoproteins (Fitzpatrick et al., 1989). The use of the R-G-D motif as a viral attachment ligand has been recently demonstrated for foot-and-mouth disease virus (Mason et al., 1994). In addition, it is known that gC homologues can bind serum complement component C3b (Friedman et al., 1984). A more recent study suggested that one of the pathways of HSV-1 entry into the cells begins with the gC-dependent attachment of the virus to C3b receptor present on the cell surface (Sears 1994). We show here that heparin and heparinase treatment not only prevented gC from binding to cells but were also able to remove gC that had bound to cells, indicating that gC, under the conditions tested, binds only to cellular HS. The contention that gC binds only to HS was further corroborated by the single
binding kinetics exhibited by gC. While it is still formally possible that gC may have non-HS binding sites on other cell types, our results favour the hypothesis that gC does not have non-HS cellular receptor on MDBK cells.

The observations made with BHV-1 gD in the present study are consistent with what has been previously described for soluble HSV-1 gD (Johnson et al., 1990). Like that of HSV-1 gD, binding of BHV-1 gD to MDBK cells is independent of cellular HS (Fig. 4-2). According to the binding affinity analysis, BHV-1 gD was estimated to have a dissociation constant of $1.5 \times 10^{-7}$ to $4.2 \times 10^{-7}$ M and about $9.5 \times 10^{5}$ to $9.8 \times 10^{5}$ sites per MDBK cell, which also compare favourably with the dissociation constant of $1 \times 10^{-7}$ to $1.8 \times 10^{-7}$ M and the number of cell binding sites of $4 \times 10^{5}$ to $5 \times 10^{5}$ per cell reported for HSV-1 gD (Johnson et al., 1990).

Both BHV-1 gC and gD appear to have relatively low binding affinities compared to those of other known virus-cell interactions, which usually range between $10^{-9}$ M to $10^{-10}$ M (Armstrong et al., 1984; Lasky et al., 1987; Tanner et al., 1988). It is intriguing that gC has a relatively low binding affinity yet plays a dominant role in virus attachment. It appears, therefore, that factors other than binding affinity must be responsible for the function of gC in virus attachment. Among the three proteins tested, gC has the largest number of binding sites on cells, and it has been shown that gC homologues contain multiple heparin binding
sites (Liang et al., 1993; Okazaki et al., 1994). It is conceivable that the multiple binding epitopes present on a single molecule may provide a mechanism to enhance the overall avidity of binding to its receptors. Furthermore, gC molecules constitute the longest projections present on virions (Stannard et al., 1987). All these factors may collectively contribute to the preeminent cell-binding function of gC.

**Interaction of gB with MDBK cells.** Previously, it has been shown that gB of HSV-1 can also bind to cellular HS (Herold et al., 1994, 1991). However, direct binding of gB molecules of other alphaherpesviruses to HS has not been established. On the basis of protein fractionation of radiolabeled virions via heparin affinity chromatography, it was shown that gB of PRV binds to heparin-coated beads only in conjunction with gC (Mettenleiter et al., 1990), whereas gC of BHV-1 represents the only HS binding protein (Okazaki et al., 1991). In the present study, we showed that both soluble and authentic gB bind to cellular HS (Fig. 4-2). The binding of gB to cellular HS seems to be a weak interaction in comparison to gC-HS interaction because gB was considerably more sensitive to heparin inhibition than gC (Fig. 4-2). We also found that gC was able to compete against gB binding, but gB was unable to compete against gC binding (Fig. 4-3). According to estimated numbers of cell binding sites, gB appears to have about 10 times fewer HS binding
sites than gC. This may indicate that the gB and gC do not recognize the same spectrum of HS residues but, rather, that gB may recognize only a small fraction of the HS residues that are recognized by gC. However, the possibility that gB and gC have different specificities in response to heparin competition can not be formally excluded, and this remains to be further determined. The relatively weak interaction between gB and HS may provide a potential explanation for the failure to detect BHV-1 gB by heparin affinity chromatography (Okazaki et al., 1991). The same explanation may be also applicable to the binding of PRV gB to heparin-coated beads (Mettenleiter et al., 1990). It is possible that initial binding of gC to heparin may increase the local concentration of gB coexisting with gC on the same virion, resulting in a more efficient interaction of gB with heparin.

The most significant finding of the present study is the delineation of a dual binding function of gB, i.e., binding of gB to cellular HS is coupled to an additional binding to a non-HS receptor. This conclusion was established on the basis of the following observations. First, the addition of exogenous heparin or treatment of cells to remove cellular HS prevented both authentic and soluble gB from binding to cells (Fig. 4-2); however, once bound to cells a significant portion of authentic gB became resistant to either heparin competition or heparinase treatment. Secondly, according to binding affinity analysis, authentic gB showed
dual binding kinetics in that one binding component had a
dissociation constant similar to that of soluble gB and the
other had a significant higher binding affinity. Finally, we
found that authentic gB, but not soluble gB, was able to
inhibit virus plaque formation. Since soluble gB and
authentic gB both bind to cellular HS, the ability of the
authentic gB to inhibit viral plaque formation must be the
result of its competition against the virus for the non-HS
binding sites but not from its competition for cellular HS
binding sites.

According to the dual binding mechanism, binding of
gB to its high-affinity cellular receptor requires it to be
first bound with HS. Thus, one would expect that soluble gB
which binds to cellular HS would also be able to prevent
viral gB from binding to HS, by which means it would block
viral gB binding to its high-affinity receptor and
consequently inhibit viral plaque formation. Nevertheless, we
found that soluble gB, under the conditions tested, was
incapable of inhibiting viral plaque formation. A possible
explanation for this is that gB binding to HS per se is a
rather inefficient process as discussed above, whereas for
authentic gB or the gB present on the virions, the weak
interaction between gB and cellular HS is stabilized by gB’s
secondary binding to the high-affinity receptors as well as
other binding forces such as those mediated by gD. As a
result, the soluble gB which binds to HS only would not

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effectively compete against the gB present on virions for cell binding sites.

The secondary binding of gB to MDBK cells has an estimated Kd of $4.1 \times 10^{-9}$ M and $8.4 \times 10^3$ binding sites per cell, which agrees well with the values obtained from other virus-cell interactions. For example, human immunodeficiency virus gp120 binds to CD4-positive cells with a Kd of $3 \times 10^{-3}$ to $4 \times 10^{-9}$ (Lasky et al., 1987); reovirus binds to cells with a Kd of $3 \times 10^{-9}$ M (Armstrong et al., 1984); and Epstein-Barr virus gp350/220 binds to cells with a Kd of $1.2 \times 10^{-9}$ M (Tanner et al., 1988). Because of the presence of the hydrophobic transmembrane domains, authentic gB molecules are expected to exist in solution as micelles. This physical property may have some effect on the accuracy of the estimation of its binding constant, but it is unlikely to affect the overall binding kinetics of gB, i.e., the dual binding property. In fact, binding affinity studies for a number of viruses have been carried out using whole virus particles (Armstrong et al., 1984; Bibb et al., 1994). In the present study, we showed that the authentic and soluble forms of gC and gD, which were tested in parallel with gB, exhibited similar binding constants. Furthermore, the dual binding property of gB as revealed by binding kinetic is fully consistent with the observations made with the heparin and heparinase inhibition studies and plaque inhibition experiments, as described above.
We previously observed that purified gC inhibited not only adsorption of wt BHV-1 but also that of gC mutant virus, suggesting that gC may share binding sites with other viral attachment proteins (Liang et al., 1991). Here we have shown that gB also binds to cellular HS and, in addition, the binding to HS constitutes a prerequisite for gB to further bind to a non-HS component. Therefore, it appears that the inhibitory effect of gC on binding of gC-negative virus is due to blockage of gB binding cellular HS, which in turn blocks gB binding to its high-affinity receptor. The ability of gC to inhibit gB binding may also explain why among three glycoproteins tested, gC is most effective in inhibiting viral plaque formation.

We found that although heparin was able to completely block infectivity of a gC negative BHV-1 mutant, heparinase treatment only inhibited about 50% of gC-negative virus plaque formation under the conditions tested (Fig. 4–6). The lack of complete inhibition by heparinase treatment of the mutant virus plaque formation could be an indication that gB-HS interaction might not be essential for virus infection. However, given the fact that heparin was able to completely inhibit mutant virus infectivity, a more plausible explanation would be that the role of gB-HS interactions is not to provide a dominant binding force for gC negative virus but rather is to provide a mechanism leading to productive virus entry. According to this premise, a gC negative mutant
would still bind to cells lacking HS, perhaps with a reduced efficiency, but because of the lack of gB-HS interaction it could not produce productive virus entry and therefore plaque formation. By the same premise, a certain fraction of the bound virions might become infectious after interacting with newly synthesized HS. As to exogenous heparin, it may act not simply by inhibiting binding of gC-negative virus; the association of gB with the exogenous heparin molecules may prevent gB from interacting with cellular HS residues, by which it inhibits gB binding to its high-affinity receptor. Nevertheless, further studies are necessary to ascertain whether gB binding is an essential function for virus entry. Previously, it has been shown that HSV-1 was able to infect mutant cells which are defective in HS synthesis (Gruenheid et al., 1993). In addition, the infectivity of gC-negative PRV has been shown to be refractory to the inhibition by heparin or heparinase treatment (Sawitzky et al., 1990).

Recently Shieh and Spear showed that HSV-1 gB-mediated cell fusion requires the presence of HS (Shieh and Spear 1994). In light of the fact that binding of BHV-1 gB to its high-affinity receptor also requires it to be first bound with HS, it is very tempting to speculate that the HS-dependent fusion activity exhibited by HSV-1 gB and the high-affinity binding of BHV-1 gB revealed in this study are related. However, a difference has also been noticed. For HSV-1 gB, the fusion activity can be activated by the
addition of exogenous heparin, whereas for BHV-1 gB, high-affinity binding was not detected by the addition of exogenous heparin to heparinase-treated cells.

The observation that the full-length gB and the truncated gB show different cell binding activities is intriguing, and the reason for this has yet to be determined. Could the high-affinity binding activity observed with the full length gB be caused by a non-specific interaction between an aberrant hydrophobic structure of gB and the cell surface? It seems unlikely for the following reasons. First, the gB high-affinity binding is saturable (Fig. 4-5), a property which is characteristic of specific interaction between a ligand and its receptor. Second, the full length gB was able to inhibit viral plaque formation. This suggests that the purified gB was able to compete against the gB present on the virus surface for the same cell binding sites and thus the binding function of the purified gB resembles to that of the authentic gB present on virions. Finally, heparin or heparinase treatment was able to completely prevent the authentic gB from binding to cells (Fig. 4-1). It is unlikely that a hydrophobic non-specific binding could be affected by heparin or heparinase treatment. The fact that binding to HS is required for authentic gB to engage in the secondary high-affinity binding suggests that binding to HS may cause conformational change of authentic gB resulting in exposure of its high-affinity binding sites. Therefore, a more
plausible explanation would be that the ability of gB binding to its high-affinity receptor is a conformation-dependent property, and as to the soluble gB, the truncation of its carboxyl terminal amino acid sequence may have caused a defect in its overall conformation state, which may preclude the proper exposure of its high-affinity binding site in response to HS binding. In support of this hypothesis, it has been shown that the carboxy-terminal sequences of HSV-1 gB and PRV gB have a profound effect on the overall gB structural and functional properties, including oligomerization and fusion activity of gB molecules (Baghian et al., 1993; Navarro et al., 1992; Whealy et al., 1990). Studies are currently underway to investigate this possibility.
5.0 GLYCOPROTEIN Bb, THE N-TERMINAL SUBUNIT OF BOVINE HERPESVIRUS 1 gB, CAN BIND TO HEPARAN SULFATE ON THE SURFACE OF MADIN DARBY BOVINE KIDNEY CELLS

5.1 INTRODUCTION

Bovine herpesvirus 1 (BHV-1), a member of the Alphaherpesviridae subfamily, is an economically important pathogen in cattle (Gibbs and Rweyemamu 1977). The genome of BHV-1 encodes more than 33 structural proteins, among which there are at least nine membrane associated glycoproteins (Bolton et al., 1983; Fitzpatrick et al., 1989; Khadr et al., 1996; Khattar et al., 1995; Leung-Tack et al., 1994; Marshall et al., 1986; Meyer et al., 1991; Misra et al., 1981, 1988; Tikoo et al., 1990; Zamb 1987). The three major glycoproteins, gB, gC, and gD, have been shown to play a role in the virus entry process (Liang et al., 1991). gC is the dominant heparin-binding glycoprotein involved in the initial step in virus adsorption to cells, whereas gB and gD are important for subsequent interactions (Liang et al., 1993; Spear 1993). Previous studies have shown that BHV-1 gC but not gB could bind heparin (Okazaki et al., 1991). However,
during our characterization of cell-binding properties of the three major BHV-1 glycoproteins, we found that the binding of both gB and gC to MDBK cells could be inhibited by exogenous heparin, suggesting that BHV-1 gB is also involved in heparin binding (Chapter 4.0). This is consistent with observations for herpes simplex virus 1 (HSV-1) and a recent report for BHV-1 (Byrne et al., 1995; Herold et al., 1991). In order to gain a better understanding of the function of gB in BHV-1 entry, we carried out further studies on the heparin-binding property of gB.

BHV-1 gB consists of 933 amino acids with features characteristic of type I integral membrane proteins, including a putative signal peptide sequence located between amino acid residues 50 and 67 and a transmembrane domain between residues 759 and 828 that is followed by a 104-amino-acid cytoplasmic tail (Whitbeck et al., 1988; Zamb 1987). Like the gB homologues of most other herpesviruses, including pseudorabies virus (PRV) (Lukacs et al., 1985), varicella-zoster virus (VZV) (Montalvo and Grose 1987), human cytomegalovirus (HCMV) (Britt and Vugler 1989) and equine herpesvirus (EHV) (Meredith et al., 1989; Whalley et al., 1989), mature BHV-1 gB exists predominantly as a covalently linked heterodimeric complex, derived from a common primary translation product by proteolytic cleavage (Lukacs et al., 1985; Montalvo and Grose 1987; van Drunen Littel-van den Hurk et al., 1989). This cleavage event does not occur in HSV gB.
As one of the essential viral glycoproteins, gB plays an important role in virus entry including initial virus attachment and subsequent penetration involving membrane fusion. We previously demonstrated that gB may participate in BHV-1 attachment and fusion processes (Fitzpatrick et al., 1988, 1990; Liang et al., 1991; van Drunen Littel-van den Hurk et al., 1992). Others showed that gB-specific antibodies possess postadsorption neutralization activity, indicating the involvement of gB in viral penetration (Dubuisson et al., 1992). However, the working mechanism for this glycoprotein is not clear.

In this section, we present the heparin-binding profiles of gB, gC and gD of BHV-1 using heparin affinity chromatography. We confirmed that both gC and gB, but not gD are heparin-binding proteins. In order to further study the heparin-binding function of gB and identify the region involved in such binding, we expressed the extracellular portion of gB, gBt, the large subunit of gB, gBb, and the truncated C-terminal subunit, gBct, in a heat-inducible, bovine heat-shock protein 70A (HSP70A) gene promoter based expression system (Kowalski et al., 1993). Both gBt and gBb were expressed at high levels in this novel expression system. They were fully processed and secreted, and retained structural and antigenic properties similar to the authentic BHV-1 gB. Furthermore, both gBt and gBb possessed heparin-binding properties similar to authentic gB, whereas gBct did
not. We localized the heparin-binding domain(s) of gB to the N-terminal subunit, gBb.

5.2 MATERIALS AND METHODS

5.2.1 Virus, cells and reagents.

The BHV-1 Cooper strain was propagated in Madin Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO). Heparin of bovine intestinal mucosa (molecular weight about 3000 dalton) and heparinase of flavobacterium heparinum were purchased from Sigma (St.Louis, MO); heparin CL6B Sepharose from Pharmacia (Ste-Anne-De-Bellevue, Quebec); Trans[^35S]-label, methionine and cysteine, from ICN (Mississauga, Ontario); and Na[^23I] from Amersham (Oakville, Ontario).

5.2.2[^35S] labeling of BHV-1 glycoproteins.

Confluent MDBK cells were infected with wild-type BHV-1 at an MOI of 5 for 1 h at 37°C, then incubated with MEM containing 5% FBS at 37°C for 4 h. After starving the cells in Met/Cys free MEM (GIBCO) for 30 min, they were labeled in 50 μCi/ml of[^35S]-Met/Cys in Met/Cys free MEM at 37°C for 6-10 h. The cells were collected and washed in PBS, then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Sodium Deoxycholate, 1% Triton X-100, 0.1% SDS, 0.02% NaN₃).
on ice for 30 min. After centrifugation at 17,000 g for 30 min, the supernatant was used as the labeled BHV-1 glycoprotein fraction. For each 75 cm² tissue culture flask, 5 ml of cell lysate was prepared.

To label gB products from the transfected MDBK cells, the cells were first subjected to heat-shock for 4 h at 43°C, then starved and labeled as above. After labeling, the cells and supernatant were harvested separately for immunoprecipitation assays.

5.2.3 Heparin affinity chromatography.

To assess the binding of radiolabeled viral protein to heparin, 0.5 ml of labeled sample was incubated with 0.5 ml of packed heparin-Sepharose saturated in RIPA buffer. After 1 h at 4°C, the supernatant was collected as the unbound fraction and the heparin-Sepharose was extensively washed with RIPA. The bound proteins were dissociated from the heparin-Sepharose by incubation with RIPA, 2% SDS, 2 mg/ml heparin at 60°C for 5 min. All the samples (original lysates, bound and unbound fraction) were subjected to immunoprecipitation.

To demonstrate the specificity of binding to heparin-Sepharose, radiolabeled samples were incubated with different amounts of soluble heparin, chondroitin sulfate (Sigma), or dermatan sulfate (Sigma) at 4°C for 1 h, before incubation with a specified amount of heparin-Sepharose. After
incubation and washing, the bound proteins were dissociated from the heparin-Sepharose and subjected to immunoprecipitation to compare the inhibition of binding caused by soluble heparin.

5.2.4 Immunoprecipitation and autoradiography.

Samples were incubated for 1 h at room temperature with an appropriate mixture of monoclonal antibodies (van Drunen Littel-van den Hurk et al., 1992), followed by incubation with protein A Sepharose for 1 h. After 5 washes with RIPA, the precipitated proteins were dissociated by boiling in 50 μl SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 1.25% SDS, 12.5% glycerol, 0.001% bromphenol blue). All the samples were loaded onto 7.5% SDS-polyacrylamide gels. Following electrophoresis, the gels were fixed for 30 min, then soaked in Amplify (Amersham) for 30 min, vacuum dried, and exposed to X-ray film.

5.2.5 Construction of transfer vectors and transfected cell lines.

Expression of BHV-1 gB ectodomain derivatives was described previously (Chapter 3.0).

5.2.6 Protein iodination and binding assay on MDBK cells.

Protein iodination was carried out by the
lactoperoxidase method (Thorell and Johansson 1971). About 5 
µg of affinity-purified protein was used for iodination, the 
labeled protein was separated from free Na\(^{125}\)I by 
chromatography on a Sephadex G-25 column and then stored at -
70°C in small aliquots. In the binding study, confluent MDBK 
cells in 48-well plates were first cooled to 4°C, then 
incubated with approximately 2 x 10\(^5\) cpm of iodinated protein 
diluted in MEM-FBS at 4°C for 4 h. The effects of heparin and 
heparinase treatments on protein binding were evaluated as 
described previously (Chapter 4.0). After incubation, cells 
were washed three times with MEM-FBS and lysed with lysis 
buffer (PBS, 1% Triton X-100, 1% SDS). Total cell lysate was 
collected and counted for radioactivity in a gamma counter. 
In each plate, wells containing iodinated protein together 
with 100 µg/ml of unlabeled protein were included and used as 
controls for non-specific background binding.

5.3 RESULTS

5.3.1 Heparin affinity chromatography of BHV-1 gB, gC 
and gD.

By using a plaque inhibition assay, we previously 
found that heparan sulfates are involved in the attachment of 
both wild-type and gC-negative BHV-1 to MDBK cells, 
suggesting that gC is not the only viral glycoprotein capable 
of binding cellular heparan sulfates (Chapter 4.0). We also
demonstrated that binding of gC and gB to MDBK cells could be prevented by the addition of exogenous heparin or heparinase treatment of cells, indicating gB is also a heparin-binding protein (Chapter 4.0). A recent report by Byrne et al. showed that BHV-1 gB can bind to heparin (1995). These results are contradictory to a previous report by Okazaki et al., which showed that in BHV-1, gB does not bind to heparin (1991). In order to confirm that gB can bind to heparin, we used heparin affinity chromatography, which has also been used to identify the heparin-binding proteins in other herpesviruses (Byrne et al., 1995; Compton et al., 1993; Herold et al., 1991; Mettenleiter et al., 1990; Okazaki et al., 1991; Vanderplasschen et al., 1993). In this experiment, binding of gC and gD was also tested as appropriate controls for the gB study. As shown in Fig. 5-1, all three glycoproteins were present in the original cell lysates (Fig. 5-1 A). After incubation of infected cell lysates with heparin-Sepharose, the amounts of both gC and gB were greatly reduced in the unbound fraction but the amount of gD was not (Fig. 5-1 B). In addition, gC and gB, but not gD, could be recovered from the bound proteins released from the heparin-Sepharose (Fig. 5-1 C). These data confirm that gC and gB have the ability to bind heparin.
Fig. 5-1. Binding of BHV-1 glycoproteins to heparin Sepharose. $^{35}$S-labeled BHV-1-infected MDBK cell lysates were incubated with heparin-Sepharose. The samples of original lysates before heparin-Sepharose incubation (A), unbound cell lysates after incubation with heparin-Sepharose (B), and bound proteins dissociated from heparin-Sepharose (C) were subjected to immunoprecipitation with pooled monoclonal antibodies against gB, gC or gD. Molecular mass standards (kilodaltons, kDa) are indicated on the right.
5.3.2 Binding specificities of gC and gB to immobilized heparin.

In order to demonstrate that under our experimental conditions heparin binding of gC and gB was indeed a specific event, we tried to block this binding by using soluble heparin, as well as chondroitin sulfate and dermatan sulfate, two other glycosaminoglycans found on cell surfaces. We found that at a concentration of 100 µg/ml, soluble heparin could block nearly all of the gC binding and 50% of the gB binding to heparin-Sepharose (Fig. 5-2). However, even at a concentration of 1,000 µg/ml, chondroitin sulfate and dermatan sulfate could not inhibit binding of gB or gC to heparin-Sepharose. In this experiment, soluble heparin showed a different capacity to inhibit binding of gB and gC to heparin-Sepharose. The highest heparin concentration used could completely block binding of gC to heparin-Sepharose, but blocked only 75% of the binding of gB (Fig. 5-2 C). This difference may suggest that BHV-1 gB and gC have different avidities in heparin binding. The precursor form of gB was also found to bind to heparin-Sepharose (Fig. 5-2 A), indicating that the binding of gB to heparin does not require intracellular modifications such as glycosylation and proteolytic cleavage.

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Fig. 5-2. Heparin inhibition of binding of gB and gC to heparin-Sepharose. $^{35}$S-labeled BHV-1-infected MDBK cell lysates were first incubated with different amount of heparin, chondroitin sulfate (C.S.), or dermatan sulfate (D.S.) at 4°C for 1 h and then further incubated with heparin-Sepharose for another hour. After extensive washing, bound proteins were dissociated and subjected to immunoprecipitation to detect gB (A.) and gC (B.). In heparin incubation groups, all bands were scanned by a BioRad video densitometer (model 620). The amounts in each band are indicated as a percentage of the control which has no heparin as an inhibitive reagent (C.). Concentration units in panels A and B are microgram per milliliter ($\mu$g/ml).
5.3.3 Expression and characterization of gB ectodomain derivatives in MDBK cells.

In order to obtain reagents for further study of the heparin-binding properties of gB, we expressed the extracellular portion (gBt), the N-terminal subunit of gB (gBb), and the truncated C-terminal subunit (gBct) in mammalian cells under the control of the bovine heat-shock protein 70A gene promoter (Chapter 3.0). Immunoprecipitation studies employing these recombinant gB expressing cells showed that the expression of the gB products could be induced by heat shock treatment (Fig. 5-3 A). Under the conditions tested, gBt and gBb were readily detected in the culture medium (lanes M) with minimal quantities in the cell lysate (lanes C), whereas gBct was mainly retained intracellularly (data not shown). gBt existed as a single band with a molecular weight of about 115 kDa under non-reducing conditions (Fig. 5-3 A); when reduced, the 74 kDa and 38 kDa bands were dominant, although the 115 kDa band was still visible (Fig. 5-3 B). With respect to gBb, the product expressed had a mobility similar to authentic gBb. Based on these studies, we anticipated that the expressed gBt and gBb from the bovine heat-shock expression system were structurally similar to authentic gB.

In order to further compare the recombinant gBt and gBb with the authentic one, a panel of gB-specific monoclonal antibodies were used. We found that both gBt and gBb reacted
in an ELISA with all eight gB-specific monoclonal antibodies tested, and they all showed a similar degree of reactivities to that exhibited by authentic gB (Table 5–1). Similar results were observed by immunoprecipitation assays (data not shown). Among these monoclonal antibodies, some can only recognize epitopes properly configured or glycosylated (van Drunen Littell-van den Hurk and Babiuk 1985, 1986). Therefore, our results suggested that both gBt and gBb retain not only linear but also conformation- and glycosylation-dependent epitopes.

<table>
<thead>
<tr>
<th>Table 5–1. Reactivity of monoclonal antibodies with gB, gBt and gBb.</th>
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<tr>
<td><strong>MAb</strong></td>
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<tr>
<td>3F3</td>
</tr>
<tr>
<td>1E11</td>
</tr>
<tr>
<td>1F8</td>
</tr>
<tr>
<td>3C7</td>
</tr>
<tr>
<td>3G11</td>
</tr>
<tr>
<td>5G11</td>
</tr>
<tr>
<td>6G11</td>
</tr>
<tr>
<td>1F10</td>
</tr>
</tbody>
</table>

*G* B mAbs and epitopes were described previously (van Drunen Littell-van den Hurk and Babiuk 1985, 1984).

*Titers are the reciprocals of the highest dilution of gB products which show positive ELISA values (an optical density at 405 nm of at least 0.1). A 1:10 dilution corresponds to 0.04μg of protein per well (Kowalski et al., 1993).
Fig. 5-3. Immunoprecipitation of gBt and gBb from transfected cell lines. Cells grown in T-25 flasks were heat shocked at 43°C for 6 h (+HS) or left untreated (-HS) and labeled with [35S]Met-Cys. Cells and culture medium were collected separately and each precipitated with gB-specific monoclonal antibodies. The samples were separated on SDS-8.5% polyacrylamide gels under non-reducing (A) or reducing (B) conditions. Lanes BHV1, gB precipitated from the wild-type BHV-1-infected MDBK cells; lanes C, cellular fraction; lanes M, medium; HS, heat shock. Molecular mass standards (kDa) are indicated on the right.
5.3.4 Binding of recombinant gB derivatives to heparin-Sepharose.

Since the recombinant gBt and gBb were structurally and antigenically similar to the authentic gB, they were used as reagents for further studies to identify the region of gB that contains the heparin-binding activity. By using heparin affinity chromatography, both gBt and gBb were found to bind to heparin-Sepharose (Fig. 5-4 upper panel). These results are consistent with those using BHV-1 infected cell lysates, indicating that authentic gB, gBt and gBb could all bind heparin.

We previously expressed the truncated part of the C-terminal subunit, gBct, in the same expression system (Chapter 3.0). It was an endo-H-sensitive precursor form which was intracellularly retained. However, since we found that heparin binding of gB does not require complete glycosylation of the protein (Fig. 5-1, 5-2), we also tested the heparin binding ability of the recombinant gBct. After incubation with heparin-Sepharose, the labeled gBct remained in the supernatant (Fig. 5-4 bottom panel, lane B) and could not be released from the heparin-Sepharose (Fig. 5-4 bottom panel, lane C). Therefore, our results suggest that the gB-heparin binding domain(s) are located in the N-terminal subunit of BHV-1 gB.
Fig. 5-4. Binding of radiolabeled recombinant gB derivatives to heparin-Sepharose. $^{35}$S-labeled BHV-1 infected MDBK cell lysates and samples from recombinant gB expressing cells were incubated with heparin-Sepharose. The original samples before heparin-Sepharose incubation (A), unbound samples after incubation with heparin-Sepharose (B), and bound proteins dissociated from heparin-Sepharose (C) were subjected to immunoprecipitation with pooled monoclonal antibodies against gB, gC or gD. In lanes BHV, immunoprecipitations were done with combined monoclonal antibody pools (gB-gC-gD), or with indicated individual pools (C). Molecular mass standards (kDa) are indicated on the right. Upper panel: detection of BHV-1-infected cell lysates and samples from gBt or gBb expressing cells. Bottom panel: detection of samples from gBct expressing cells.
5.3.5 Binding of gBt and gBb to heparan sulfate on MDBK cells.

To demonstrate that gBt and gBb could bind heparan sulfates on permissive MDBK cells, we incubated MDBK cells with affinity purified iodine labeled gBt or gBb as previously reported (Chapter 4.0). We found that soluble heparin blocked binding of glycoprotein to cells in a dose-dependent manner (Fig. 5-5 A), indicating that binding is mediated by heparan sulfates (HS). This result was further supported by experiments in which cells depleted of HS by heparinase treatment were not able to bind labeled proteins (Fig. 5-5 B). Although both gBt and gBb can specifically bind heparan sulfates on the surface of MDBK cells, they may have different binding capacities, since at low heparin concentration, 0.1 to 10 µg/ml, gBb expressed higher binding to cells than gBt, and it also showed slightly higher binding in the heparinase treatment experiment (Fig. 5-5 B). These data suggest that gBb subunit has the heparin-binding activity.

5.4 DISCUSSION

Heparan sulfate, an unbranched glycosaminoglycan, exists ubiquitously on mammalian cell surfaces (Couchman and Woods 1993; Kjellen and Lindahl 1991). This cellular component has been shown to be used by herpesviruses in the
Fig. 5-5. Effects of heparin and heparinase treatment on binding of iodinated gBt and gBb to MDBK cells. For the heparin inhibition assay (A), confluent MDBK cells were incubated with heparin at indicated concentrations at 4°C for 1 h, which was followed by addition of \(^{125}\)I-labeled gBt or gBb and incubation at 4°C for 4 h. Cell-associated radioactivity was measured in a gamma counter. For heparinase treatment (B), cells were first treated with heparinase at indicated concentrations at 37°C for 1 h. Then the cells were incubated with \(^{125}\)I-labeled gBt or gBb for 4 h at 4°C, which was then followed by washing and counting. The counts per minute bound in the presence of 100 μg/ml of the unlabeled protein was defined as background, which has been subtracted from all sample counts. The counts per minute bound in the absence of inhibitive factor was defined as 100%. The curves were drawn from triplicated sample counts where standard deviation bars are also indicated.
initial attachment process (Spear 1993; WuDunn and Spear 1989). In the proposed model of HSV-1 entry, the virus first attaches to the cell via interaction between viral gC, gB and cellular heparan sulfate to establish unstable attachment, which can be dissociated in the presence of heparin (heparin-sensitive). The unstable interaction can be converted to a stable heparin-resistant attachment through further interactions between other viral glycoproteins and their corresponding cellular receptors (Karger and Mettenleiter 1993; Lee and Fuller 1993; McClain and Fuller 1994). During this biphasic attachment process, a cooperative interaction between multiple viral and cellular components has been established, which is the prerequisite for the next step in the infection process, penetration and fusion. It is believed that viral glycoproteins gB, gD, gH, gK, and gL are involved in this activity (Cai et al., 1988; Desai et al., 1988; Hutchinson et al., 1995; Lee and Fuller 1993; Roop et al., 1993). However, the corresponding cellular components which interact with these glycoproteins remain unknown.

Studies of HSV-1 and PRV have shown that the interaction between gC and cellular heparan sulfates plays the dominant role in the initial viral attachment (Karger and Mettenleiter 1993; McClain and Fuller 1994). In mutants with shortened gC or without gC, the attachment was significantly impaired (Flynn and Ryan 1995; Herold et al., 1991; Liang et al., 1991; Zsak et al., 1991; Zuckermann et al., 1989).
However, gC-negative HSV-1 has the same heparin-dependent binding pattern as the wild-type virus, whereas for gC-negative PRV, a proteoglycan-independent attachment was reported (Karger et al., 1995; Mettenleiter et al., 1990), indicating that different mechanisms could exist for the early events in entry of different herpesviruses. In BHV-1, our previous data showed that HS is involved in the attachment of gC-negative BHV-1 (Chapter 4.0), which suggests that other viral component(s) can bind HS. In HSV-1, gB has been found to bind to heparin-Sepharose columns (Herold et al., 1991), whereas gB of PRV can bind heparin, but only in the presence of gC (Mettenleiter et al., 1990). In contrast, in BHV-1, gC was previously reported to be the only heparin-binding protein (Okazaki et al., 1991). In our study, we found that BHV-1 gB could also bind to immobilized heparin as well as heparan sulfate on MDBK cells (Chapter 4.0 and this chapter). We suspect that differences in experimental conditions between Okazaki’s and our studies are responsible for the contradictory results. In order to demonstrate the specificity of gB-heparin interaction, three different glycosaminoglycans were used to block gB binding in our heparin affinity chromatography study. As shown in Fig. 5-2, only heparin could effectively block gC and gB binding to heparin-Sepharose, whereas chondroitin sulfate and dermatan sulfate did not. It is of interest that soluble heparin inhibited binding of gC to heparin-Sepharose more efficiently
than binding of gB (Fig. 5-2). This difference provides further evidence that gC and gB have different avidities in heparin binding. In this study, a low molecular weight heparin was used as a soluble inhibitory reagent. This soluble heparin is different from the immobilized heparin on the Sepharose which has a higher molecular weight. It has been found previously that heparin with shorter saccharide chains may be less efficient in interacting with viral heparin-binding proteins (Lycke et al., 1991). Therefore, the immobilized heparin might have higher avidity than the soluble heparin in gB binding, which results in the incomplete inhibition of binding of gB to heparin-Sepharose by soluble heparin (Fig. 5-2). The difference of the binding avidity of gB to soluble heparin and immobilized heparin may also explain the previous failure to recognize gB as a heparin-binding protein (Okazaki et al., 1991), because soluble heparin may not be able to recover the bound gB from heparin-Sepharose under certain experimental conditions. The different structures of heparin may also be responsible for the binding difference between HSV-1 gB and gC (Herold et al., 1994, 1995; Sears et al., 1991). It was suggested that gB interacts with more highly sulfated regions of heparan sulfate, which may cause gC-negative HSV-1 mutants to be more resistant to heparin washing than wild-type viruses.

In this section, we describe the production and characterization of different gB ectodomain derivatives. gBt
and gBb, two reagents for further study of gB-heparin binding, are efficiently secreted from the cells and possess structural and antigenic properties similar to authentic gB. The gBt, like authentic gB, was cleaved into two subunits, forming covalently linked heterodimers. Both gBt and gBb had apparent molecular weights as predicted. Based on their reactivities to a panel of gB-specific monoclonal antibodies, the expressed gBs were shown to possess all the tested antigenic epitopes present on authentic gB, including the glycosylation- and conformation-dependent epitopes. Furthermore, like authentic gB, both gBt and gBb were able to elicit antibody responses in mice to a comparable extent (Chapter 3.0). Taken together, these results suggest that our recombinant gBs have proper post-translational modifications, including intra-chain disulfide bond formation, proteolytic cleavage and glycosylation. Both gBt and gBb were able to bind heparin-Sepharose (Fig. 5-4); in addition, the iodinated proteins could bind to MDBK cells in a heparin-sensitive manner. The soluble heparin could effectively block binding of iodinated gBt and gBb to MDBK cells (Fig. 5-5). The different binding capacities of the two gBs to cellular heparan sulfate may be caused by a conformational change, that may results in better exposure of the heparin binding domain(s) on gBb. To explore the heparin-binding ability of other part of gBt, the C-terminal subunit of gBt was also expressed and tested by heparin affinity chromatography. Our
results suggest that the gBc subunit is not needed for the heparin-binding activity of gB (Fig. 5-4).

Table 5-2. Basic amino acid clusters in BHV-1 gB*

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<th>Sequence</th>
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<td>212-221</td>
<td>LVDKKKWRCLS</td>
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<tr>
<td>225-234</td>
<td>YLRSGRKKVVA</td>
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<tr>
<td>343-352</td>
<td>ATGRRLKEPV</td>
</tr>
<tr>
<td>368-377</td>
<td>WVPKRRKNVCS</td>
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</table>

* Sequence from T. Zamb (1987). Boldface indicates clusters of lysine and arginine.

It has been suggested that clusters of basic amino acids including lysine and arginine are involved in the binding of polyanionic heparin-like structure by viral glycoproteins (Liang et al., 1993; Spear et al., 1992). In HSV-1 gB, a short lysine-, proline-rich region located near the N-terminus of the polypeptide was thought to be the heparin-binding domain (Spear et al., 1992). The basic residue clusters, B-B-X-B and B-B-B-X-X-B, found in other heparin-binding proteins have been recognized as heparin-binding motifs (Manley et al., 1992), where the basic amino acids (B) and the hydrophobic amino acids (X) are specifically arranged. Although in BHV-1 gB, there is no such obvious lysine-rich region like in HSV-1, we found several basic residue clusters (Table 2) and a proline rich region in the N-terminal portion of gB. These regions may be the
heparin-binding domain(s). Since the precursor form of gB was able to bind heparin (Fig. 5-2 A; Fig.5-4), the primary sequence but not posttranslational modification appears to be important for heparin-binding of gB. In the C-terminal subunit, gBct, there are few basic residue clusters, and experimental data also confirmed that this part does not bind heparin-Sepharose. By comparing the binding of gBt and gBb to MDBK cells, we also found that the heparin binding activity was mostly correlated with gBb (Fig. 5-5). On the basis of these observations, the potential heparin-binding domain(s) of gB are probably located in the N-terminal gBb region.
6.0 THE CYTOPLASMIC DOMAIN OF BOVINE HERPESVIRUS 1
GLYCOPROTEIN B IS IMPORTANT FOR MAINTAINING CONFORMATION
AND THE HIGH-AFFINITY BINDING SITE ON gB

6.1 INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) is a major viral pathogen in cattle. Each year, BHV-1 related infections such as the complex respiratory disease syndrome (shipping fever) can cause significant economic losses (Yates 1982). BHV-1 is a member of the alphaherpesviruses, which all have a similar enveloped virion structure. On the envelope, there are different viral glycoproteins, which may be involved in the entry of the virus into permissive cells (for a comprehensive review, see Spear 1993). In BHV-1, three major glycoproteins, gB, gC and gD, have been found to bind to receptors on permissive cells (Chapter 4.0; Liang et al., 1991). During the early entry process of BHV-1, gC plays the dominant role for virus binding to the cells by interacting with cellular heparan sulfate proteoglycans (HSPG, HS) (Liang et al., 1993; Okazaki et al., 1991). BHV-1 gD can bind to a non-HS component, whereas BHV-1 gB can bind to two different types
of cellular receptor, the HS and non-HS components (Chapter 4.0). Previously, the binding of gB to a non-HS cellular receptor has been found to be important for the infectivity of BHV-1 (Chapter 4.0).

BHV-1 gB is a type I integral membrane glycoprotein which consists of 933 amino acids, including a putative signal peptide sequence between residues 1-67 and the transmembrane domain between residues 759-828, which is followed by a 104 amino acid cytoplasmic tail (van Drunen Littet–van den Hurk et al., 1992; Whitbeck et al., 1988; Zamb 1987). Like the gB homologues of most other herpesviruses (Britt and Vugler, 1989; Lukacs et al., 1985; Meredith et al., 1989; Montalvo and Grose, 1987; Whalley et al., 1989), mature BHV-1 gB exists predominantly as a covalently linked heterodimeric complex (Lukacs et al., 1985; Meredith et al., 1989; van Drunen Littet–van den Hurk and Babiuk, 1986). Its N-terminal subunit, gBb, has been shown to be required for heparin-binding (Chapter 5.0). However, the structure responsible for the high-affinity binding to the non-HS receptor has not been identified yet.

As one of the essential viral glycoproteins, gB homologues of herpesviruses play an important role in virus entry including initial virus attachment and subsequent penetration involving membrane fusion (Pereira 1994; Spear 1993). There is evidence that gB-specific antibodies possess post-adsorption neutralization activity (Dubuisson et al.,
1992; Highlander et al., 1988; Navarro et al., 1992) and expression of gB in transfected cells can induce syncytium formation and membrane fusion (Ali et al., 1987; Fitzpatrick et al., 1988, 1990; Tugizov et al., 1994; van Drunen Littel-van den Hurk et al., 1992). The functions of gB have been shown to have strict structural requirements (Baghian et al., 1993; Navarro et al., 1992; Whealy et al., 1990), which may include oligomerization and proper folding. It has been shown that in herpes simplex virus 1 (HSV-1), pseudorabies virus (PRV) and human cytomegalovirus (HCMV), gB homologues exist as a dimer (Britt and Vugler, 1992; Claesson-Welsh and Spear, 1986; Eberle and Courtney, 1982; Sarmiento and Spear, 1979; Whealy et al., 1990). In temperature-sensitive HSV-1 isolates, gB failed to form dimers at the nonpermissive temperature, suggesting that oligomerization is necessary for the production of infectious virus (Chapsal and Pereira, 1988; Haffey and Spear, 1980). Truncations in the C-terminal region may preclude overall oligomer formation (Navarro et al., 1993), which in turn may influence the function of gB. Therefore, we focused on the oligomerization and conformation of different truncated forms of gB to study the structural requirements for the existence of the high-affinity binding site on BHV-1 gB.
6.2 MATERIALS AND METHODS

6.2.1 Virus, cells and reagents.

The BHV-1 Cooper strain was obtained from the National Veterinary Services Laboratories, Ames, Iowa, and propagated in Madin Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO). The Lipofectin and G418 were from GIBCO; Tran[^35S]-label, which includes radiolabeled methionine and cysteine (Met/Cys), was from ICN (Mississauga, Ontario).

6.2.2 Construction of transfer vectors and establishing transfected cell lines.

BHV-1 gB is synthesized as a 933-amino acid precursor, which is subsequently cleaved into two subunits, i.e., the amino terminal fragment gBb and carboxyl terminal fragment gBc. According to amino acid sequence analysis, the predicted transmembrane domain of gB is located between residues 759-828 (Whitbeck et al., 1988; Zamb 1987) and the proteolytic cleavage recognition site between residues 505-506. The location of the cleavage site has been confirmed by amino terminal sequencing of gBc showing that the alanine at position 506 is the amino terminal residue (van Drunen Littel-van den Hurk et al., 1992). On the basis of this information, the gBb, gBt and gBtM genes were constructed so
that they contained the sequences coding for residues 1-505, 1-763 and 1-807, respectively. Fig. 6-1A depicts the sequences of authentic gB, gBb, gBt and gBtM. Plasmid p3KHSPG4HU (Kowalski et al., 1993), which contains a truncated BHV-1 gD gene under the control of the bovine heat-shock protein 70A (hsp70A) gene promoter and the aminoglycoside phosphotransferase gene under the control of the SV40 early promoter, was used as the parental plasmid for constructing transfer vectors.

To establish recombinant gB expressing cell lines, approximately 4 x 10^6 MDBK cells were transfected with 5 µg of the transfer vector using Lipofectin; after transfection, the cells were grown in the presence of 666 µg/ml of G418. The G418-resistant cells were screened for gB production by an immune dot blot assay using gB-specific monoclonal antibodies (Kowalski et al., 1993). The positive cells were isolated and subjected to single cell cloning.

6.2.3 [35S] labeling of BHV-1 glycoproteins.

Confluent MDBK cells were infected with wild-type BHV-1 at an MOI of 5 for 1 h at 37°C, then incubated with MEM containing 5% FBS at 37°C for 5 h. After starving the cells in Met/Cys free MEM (GIBCO) for 30 min, they were labeled in 50 µCi/ml of [35S]-Met/Cys in Met/Cys free MEM at 37°C for 6-10 h. The cells were collected and washed in phosphate buffered saline (PBS, pH 7.2), then lysed in
radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.02% NaN₃) on ice for 30 min. After centrifugation at 15,000 g for 30 min, the supernatant was used as the labeled BHV-1 glycoprotein fraction.

To label gB products from the transfected MDBK cells, the cells were first heat-shock treated (43°C for 4 h), then starved and labeled as above. After labeling, the cells and supernatant were harvested separately for immunoprecipitation assays.

6.2.4 Immunoprecipitation and autoradiography.

Samples were incubated for 1 h at room temperature with an appropriate mixture of monoclonal antibodies (van Drunen Littel-van den Hurk et al., 1992), or with individual monoclonal antibodies, followed by incubation with protein A Sepharose for 1 h. After 5 washes with RIPA, the precipitated proteins were dissociated by boiling in 50 µl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 1.25% SDS, 12.5% glycerol, 0.001% bromophenol blue). All the samples were loaded onto SDS-7.5% or 8.5% polyacrylamide gels. Following electrophoresis, the gels were fixed for 30 min, then soaked in Amplify (Amersham, Oakville, Ontario) for 30 min, vacuum dried, and exposed to X-ray film at -70°C.
6.2.5 Pulse-chase experiment.

BHV-1 infected MDBK cells or gB-expressing cells grown in 6-well plates or T-25 flasks were used in this study. For BHV-1 infected cells, pulse-chase labeling was conducted at 6 h post-infection. For gB-expressing cells, a heat-shock treatment was required before labeling. After starving for methionine and cysteine in Met/Cys free medium at 37°C for 30 min, cells were pulse-labeled by the addition of 200 μCi of [35S]-Met/Cys per ml of medium at 37°C for 10 min. After labeling, supernatants were removed and cells were further incubated in Opti-MEM (GIBCO) containing 2 mM methionine and cysteine. At indicated time points, supernatants and cells were collected separately, and samples were precipitated with the gB specific monoclonal antibody pool and separated by SDS-7.5% polyacrylamide gel electrophoresis under non-reducing conditions.

6.2.6 Chemical cross-linking.

Cross-linking analysis was performed essentially as described by Russel et al. (1994) with minor modifications. The cross-linker, ethylene glycolbis(succinimidylsuccinate) (EGS) (PIERCE, Rockford, Illinois), was initially dissolved in dimethylsulfoxide. The overnight radiolabeled infected cells were washed and collected in cold PBS, then dissolved in the cross-linking buffer (PBS, 0.5% NP-40, 0.5% sodium deoxycholate, desired concentration of EGS). The reaction was
carried out at 4°C for 1 h, then quenched with glycine at a final concentration of 100 mM. After cross-linking, the sample was further lysed by RIPA buffer and processed in a normal immunoprecipitation assay.

6.2.7 Sucrose density gradient centrifugation.

To assess the oligomerization status of gB, sucrose density centrifugation was applied as described by Whealy et al. (1990). Briefly, one T-25 flask of radiolabeled, BHV-1-infected or recombinant gB expressing MDBK cells, were washed in PBS, and lysed in 0.3 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100) on ice for 30 min, then pelleted for 15 min at 4°C. The supernatant was layered onto 5 ml continuous 5-20% (W/V) sucrose gradients in lysis buffer. The centrifugation was for 16 h at 240,000 g in a SW50.1 rotor (Beckman Inc., Mississauga, Ontario) at 4°C. A parallel tube with protein mass markers was included in each centrifugation. After centrifugation, 15 fractions were collected from the bottom. The entire sample in each fraction was subjected to immunoprecipitation. To study purified gB products, 0.2 ml (about 10-20 µg) of affinity purified proteins were layered onto 5 ml continuous 5-15% sucrose gradients. After centrifugation, 10 fractions were collected. The protein mass markers in our study were ferritin (440 kDa), catalase (250 kDa), alcohol dehydrogenase (150 kDa), hexokinase (100 kDa), and bovine serum albumin (67 kDa).
(Sigma, St. Louis, Mo).

6.2.8 Enzyme-linked immunosorbent assay (ELISA) and Western blot.

An indirect ELISA, which was described by Kowalski et al. (1993), was performed to assess the antigenic reactivities of the purified proteins. The Western blot method was described by van Drunen Littel-van den Hurk and Babiuk (1986).

6.2.9 Inhibition of virus adsorption.

BHV-1 gB and truncated forms of gB were purified by affinity chromatography as previously described (Chapter 4.0; van Drunen Littel-van den Hurk and Babiuk, 1985). The proteins were diluted with MEM-10% FBS to specified concentrations and incubated with precooled MDBK cells in 24-well plates at 4°C for 1 h, then incubated with virus inoculum for another hour. After adsorption, the plates were washed three times with MEM and overlaid with MEM containing 1% methylcellulose and 2% FBS. After incubation at 37°C for 3 days, the plaques were stained with crystal violet and counted. The number of viral plaques formed in the absence of viral protein was taken as 100% plaque formation and used as a reference for calculating the percentage of viral plaques formed in the presence of the viral protein.
6.3 RESULTS

6.3.1 Expression of different derivatives of BHV-1 gB in MDBK cells under control of the hsp70A gene promoter.

The cleavage site in BHV-1 gB can divide this molecule into a 74 kDa N-terminal subunit and a 55 kDa C-terminal subunit, which are linked by intramolecular disulfide bonds to enable the intact protein to exist as a 130 kDa heterodimer (van Drunen Littel-van den Hurk and Babiuk, 1986). Previously, based on the location of the putative transmembrane region between residues 759-828 (Whitbeck et al., 1988; Zamb 1987), we expressed gBt, a truncated gB without the putative transmembrane and cytoplasmic regions, in MDBK cells by using the bovine hsp70A gene promoter. The recombinant gBt possessed authentic gB-like structure and antigenicity and it also retained the heparin-binding, but not the secondary high-affinity binding function (Chapters 4.0 and 5.0). In order to further study the structural requirements for the high-affinity binding site in BHV-1 gB, an extended form of truncated gB, gBtM, was expressed. This truncated gB stops after residue 807, such that it contains two third of the putative transmembrane region of gB. Studies of HSV have demonstrated that the 69 amino acid-transmembrane region can be divided into three segments where the C-terminal segment (segment 3) is sufficient to be the membrane anchor and segment 2 has a weak
ability to retain the protein in the membrane (Gilbert et al., 1994; Rasile et al., 1993). From the sequence analysis, the corresponding segment 3 in BHV-1 gB has the highest hydrophobicity among the three potential membrane spanning segments, which may be used as the real membrane anchor. Therefore, gBtM may represent the entire extracellular portion of BHV-1 gB.

The expression of recombinant forms of gB is under the control of the bovine hsp70A gene promoter (Kowalski et al., 1993). After lipofectin mediated transfection, the gB expressing cells were cloned and subcloned. All the gB expressing cell lines exhibited morphology and growth properties similar to the parental MDBK cells. Expression of gB products can be enhanced by heat-shock treatment (Kowalski et al., 1993). Under non-reducing conditions, the recombinant gBb, gBt, and gBtM have expected molecular weights of about 74 kDa, 115 kDa, and 120 kDa respectively (results not shown). Cleavage does occur in the recombinant gBt and gBtM (Fig. 6-1B) and the cleaved N-terminal subunits are in the same position as the authentic gBb (lane gB) as well as the recombinant gBb (lane gBb). Differences between recombinant forms of gB were identified on SDS-PAGE gels, as cleaved C-terminal did not migrate to the same position. The C-terminal subunit gBc of authentic gB has a molecular weight of about 55 kDa. For gBt, a 38 kDa band was found, whereas for gBtM, a 42 kDa band was observed. The reduced molecular weights of
gBt and gBtM were attributed to the truncation of the respective C-terminal regions. Based on their mobilities under reducing and non-reducing conditions, we suspected that these recombinant forms of gB are structurally similar to authentic gB.

Immunoprecipitation studies showed that authentic gB is detected in the cell lysate, whereas all the recombinant forms of gB are detected both in the cell lysate and in the medium. After overnight labeling, gBt, gBb, and gBtM were mostly present in the medium (Fig. 6-1B, lanes M), whereas only trace amounts of protein, primarily the precursor forms, were present in the cells (Fig. 6-1B, lanes C), indicating that gBt, gBb, gBtM are efficiently secreted from the cells. This confirms that the first two segments in the transmembrane region can not stably retain the protein in the cell membrane and that gBtM represents the whole ectodomain of gB as we predicted.

6.3.2 Maturation and transport of the expressed gB products.

Preliminary characterization showed that recombinant gBb, gBt, and gBtM are secreted and contain the expected structure. In order to further study their synthesis and transport, a pulse-chase experiment was designed. BHV-1 infected MDBK cells were included in this study as the authentic gB control, which also could be used to compare the
**Fig. 6-1. Schematic diagram and immunoprecipitation of BHV-1 gB and truncated derivatives.** (A) BHV-1 gB is represented as a box structure where the signal sequence (S) and transmembrane anchor region (TMA) are shaded. Amino acid sequences for both termini of the truncated forms of gB are presented with the corresponding residue number. The amino acids in parentheses of gBtM are exogenous residues added during construction of the molecule. (B) After labeling with $[^35]S$-Met/Cys, the labeled cells (C) and the media (M) were collected separately and precipitated with a gB-specific monoclonal antibody pool (van Drunen Littel-van den Hurk et al., 1992). The samples were separated on SDS-8.5% polyacrylamide gels under reducing condition. Lane gB is the sample from wt BHV-1-infected MDBK cells; lanes gBb, gBt, and gBtM are samples from the corresponding transfected MDBK cell lines which express gBb, gBt, and gBtM respectively. Molecular mass markers (kDa) are shown on the right.
Fig. 6-1. Schematic diagram and immunoprecipitation of BHV-1 gB and truncated derivatives (continued).
conversion rate from the precursor to the glycosylated forms of the recombinant forms of gB. To reduce variability in processing, which occurs at different times post-infection (Sommer and Courtney, 1991), labeling was conducted at a fixed time point (6 h post-infection). After labeling for 10 min with [35S]-Met/Cys, the cells were chased for different times with medium containing an excess amount of nonlabeled Met/Cys. At each time point, samples of cells and medium were collected and precipitated with a gB-specific monoclonal antibody pool. In BHV-1 infected cells, after 10 min pulse labeling, a dominant, 117 kDa band was detected in the cells, which represents the high-mannose oligosaccharide-containing gB precursor (Fig. 6-2, BHV-1 gB, p) (van Drunen Littet-vanden Hurk et al., 1989). This gB precursor was subsequently chased into a 130 kDa fully glycosylated mature gB (Fig. 6-2, BHV-1 gB, M). Similar pulse-chase patterns were observed in gBb, gBt, and gBtM expressing cells. The precursor of gBb was 65 kDa, which was chased into a 74 kDa mature gBb. For gBt and gBtM, the precursors were 90 kDa and 93 kDa and the mature products were 115 kDa and 120 kDa respectively (Fig. 6-2). In all these cells, after 10 min of pulse labeling, the gB products were primarily precursor forms derived from the endoplasmic reticulum (ER). After a 15 min chase, the glycosylated mature products started to be visible, indicating transport to the Golgi where further glycosylation occurs. After a 180 min chase, it was difficult to detect the
precursor except in gBtM cells (Fig. 6-2). The conversion rates of precursor to mature protein were similar in authentic gB, recombinant gBb and gBt as detected by densitometer scanning (Chapter 3.0). This experiment further supports our contention that, with exception of gBtM, the gB forms produced in the heat-shock system are processed in a manner similar to authentic gB.

From this pulse-chase experiment, all three recombinant forms of gB seemed to be secreted efficiently into the medium. It is of interest that gBtM follows a similar pattern of maturation and secretion as gBt with a slightly slower conversion rate. Immunofluorescence studies also indicates that gBtM is retained more strongly in the cellular and nuclear membranes than gBt or gBb (Chapter 7.0). These results suggest that the first two segments of the transmembrane region may provide unstable membrane retention for the protein without the third segment of the transmembrane region and the cytoplasmic domain, which is consistent with studies of HSV-1 and HCMV (Rasile et al., 1993; Reschke et al., 1995). However, after a 3 to 6 h chase, most of the recombinant gB products were found in the medium, which can be collected for protein purification.
Fig. 6-2. Maturation and transport of truncated gB derivatives examined by a pulse-chase experiment. MDBK cells were infected by wt BHV-1, and pulse-labeled with 200 μCi/ml of [35S]-Met/Cys for 10 min at 6 h post-infection, then chased for different time periods. The truncated gB expressing cells were heat-shock treated and pulse-labeled followed by chase with unlabeled media. After each chase interval, cells and media were collected separately and immunoprecipitated with the gB-specific monoclonal antibody pool. Authentic gB (BHV-1 gB) and the truncated forms of gB are identified at the left. M: mature form. p: precursor form. Molecular mass markers (kDa) are shown on the right.

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6.3.3 Oligomerization study of BHV-1 gB.

In HSV-1, PRV, and HCMV, gB homologues have been identified as dimeric oligomers (Britt and Vugler, 1992; Claesson-Welsh and Spear, 1986; Highlander et al., 1991; Whealy et al., 1990). Previous studies in HSV-1 showed that the gB homodimer was heat-dissociable and SDS-resistant, and that its dimerization was not dependent on glycosylation or expression of other viral proteins (Claesson-Welsh and Spear, 1986; Sarmiento and Spear, 1979). In a proposed synthesis pathway, gB homologues may be initially synthesized as a monomer in the ER, then rapidly converted to an oligomeric form, and transported to the Golgi for further processing (Whealy et al., 1990). However, thus far there is no evidence for oligomerization of BHV-1 gB. To examine the oligomeric status of BHV-1 gB, we first subjected the BHV-1 infected MDBK cells to a non-reversible cross-linking agent, EGS. This cross-linker has a spacer arm length of 6.1Å, and has been used to study protein oligomerization in the presence of detergent (Russel et al., 1994). In the absence of the cross-linker, the monomeric precursor form and mature gB are both detected by SDS-PAGE. With increasing concentrations of the cross-linker, a higher molecular weight band appeared at a position corresponding to the dimer of gB (about 250 kDa) (Fig. 6-3, arrow D). This band was located in the separating gel, and no other protein bands could be seen above or at the interface between the stacking and separating gels.

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Therefore, it is believed that for BHV-1 gB, there are no other oligomeric forms except a dimer. It also appeared that in BHV-1 infected cells, after overnight labeling, most of the gB product existed as an oligomer. No monomeric form was found when the EGS concentrations were higher than 5 mM.

To confirm the oligomerization status of BHV-1 gB, sucrose gradient sedimentation was used. BHV-1 infected cells were pulse-labeled for 10 min with $^{35}$S-Met/Cys, at 6 h post-infection, and either harvested immediately or chased for 15 or 60 min with unlabeled medium. Immediately after pulse-labeling, the majority of labeled gB existed as a monomeric precursor at a position of approximately 100 kDa (Fig. 6-4, fraction 11). After a 15 min chase, some of the monomeric precursor shifted to a position of approximately 200 kDa, where the completely glycosylated gB was also apparent (fractions 6 and 7). After a 60 min chase, most labeled gB was present as a dimer at the 250 kDa position. This result is consistent with a previous study of PRV (Whealy et al., 1990), which showed that oligomers formed shortly after the polypeptide was synthesized, the dimerized precursor was transported to the Golgi and glycosylated to become the mature protein which remained oligomerized (Fig. 6-4, fraction 6, 7, 8). Our data also indicate that after glycosylation in the Golgi, the protein could be cleaved and the different subunits were held together by disulfide bonds (data not shown).
Fig. 6-3. Cross-linking of BHV-1 gB with ethylene glycolbis(succinimidy1succinate) (EGS). Radiolabeled wt BHV-1 infected MDBK cells were treated with different concentrations of EGS at 4°C for 1 h. After lysis with RIPA, the samples were immunoprecipitated by the gB-specific monoclonal antibody pool and separated on a SDS-5% polyacrylamide gel under non-reducing conditions. The molecular mass markers (kDa) are shown on the right. D: dimer. M: monomer. P: precursor.
Fig. 6-4. Sucrose gradient sedimentation study of BHV-1 gB. Wild-type BHV-1 infected MDBK cells were pulse-labeled with 200 μCi/ml [35S]-Met/Cys for 10 min at 6 h post-infection, then chased for different intervals. The radiolabeled cells were washed in PBS, and lysed in 0.3 ml lysis buffer on ice for 30 min. The samples were layered onto 5 ml continuous 5–20% (W/V) sucrose gradients prepared in lysis buffer and centrifuged for 16 h at 240,000 g (45,000 rpm). After centrifugation, 15 fractions were collected from the bottom (fraction 1 is the first bottom fraction). All the fractions were subjected to immunoprecipitation and the precipitates were separated on a SDS-7.5% polyacrylamide gel. Protein markers were run in parallel and included: ferritin (440 kDa), catalase (250 kDa), hexokinase (100 kDa). Fractions containing the protein markers are indicated with arrows. The different chase intervals (min) are indicated on the left and the molecular mass markers (kDa) are shown on the right.
6.3.4 Sucrose gradient sedimentation of truncated gB derivatives and oligomerization domain of BHV-1 gB.

We demonstrated that BHV-1 gB forms a dimer by both chemical cross-linking and sucrose gradient centrifugation (Fig. 6-3 and 6-4). In order to find the structural requirement for the high-affinity binding site of gB, we compared the oligomerization status of authentic gB and the recombinant derivatives. BHV-1 infected MDBK cells and gB expressing MDBK cells were pulse-labeled for 10 min, then chased for 15 or 60 min. From the result in Fig. 6-2, it is evident that at 60 min, a fair amount of the gB products was still intracellularly located. Therefore, cells were collected for sucrose gradient centrifugation after 15 and 60 min chase periods. The result using authentic gB (Fig. 6-5, BHV-1 gB) was similar to the result in Fig. 6-4, in that the monomeric precursor was oligomerized into a dimer, and then became the fully glycosylated dimer. The gBt and gBtM expressing cells had similar patterns in that after a 60 min chase, both glycosylated gB products were found in fraction 7 as a dimer, whereas the monomeric precursor forms were in fraction 11. However, in the gBb expressing cells, a different pattern was observed, which showed that the fully glycosylated product remained in the same fractions as the monomeric precursor form (Fig. 6-5, gBb, fraction 11-13), suggesting that gBb did not form an oligomeric structure. Comparison of the ability of gBt and gBb to form oligomers
Fig. 6-5. Sucrose gradient sedimentation study of truncated gB derivatives. The study of wt BHV-1 gB (BHV-1 gB) was described in the legend of Fig. 6-4. All the truncated gB expressing cells were heat-shocked for 4 h at 43°C, followed by starving and pulse-labeling for 10 min. The samples were processed as described in Fig. 4, and the odd numbered fractions were subjected to immunoprecipitation.
would suggest that the fragment between amino acids 506 to 763 is important to BHV-1 gB oligomerization.

The relatively poor resolution of some of the protein bands and the appearance of gB in higher molecular weight fractions in this experiment may be due to differential glycosylation or the association of gB with other unknown components, which have been suggested in previous studies (Roberts et al., 1991; Whealy et al., 1990).

6.3.5 Recognition of the recombinant gB products by monoclonal antibodies.

Previously, a panel of gB-specific monoclonal antibodies has been established and characterized (van Drunen Littel-van den Hurk et al., 1984; van Drunen Littel-van den Hurk and Babiuk, 1985, 1986a; Fitzpatrick et al., 1990a). Among these antibodies, some can only recognize epitopes following proper glycosylation or folding, which can be used to identify the proper conformation of the proteins. In order to compare authentic gB with the recombinant forms of gB, ten gB-specific monoclonal antibodies were used to precipitate the different gB products. Of the ten monoclonal antibodies, one antibody, 1B10, which recognizes epitope I of gB, had dramatically different reactivities with different forms of gB (Fig. 6-6). This antibody reacted with authentic gB, whereas it failed to react with the recombinant forms of gB (Fig. 6-6). An ELISA test further suggested that the
Fig. 6-6. Immunoprecipitation of truncated gB derivatives with individual gB-specific monoclonal antibodies. The wt BHV-1-infected MDBK cells (gB) and the truncated gB expressing cells were radiolabeled. Samples of cell lysate and medium were combined and immunoprecipitated with individual gB-specific monoclonal antibodies. The antibody's names and their corresponding epitopes are indicated on top. On the left the different gB derivatives are indicated.
Fig. 6-7. ELISA of reactivities of gB derivatives with monoclonal antibodies 1E11 and 1B10. The affinity-purified gB derivatives were serially diluted and coated to microtitre plates. A 1:10 dilution corresponded to 0.04 µg protein per well. After overnight coating at 4°C, gB-specific monoclonal antibodies 1E11 or 1B10 were used in the detection. The antigen reactivity titers are the reciprocals of the highest dilution of gB products which show positive ELISA values (at least 0.1 at OD405nm) (Kolwalski et al., 1993).
differences in reactivity of 1B10 with the recombinant forms of gB and authentic gB were not quantitive, but qualitative (Fig. 6-7). This epitope has previously been localized between residues 744 to 763 (Fitzpatrick et al., 1990a), which are present in both gBt (1-763) and gBtM (1-807). However, none of the recombinant forms of gB in this study were recognized by monoclonal antibody 1B10. Therefore, the inability of this antibody to recognize gBt and gBtM was probably caused by improper folding of the recombinant forms of gB.

Previously, mapping of gB epitopes was done by expression of truncated forms of gB in murine cells, followed by immunocytochemical staining (Fitzpatrick et al., 1990a). The cytoplasmic domain was included in all of these previously truncated forms of gB. In order to confirm that 1B10 is not located in the cytoplasmic region, we expressed gBd(486-829) in MDBK cells, followed by immunoprecipitation analysis (Fig. 6-8). This truncated gB has a deletion from amino acids 486 to 829, contains most of gBb (residues 1-485) and the entire cytoplasmic region (residues 830-933). After immunoprecipitation, a 80 kDa protein was recognized by the gB-specific monoclonal antibody pool, but not by 1B10, which confirms that epitope I is not located in regions 1-485 or 830-933.
Fig. 6-8. Immunoprecipitation of transiently expressed gBd(486-829). A truncated gB was constructed by deleting residues 486 to 829 and expressed under the control of a Rous sarcoma virus promoter (Fitzpatrick et al., 1988; 1990b). MDBK cells were transfected with this expression vector using the DEAE-dextran method as reported by Pereira et al. (1989). The cells were labeled for 16 h after 40 h transfection, and the cells and media were collected and reacted with the gB-specific monoclonal antibody pool or 1B10. BHV-1 gB is the sample of radiolabeled wt BHV-1 infected MDBK cells. The arrow on the left indicates gBd(486-829). The molecular mass markers (kDa) are shown on the right.
6.3.6 Inhibition of BHV-1 adsorption by recombinant forms of gB.

We previously demonstrated that BHV-1 gB binds to two types of receptors on permissive MDBK cells, the HS receptor and non-HS high-affinity binding receptor (Chapter 4.0). The binding of gB to the high-affinity, non-HS receptor is important for the infectivity of BHV-1. Although this receptor remains currently unknown, it can be blocked by authentic gB and thereby infection with BHV-1 is blocked (Liang et al., 1991; Chapter 4.0).

In this study, we expressed different recombinant forms of gB to study the structural requirements for the high-affinity binding site of gB. It is believed that the primary structure of this site consists of the extracellular part of gB. Previously, gBt was found to have no such site, and it also failed to block BHV-1 plaque formation (Chapter 4.0). In order to produce the entire potential ectodomain of gB, gBtM was expressed in the same expression system. Like the recombinant gBt, gBtM was also a secreted glycoprotein, with structural properties similar to authentic gB. To assess the existence of the high-affinity binding site, the affinity-purified protein was used in a BHV-1 plaque inhibition assay. Before the proteins were used in such an assay, their oligomerization status was examined by sucrose gradient centrifugation. It was found that with the exception of gBb, all the other gB products, gB, gBt, and gBtM,
Fig. 6-9. Sucrose gradient study of affinity-purified gB, gBt, gBtM, and gBb. For each affinity-purified gB derivative, 10-20 μg in 0.2 ml were layered onto 5 ml continuous 5-15% sucrose gradients. After centrifugation, 10 fractions were collected from the bottom. The protein mass makers used in a parallel centrifuge tube were ferritin (440 kDa), catalase (250 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (67 kDa). About 50 μl samples from fractions 1 to 9 were separated on SDS-7.5% polyacrylamide gels, then transferred to nitrocellulose and probed with a gB-specific monoclonal antibody pool. The fractions and their corresponding molecular markers (kDa) are indicated on top. The gB derivatives are indicated on the right and the protein mass markers are indicated on the left (kDa).
Fig. 6-10. Effect of gB derivatives in blocking BHV-1 plaque formation. Confluent MDBK cells grown in 24-well plates were cooled at 4°C and then incubated with 50 μl of a gB product at the indicated concentration per well at 4°C for 1 h. After incubation, 50 μl of diluted wt BHV-1 (about 150 PFU) were added to each well of cells in the presence of the proteins, followed by an additional 1 h incubation at 4°C. After adsorption, the plates were washed three times with MEM and overlaid with MEM containing 1% methylcellulose and 2% FBS. The plaques were stained with crystal violet and counted three days later. The number of viral plaques formed in the absence of gB products was defined as 100% plaque formation and used as a reference for calculating the percentage of viral plaques formed in the presence of gB derivatives. The data represent means plus standard deviations for triplicate samples. The different gB derivatives are indicated on the right.
remained as oligomeric forms after purification ([Fig. 6-9]). This result provides evidence that gB in BHV-1 virion exists predominantly as dimers, since the authentic gB was purified from BHV-1 virions. Results of the plaque inhibition assay showed that only authentic gB blocked BHV-1 plaque formation, whereas all the truncated forms of gB failed to inhibit BHV-1 plaque formation ([Fig. 6-10]). Therefore, the truncated gB derivatives do not have the high-affinity binding site.

6.4 DISCUSSION

Previously, it has been found that recombinant BHV-1 gB was functional, causing spontaneous fusion in the absence of other viral components ([Fitzpatrick et al., 1988; 1990a]). The protein binding study also suggested that the existence of the high-affinity binding site on gB is not dependent on the presence of other viral proteins (Chapter 4.0). In order to study the function of gB in BHV-1 infection, especially the role of the high-affinity binding site, we independently expressed different truncated gB derivatives in mammalian cells. By expressing mutant forms of gB, it was possible to study its structural and functional properties without constructing viral mutants. This strategy has been used in studying HSV-1 and HCMV gB ([Navarro et al., 1993; Tugizov et al., 1994; 1995]).

In our previous studies, we expressed the
extracellular portion of gB, gBt, and the gB large subunit, gBb, in the bovine hsp70A gene promoter-based expression system (Chapters 3.0, 4.0, and 5.0). Both proteins were fully processed and secreted, and retained structural and antigenic properties similar to authentic BHV-1 gB. They all possess heparin-binding properties like authentic gB and the heparin-binding domain(s) was localized to the N-terminal subunit, gBb (Chapter 5.0). However, the recombinant gBt, which contains the majority of the ectodomain of gB does not have the high-affinity binding site. To study the structural requirement for such a site in gB, we further expressed the entire potential ectodomain of BHV-1 gB, gBtM. This truncated glycoprotein contains amino acids 1-807 of gB, which terminates in front of the last segment (residues 808-828) of the transmembrane anchor region. As shown in Fig. 6-2, both gBt and gBtM were efficiently secreted. This result provides evidence that BHV-1 gB may use segment 3 of the transmembrane region as its major anchor to target and retain gB in the membrane (Rasile et al., 1993; Reschke et al., 1995). Therefore, gBtM may represent the entire ectodomain of BHV-1 gB as we expected.

Since membrane proteins require oligomerization and correct folding for transport through the exocytosis pathway (Doms et al., 1993), we compared the oligomeric status of all the recombinant forms of gB. Like most herpesviruses, BHV-1 gB was found to be oligomerized (Fig. 6-3, 6-4, 6-5). The
cytoplasmic and membrane anchor regions do not appear to be required for the oligomerization of BHV-1 gB, which is consistent with reports on HSV-1 (Ali, 1990; Navarro et al., 1993; Qadri et al., 1991). In HSV-1 gB, two regions of the ectodomain, amino acids 93-282 and 596-711, have been found to be necessary for dimer formation (Highlander et al., 1991). Others confirmed that the region between amino acids 600-690 was important for dimerization (Ali, 1990; Navarro et al., 1993; Qadri et al., 1991). In this study, by comparing different truncated gB derivatives, we found that a fragment between amino acids 506 to 763 is required for BHV-1 gB oligomerization. The potential oligomerization domain(s) may be located in this region or it may provide a conformational requirement for oligomer formation. A recent study provided strong evidence that in HSV-1 gB, a 28-amino acid domain, residues 626 to 653, is responsible for gB oligomerization (Lanquerre et al., 1996). Taken together, this suggests that in BHV-1 gB, an oligomerization domain may be located in the region between residues 506 to 763.

From our previous results and current data, we can propose a pathway for maturation of BHV-1 gB. The newly synthesized gB precursor quickly forms an oligomer in the endoplasmic reticulum, which is transported to the Golgi for further glycosylation and then proteolysis. gBt and gBtM are processed in a similar way and are structurally similar to authentic gB. Both recombinant gB products form oligomers,
however, unlike authentic gB, they do not have the high-affinity binding site. The most likely reason is that the cytoplasmic domain is important in modulating the conformation of gB. It is believed that the conformation of gB can be influenced by many factors at every step of the export pathway of gB. This contention is supported by one of the gB-specific monoclonal antibodies, 1B10, which is located at the C-terminal end, and has been previously proven to be important for fusion activity of gB (Fitzpatrick et al., 1990a; van Drunen Littel-van den Hurk et al., 1992). The epitope recognized by 1B10 has been localized to a segment of gB between amino acids 744 to 763 (Fitzpatrick et al., 1990a). This region represents a major hydrophilic peak in the extracellular region of the carboxy-terminal fragment of gB. Both gBt and gBtM contain this epitope. However, these gB products are not recognized by this antibody in immunoprecipitation or ELISA. Based on previous epitope mapping, we further excluded the possibility that epitope I might be located in the cytoplasmic region by expression and detection of gBd(486-829) in MDBK cells (Fig. 6-8). This gives further support to our contention that epitope I may be conformation-dependent and was lost from truncated gB products which have no cytoplasmic region. Although the entire cytoplasmic domain and the third segment of the putative transmembrane region are all missing from gBtM, further studies showed that inclusion of the third segment in
recombinant gB was still not able to reconstitute epitope I (Chapter 7.0). Therefore, we conclude that the cytoplasmic region is a major contributor to the local conformation around epitope I. For the present study, we only have one monoclonal antibody recognizing the C-terminal subunit, gBc, therefore, more reagents are required for further characterization of gB conformation, especially in the juxtamembrane region.

In general, the cytoplasmic domain of viral glycoproteins may have a signal that influences their targeting to vesicular carriers and the transport of glycoproteins through the exocytosis pathway (Rose and Doms, 1988). In vesicular stomatitis virus G glycoprotein and paramyxovirus hemagglutinin-neuraminidase, the mutations in the cytoplasmic domain can slow or prevent protein transport from the ER to the Golgi (Guan et al., 1988; Park and Lamb, 1990). The cytoplasmic region in HSV-1 and HCMV gB have also drawn much attention because of the potential impact on the function of gB (Navarro et al., 1993; Tugizov et al., 1995). As a hydrophilic region with more than 100 amino acids, this part has been shown, by flow cytometry staining, to be inside the cells (Basgoz et al., 1992). However, this particular region also plays an essential role in the function of gB. In HSV-1 gB, although some C-terminal residues (about 40 amino acids) are functionally dispensable (Huff et al., 1988), the cytoplasmic region is found to be essential for the role of
gB in membrane fusion. Truncation after residue 851 causes failure of the protein’s fusion function (Cai et al., 1988). Within the cytoplasmic region, many syncytium mutations have been identified (Bzik et al., 1984; Cai et al., 1988; Engel et al., 1990; Tugizov et al., 1994; 1995). Furthermore, a spontaneously mutated HCMV gB, which had a termination codon at aa 669 (in the ectodomain) was found to have decreased fusion activity in transfected cells (Tugizov et al., 1995). All these data suggest that the cytoplasmic region is involved in the function of gB. Since the cytoplasmic region of gB may not actually be exposed to the extracellular environment, it may function indirectly by facilitating the proper folding of a functional fusion domain and maintaining gB’s high-affinity binding site.

There are several proposed mechanisms for the relationship between the cytoplasmic region of gB and its function. In alphaherpesviruses, gB sequence alignment shows the existence of two conserved alpha-helical regions in the cytoplasmic domain. Syncytium mutations can be found in these regions when residues are altered (Gage et al., 1993), suggesting that these regions may provide a signal for the conformation and function of gB. It has also been suggested that a down-regulating domain in the cytoplasmic region of HSV-1 gB may interact with other HSV-1 proteins to control fusion activity (DeLuca et al., 1982; Cai et al., 1988). Another proposed mechanism is that the cytoplasmic region of
gB may send a signal, after being phosphorylated, which may change the protein conformation or local hydrophobic properties (Tugizov et al., 1995). However, at this moment, the function of the cytoplasmic domain of gB is far from clear. Our results suggest that this region may be important for maintaining the conformation of gB as well as the integrity of the high-affinity binding site on this molecule. Although they form oligomers, both gBt and gBtM products are conformationally different from authentic gB, possibly resulting in the loss of the high-affinity binding site.

The high-affinity binding site of BHV-1 gB was previously found to bind to a specific non-HS receptor on MDBK cells, which is a critical event in BHV-1 infection (Chapter 4.0). In a productive infection, the attached BHV-1 can penetrate the cell membrane and gain entry to the host cell. During these processes, although other viral and cellular components may be involved, gB and gD are essential for membrane fusion and fusion modulation respectively (Fitzpatrick et al., 1988; 1990; Liang et al., 1995). Our studies further suggest that gB can function as a fusion protein, and therefore plays a key role in penetration (Chapter 7.0). However, gB induced fusion may be controlled by a complex series of events. We speculate that during virus entry, in order to achieve the correct configuration for inducing fusion, gB has to bind to the non-HS receptor via its high-affinity binding site. Therefore, the blockage of
high-affinity binding may preclude the fusion process, which in turn may block virus infection. Without the cytoplasmic region, the truncated forms of gB can not induce the correct conformation of the high-affinity binding site. Thus they can not block the corresponding receptor on MDBK cells.
7.0 FUNCTIONAL ANALYSIS OF THE TRANSMEMBRANE ANCHOR REGION OF
BOVINE HERPESVIRUS 1 GLYCOPROTEIN B

7.1 INTRODUCTION

Entry of enveloped viruses into host cells requires fusion of the viral envelope with the host cell membrane, which is mediated by viral fusion proteins in the envelope (White 1990, 1992). Among the thirteen families of enveloped RNA or DNA viruses, eight have been found to have membrane fusion proteins (White 1990). The best example is hemagglutinin of influenza virus, which undergoes a conformational change to expose the fusion domain and fuse the membrane under low pH condition (Bentz et al., 1993). Such pH-dependent fusion can also been found in Rhabdoviridae, Alphaviridae, and Flaviviridae (Stegmann et al., 1989). In some other virus families, including Coronaviridae, Paramyxoviridae, and Retroviridae, the fusogenic glycoproteins can mediate fusion at neutral pH (White 1990). By fusing the viral envelope with the plasma membrane of host cells, viruses may release its genome into the cells, and initiates infection.

The Herpesviridae is a large family of DNA viruses,
which can cause diseases in humans and different animals. Currently, the entry pathway for many herpesviruses is found to be similar in that the virus first binds to heparan sulfate proteoglycans (HS) on permissive cells. This initial interaction is mediated by glycoproteins gC and/or gB, which is followed by sequential interactions between viral glycoproteins and the corresponding cellular receptors (for a comprehensive review, see Spear 1993). Through cooperative interactions between various virus and host cell components, the virus fuses with the cell membrane in a pH-independent way and releases the nucleocapsids into the cytoplasm of the cell (Spear 1993). It has been found that gB, gD, gH, gK, and gL are essential for herpes simplex virus type 1 (HSV-1) infection (Cai et al., 1988; Forrester et al., 1992; Fuller et al., 1989; Hutchinson et al., 1995; Ligas and Johnson 1988; Roop et al., 1993). These glycoproteins appear to be involved both in viral attachment and/or penetration. In mutant viruses depleted of one of these glycoproteins, virions still attach to permissive cells, but do not enter cells. Since the attached virions can gain entry into cells in the presence of the chemical fusogen, polyethylene glycol, it has been suggested that these glycoproteins are involved in fusion activity (Fuller et al., 1989; Fuller and Spear 1987). Direct evidence of fusogenic glycoproteins in herpesviruses comes from virus mutants and the expression of viral glycoproteins in mammalian cells. In HSV-1, mutations
in gB and gK can generate syncytium mutants (Bzik et al., 1984; Gage et al., 1993; Highlander et al., 1989, Hutchinson et al., 1992), but gK is believed to be a fusion inhibitor, which modulates fusion, rather than a real fusion protein (Hutchinson et al., 1992, 1993, 1995, 1995a). Expression of gB and gD in mammalian cells shows that they can cause cell fusion (Ali et al., 1987; Campadelli-Fiume et al., 1988; Tikoo et al., 1990), however, later studies challenged the fusogenic function of gD (Fehler et al., 1992; Liang et al., 1995; Peeters et al., 1992). Therefore, the only confirmed fusogenic glycoprotein so far is gB. This is a dominant glycoprotein in the herpesvirus envelope, and also belongs to the most conserved family of herpesvirus glycoproteins (Pereira 1994). The gB homologues in at least some herpesviruses have been shown to be functionally dispensable (Kopp and Mettenleiter 1992; Mettenleiter and Spear 1994; Rauh et al., 1991). The conservation of gB in all herpesviruses suggests that gB plays an important role in viral infection, which is most likely related to its distinct fusion activity. Studies on the fusogenic domain of gB have been mostly carried out by syncytium mutations and monoclonal antibodies (Britt et al., 1988; Bzik et al., 1984; Highlander et al., 1989, 1988; Kousoulas et al., 1988; Navarro et al., 1992, 1993; Pereira et al., 1984; Tugizov et al., 1994). However, there is no direct evidence for its existence on the gB ectodomain (Pereira 1994).
Bovine herpesvirus 1 (BHV-1) is a member of the alphaherpesviruses, and a viral pathogen for cattle. There are at least nine membrane-associated glycoproteins in BHV-1 (Bolton et al., 1983; Fitzpatrick et al., 1989; Khadr et al., 1996; Khattar et al., 1995; Leung-Tack et al., 1994; Marshall et al., 1986; Meyer et al., 1991; Misra et al., 1981, 1988; Tikoo et al., 1990; Whitbeck et al., 1988). Among them, three major glycoprotein, gB, gC and gD, have been found to mediate virus entry (Chapter 4.0; Liang et al., 1991). During BHV-1 infection, binding of gC to cellular HS may be the dominant force to bring virions and cells together, with gB initially binding to HS and then to a high-affinity non-HS receptor, followed by gD binding to a non-HS receptor (Chapter 4.0; Liang et al., 1993; Okazaki et al., 1991). The result of the interactions between viral glycoproteins and cellular receptors is that viruses gain entry by membrane fusion. Previously, it has been found that gB-specific antibodies possess post-adsorption neutralization activity which block penetration (Dubuisson et al., 1992) and expression of gB in transfected cells can induce syncytium formation caused by membrane fusion (Fitzpatrick et al., 1988, 1990; van Drunen Littel-van den Hurk et al., 1992). To gain insight into gB’s fusion activity, we expressed different gB derivatives in Madin Darby bovine kidney (MDBK) cells.

BHV-1 gB is a type I integral membrane glycoprotein which consists of 933 (or 932) amino acids, including a
putative signal peptide sequence between residues 1-67 and the transmembrane domain between residues 760-828 (or 759-827), which is followed by a 104 amino acid cytoplasmic tail (van Drunen Littel-van den Hurk et al., 1992; Whitbeck et al., 1988; Zamb 1987). A centrally located cleavage site divides the molecule into two parts, gBb and gBc. The N-terminal subunit gBb, has been shown to be required for the heparin-binding activity of gB (Chapter 5.0). Based on protein sequence analysis of the C-terminal gBc subunit, we attempted to delineate the function of the three potential membrane-spanning segments in the transmembrane region. We constructed stable cell lines expressing different gB derivatives, and characterized the recombinant gB products in the cells. The results from this study provide experimental evidence that residues 785-805 constitute the fusogenic domain for BHV-1 gB.

7.2 MATERIALS AND METHODS

7.2.1 Virus, cells and reagents.

The BHV-1 Cooper strain was obtained from the National Veterinary Services Laboratories, Ames, Iowa, and was propagated in Madin Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO). Lipofectin and G418 were from GIBCO; Tran[35S]-
label, which includes radiolabeled methionine and cysteine (Met/Cys), was from ICN (Mississauga, Ontario).

7.2.2 Construction of transfer vectors and establishing transfected cell lines.

By conventional gene manipulation techniques, we constructed different transfer vectors which have amber mutations in different locations around the putative transmembrane anchor region (residues 760-828). The plasmid p3KHSPG4HU (Kowalski et al., 1993), which contains a truncated BHV-1 gD gene under the control of the bovine heat-shock protein hsp70A gene promoter and the aminoglycoside phosphotransferase gene under the control of the SV40 early promoter, was used as the parental plasmid for constructing transfer vectors. Fig. 7-1 depicts the sequences of authentic gB and recombinant gBt, gBtM, gBtDAF, and gBtMA. Three truncated gB derivatives, gBt, gBtM, and gBtMA, stop in the beginning of segment 1, at the end of segment 2, or at the end of segment 3 of the transmembrane region respectively. Another recombinant gB, gBtDAF, was derived by fusing a 41-amino acid fragment from the human decay-accelerating factor (hDAF) carboxy tail to the C-terminus of gBt. The DAF fragment can provide a signal for the addition of a glycosyl phosphatidylinositol (GPI)-based membrane anchor for the gBtDAF chimeric protein.

To establish gB expressing cell lines, approximately
4 x 10⁶ MDBK cells were transfected with 5 µg of the transfer vector using Lipofectin (GIBCO); after transfection, the cells were grown in the presence of 666 µg/ml of G418. The G418-resistant cells were screened for gB production by an immune dot blot assay using gB-specific monoclonal antibodies (Kowalski et al., 1993). The positive cells were isolated and subjected to single cell cloning.

7.2.3 [³⁵S] labeling and immunoprecipitation.

To label BHV-1 glycoproteins, confluent MDBK cells were infected with wild-type BHV-1 at a MOI of 5 for 1 h at 37°C, then incubated with MEM containing 5% FBS at 37°C for 5 h. After starving the cells in Met/Cys free MEM (GIBCO) for 30 min, cells were labeled in 50 µCi/ml of [³⁵S]-Met/Cys in Met/Cys free MEM with 2% dialysed FBS at 37°C for 8 h. The cells were collected and washed in phosphate buffered saline (PBS, [pH 7.2]), then lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.02% NaN₃) on ice for 30 min. After centrifugation at 15,000 g for 30 min, the cell lysate was used as the labeled BHV-1 glycoprotein fraction.

To label gB products from the transfected MDBK cells, cells were first heat-shock treated (43°C, 4 h), then starved and labeled as above. After labeling, the cells and supernatants were harvested separately for
immunoprecipitation assays.

In the immunoprecipitation assay, samples were incubated for 1 h at room temperature with an appropriate mixture of monoclonal antibodies (van Drunen Littel-van den Hurk et al., 1992), or with specific individual monoclonal antibodies, followed by incubation with protein A Sepharose for 1 h. After 5 washes with RIPA, the precipitated proteins were dissociated by boiling in 50 μl of sample buffer (62.5 mM Tris-HCl [pH 6.8], 1.25% SDS, 12.5% glycerol, 0.001% bromophenol blue). All the samples were loaded onto SDS-7.5% polyacrylamide gels. Following electrophoresis, the gels were fixed for 30 min, then soaked in Amplify (Amersham, Oakville, Ontario) for 30 min, dried under vacuum, and exposed to X-ray film at -70°C.

7.2.4 Immunofluorescence assay.

MDBK cells or recombinant gB expressing MDBK cells were grown on glass chamber slides (Nunc Inc., Naperville, Ill.) to subconfluence. MDBK cells were infected with BHV-1 at a MOI of 0.1 for 12 h, whereas the recombinant gB expressing MDBK cells were treated at 43°C for 4 h then returned to 37°C for 8 h. After washing with cold PBS containing 0.25% bovine serum albumin (BSA) and 0.25% goat serum, the cells were fixed with 2% paraformaldehyde at room temperature for 15 min for surface staining, or further permeabilized with methanol at -20°C for 15 min for
intracellular staining. Cells were washed and blocked with PBS containing 2% BSA and 2% goat serum for 30 min, then incubated with gB-specific monoclonal antibody pool at a dilution of 1:500 for 1 h at room temperature. The second antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Beckton-Dickinson, Mountain View, CA), which was used at a dilution of 1:80 and incubated with the cells for 30 min in the dark. The cells were washed, desalted and dried, then mounted in citifluor glycerol (Ted Pella, Inc., Redding, Calif.), and examined with the aid of a fluorescence microscope.

7.2.5 Pulse-chase analysis of protein transport.

BHV-1 infected or gB-expressing MDBK cells grown in 6-well plates were used in this study. For BHV-1 infected cells, pulse-chase labeling was conducted at 6 h post-infection. For gB-expressing cells, a heat-shock treatment was required before labeling. After starving for methionine and cysteine in Met/Cys free medium at 37°C for 30 min, cells were pulse-labeled at 37°C for 10 min by the addition of 200 μCi of [35S]-Met/Cys per ml of medium. Supernatants were removed and cells were further incubated in Opti-MEM (GIBCO) containing 2 mM methionine and cysteine. At indicated time points, supernatant and cells were collected separately, and samples were precipitated with a gB-specific monoclonal antibody pool and separated on SDS-7.5% polyacrylamide gels.
under non-reducing conditions.

7.2.6 Flow cytometric analysis of gBtDAF surface expression.

Subconfluent gBtDAF expressing cells in T-75 flasks were heat-shocked at 43°C for 4 h, then incubated at 37°C for 6 h. The cells were carefully trypsinized and washed in MEM containing 2% heat-inactivated FBS and 0.05% sodium azide (washing solution). About 10⁷ cells were resuspended in 1 ml MEM. Subsequently, half of the cells was treated with 5 µg phosphatidylinositol-specific phospholipase C (PI-PLC) at 37°C for 1 h and the other half was used as an untreated control. After PI-PLC treatment, samples were washed with MEM, followed by PBS containing 0.2% gelatin and 0.03% sodium azide, and then resuspended in PBS. Each cell sample was divided into two parts. One part was incubated with a 1:1000 diluted gB-specific monoclonal antibody pool at 4°C for 1 h, followed by a 30 min incubation with a 1:50 diluted FITC-conjugated goat anti-mouse IgG at 4°C for surface staining; the other sample was only incubated with secondary antibody to measure background staining. The cells were washed and fixed with 2% paraformaldehyde, then analyzed with a Coulter Electronics Ltd. EPICS CS system Flow Cytometer.

7.2.7 Sucrose density gradient centrifugation.

To assess the oligomerization status of the
recombinant gB proteins, we performed sucrose density centrifugation essentially as described by Whealy et al. (1990). One T-25 flask of radiolabeled cells was washed in cold PBS, and lysed in 0.3 ml lysis buffer (50 mM Tris, [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100) on ice for 30 min, then pelleted for 15 min at 4°C. The supernatant was layered onto 5 ml continuous 5-20% (W/V) sucrose gradients in lysis buffer. The centrifugation was for 16 h at 240,000 g in a SW50.1 rotor at 4°C (Beckman Inc. Mississauga, Ontario). A parallel tube with protein mass markers was included in the centrifugation. After sedimentation, 15 fractions were collected from the bottom. The entire sample in each fraction was subjected to a immunoprecipitation assay. The protein mass markers in this study were ferritin (440 kDa), catalase (250 kDa), and hexokinase (100 kDa) (Sigma, St. Louis, MO).

7.2.8 Sequence analysis.

The PC/GENE software (Release 6.70, IntelliGenetics Inc., Switzerland) was used to analyze protein sequences.

7.3 RESULTS

7.3.1 Expression of different derivatives of BHV-1 gB in MDBK cells.

The purpose for constructing different truncated gB derivatives was to express gB with different C-termini and to
study the function of those segments in the transmembrane region. From the sequence analysis of BHV-1, there appears to be a long transmembrane helix from residues 760 to 828, which can traverse the membrane three times. The hydrophobicities of the three potential membrane spanning segments have been calculated by Eisenberg’s method (1984) to be 0.37, 0.66, and 0.78, respectively. Therefore, segments 2 and 3 have the potential to span the membrane. In order to study the fusion property of gB, gBtM and gBtMA, which represent residues 1-807 and 1-829 and may use segments 1 and 2 or segment 3 as their membrane anchors, respectively, were expressed (Fig. 7-1). A previously expressed gBt containing residues 1-763 of gB was used as a control in the present study (Chapters 4.0 and 5.0).

Since recombinant gBt represents most of the ectodomain outside the transmembrane region, we constructed a new product, gBtDAF, to try to anchor this protein on the membrane of the expressing cells. A carboxy tail of the hDAF fragment, which contains 41-amino acids, was fused to the C-terminus of gBt (Fig. 7-1). This hDAF fragment contains a signal for the addition of a glycosyl phosphatidylinositol (GPI)-based membrane anchor (Medof et al., 1986), which helps to target the chimeric gBtDAF to the cell surface (Caras et al., 1987; Liang et al., 1995).
Fig. 7-1. Schematic diagram of BHV-1 gB and the truncated derivatives. BHV-1 gB is represented as a box structure where the signal sequence (S) and transmembrane anchor region (TMA) are shaded. Amino acid sequences for both termini of the truncated forms of gB are presented with the corresponding residue number. A fragment of 41 amino acids of the human decay accelerating factor carboxyl tail is indicated in the open box. The names of the truncated gB derivatives are listed on the left.
Expression of recombinant gB derivatives was analysed by immunoprecipitation (Fig. 7-2). It was found that the expression of gB products can be enhanced by heat-shock treatment (data not shown), indicating that the recombinant gB genes were under the control of the hsp70A promoter as expected. Fig. 7-2 shows the immunoprecipitated gB products under reducing conditions. The authentic gB exists as a 130 kDa heterodimer, which contains a 74 kDa N-terminal subunit, gBB, and a 55 kDa C-terminal subunit, gBC, generated by proteolysis. The subunits are linked by intramolecular disulfide bonds to form an intact molecule, whereas some gB remains uncleaved (Fig. 7-2, van Druenen Littell-van den Hurk and Babiuk 1986). Cleavage also occurred in all recombinant forms of gB such that they all have the N-terminal subunit which is in the same position as the authentic gBB, with an apparent molecular weight of 75 kDa (lane gB). The cleaved C-terminal subunits for gBT, gBTM, gBTDAF, and gBTMA can be identified on the gel at different positions with molecular weights of 38 kDa, 42 kDa, 43 kDa, and 44 kDa respectively (lanes gBT, gBTM, gBTDAF, gBTMA). The reduced molecular weight of each of the recombinant forms of gB was attributed to the truncation of its C-terminal region. As with authentic gB, a portion of each recombinant gB remained as an uncleaved product. These results suggest that the different versions of the truncated gB derivatives are properly expressed in their respective cell lines.
Fig. 7-2. Immunoprecipitation of BHV-1 gB and the truncated derivatives. After labeling with $[^{35}S]$-Met/Cys, the labeled cells (C) and the media (M) were collected separately and precipitated with a gB-specific monoclonal antibody pool. The samples were separated on a SDS-8.5% polyacrylamide gel under reducing conditions. Lane gB is the sample from the wild-type (wt) BHV-1-infected MDBK cells; lanes gBt, gBtM, gBtDAF, and gBtMA are samples from transfected MDBK cell lines which express gBt, gBtM, gBtDAF and gBtMA respectively. Molecular mass markers (M.W., kDa) are shown on the right.
In this experiment, we also compared the samples of cell lysate (C) and medium (M). We observed that after overnight labeling, most of the gBt and gBtM was secreted (Fig. 7-2). In contrast, cells expressing gBtDAF exhibited more cell-associated recombinant protein, indicating that the hDAF fragment helps gBt to target on the cell membrane. The background in lane gBtDAF was caused by over-exposure in order to match the other lanes. The expression of gBtMA showed a different pattern in that half of the expressed product consisted of a precursor form which was retained inside the cells.

7.3.2 Intracellular localization of gB derivatives by immunofluorescence staining.

In order to analyze the distribution of the recombinant forms of gB in MDBK cells, immunofluorescence staining was carried out (Fig. 7-3). All the cells including the BHV-1 infected MDBK cells were stained for surface (Fig. 7-3, left panel; capital letters) and intracellular expression (Fig. 7-3, right panel; small letters). In BHV-1 infected MDBK cells, there was strong surface (A), and perinuclear cytoplasmic staining (a), indicating that the proteins are glycosylated in the ER and the Golgi and the mature proteins are targeted to the cell surface. For gBt expressing cells, there was very weak surface staining (B), and very little amounts of perinuclearly distributed gBt (b),
which supports our contention that gBt is predominantly secreted. Results from gBtM expressing cells were similar to gBt expressing cells but the surface (C) and perinuclear staining (C) was slightly stronger than that in gBt cells, indicating that segments 1 and 2 may provide a weak function in protein retention on the membrane. For cells expressing gBtDAF, there was strong surface staining (D), whereas intracellular staining also indicates that most proteins are plasma membrane-associated (d), which suggests that protein transport from the ER to Golgi is very efficient. In cells expressing gBtMA, both surface and intracellular staining was strong (E, e), suggesting that the transport of gBtMA is being delayed whereas a small amount of gBtMA is still transported to and became associated with the cell membrane.

7.3.3 Maturation and transport of the recombinant forms of gB.

Immunoprecipitation and immunofluorescence studies suggested that gBt is secreted whereas gBtM, gBtDAF, and gBtMA are relatively cell-associated. To further characterize the transport kinetics, a pulse-chase experiment was performed. BHV-1 infected MDBK cells were included as a control, which was pulse-labeled at 6 h post-infection. The recombinant gB expressing cells were labeled after heat-shock treatment. After chasing with an excess amount of unlabeled Met/Cys for different intervals, cells and media were
Fig. 7-3. Cell surface and intracellular localization of authentic and recombinant gB derivatives by immunofluorescence. MDBK cells were infected with BHV-1 at an MOI of 0.1 for 12 h, whereas the recombinant gB expressing MD3K cells were heat-shocked for 4 h and then incubated at 37°C for 8 h. The cells were fixed with 2% paraformaldehyde for surface staining (left panel, marked with capital letters), or further permeabilized with methanol for intracellular staining (right panel, marked with small letters). After incubation with gB-specific monoclonal antibodies, the expressed gB products were visualized by incubation with FITC-conjugated goat anti-mouse IgG followed by fluorescence microscope examination. A,a: BHV-1 infected MDBK cells. B,b: gBt expressing cells. C,c: gBtM expressing cells. D,d: gBtDAF expressing cells. E,e: gBtMA expressing cells. n: mock-infected MDBK cells.
collected separately and subjected to immunoprecipitation with a gB-specific monoclonal antibody pool. In BHV-1 infected cells, after 10 min of labeling, a dominant, cell-associated 117 kDa protein was detected, which represents the high mannose oligosaccharide-containing gB precursor (Fig. 7-4, p) (van Drunen Littel-van den Hurk et al., 1985, 1989). This gB precursor was subsequently chased into a 130 kDa fully glycosylated mature gB (Fig. 7-4, M). After a chase period of 180 min, there were no precursor forms visible and all of the labeled protein was glycosylated and associated with the cells. Previously, we found that at 6 h post-infection, it takes about 50 min for half of the precursor to become mature protein (data not shown). A similar pulse-chase pattern was observed within gBt expressing cells. The gBt precursor had an apparent molecular weight of 90 kDa, which was chased into a 115 kDa mature gBt in 60-180 min (Fig. 7-4). Previous densitometer scanning results suggest that gBt has a similar conversion rate as authentic gB (data not shown). By 360 min of chase, all of the gBt was in the medium. In gBtM expressing cells, the gBtM precursor had an apparent molecular weight of 93 kDa, and the mature product had an apparent molecular weight of 120 kDa (Fig. 7-4). Its maturation was slower than that of gBt, such that at 180 or 360 min, there still were precursor forms in the cells. By 360 min of chase, although most of the mature gBtM was secreted, there still was a visible amount of precursor and
Fig. 7-4. Maturation and transport of truncated gB derivatives examined by pulse-chase analysis. MDBK cells were infected by wt BHV-1, and pulsed-labeled with 200 μCi/ml of [35S]-Met/Cys for 10 min at 6 h post-infection, then chased with unlabeled media for different intervals. The truncated gB expressing cells were heat-shocked before pulse-chase. After each chase interval, cells and media were collected separately and immunoprecipitated with a gB-specific monoclonal antibody pool. Wild-type gB and the truncated gB derivatives are identified on the left. Molecular mass markers (M.W., kDa) are shown on the right. M: mature product. p: precursor form. The chase intervals are indicated on top in min.
mature form left inside the cells (Fig. 7-4). This result suggests that gBtM may have a weak intracellular retention, which is caused by segments 1 and 2 of the hydrophobic carboxy tail. The gBtDAF protein showed the same pattern of maturation as authentic gB and gBt. After a chase period of 180 min, all of the precursor progressed to the mature form. However, the protein secretion pattern in gBtDAF cells was different from that in the gBt cells. By 360 min of chase, most labeled protein was still retained in the cells. A trace amount of labeled product could be found in the medium, which is consistent with previous reports using the hDAF derived GPI anchor (Caras and Weddell 1989; Liang et al., 1995). In gBtMA cells, a significant amount of the precursor form was retained inside the cells even at 360 min of chase. In these cells, only small amounts of precursor was transported to the Golgi and completely glycosylated. The overall transport of gBtMA was delayed. The mature gBtMA was secreted out of the cells, indicating that its membrane retention was not very stable without its cytoplasmic region.

7.3.4 PI-PLC treatment of gBtDAF expressing cells.

Many eukaryotic proteins are tethered to the plasma membrane by GPI membrane anchors (Cross 1987). To identify such proteins, the GPI cleavable enzyme, PI-PLC can be used to release the potentially GPI-anchored proteins. In our study, we fused a fragment from hDAF to the C-terminus of gBt.
to provide a signal for GPI anchoring. In the immunofluorescence study, we observed that gBtDAF expressing cells do have stronger surface staining than gBt or gBtM expressing cells (Fig. 7-3). The transport kinetic study also suggests that gBtDAF is a cell-associated protein (Fig. 7-4). In order to demonstrate that this chimeric protein is located on the cell membrane via the GPI anchor, we studied cell surface gB products before and after PI-PLC treatment by flow cytometric analysis. BHV-1 infected MDBK cells which showed no difference in surface staining before or after PI-PLC treatment were included as a control (data not shown), demonstrating that this enzyme does not remove authentic gB from the cell surface. However, treatment of gBtDAF cells with PI-PLC showed that it can remove some of the surface expressed gB, resulting in a decrease in surface staining as expected (Fig. 7-5). It is of interest to note that after PI-PLC treatment, there is only a small shift in staining intensity (Fig. 7-5, box d). A possible explanation is that the presence of intracellular gBtDAF may contribute to the staining, which increases the detected signals. Although we repeated this experiment several times, we could not reduce the signals to background level after PI-PLC treatment, which is consistent with previous studies (Caras and Weddell 1989; Liang et al., 1995, 1993). From this study, we believe that gBtDAF is a membrane-associated glycoprotein, and that it is anchored by GPI anchor.
Fig. 7-5. Flow cytometric analysis of surface-expressed gBtDAF in response to PI-PLC treatment. Subconfluent gBtDAF expressing cells were heat-shocked then incubated at 37°C for 6 h. Cells were trypsinized and suspended in MEM containing 2% heat-inactivated FBS and 0.05% sodium azide. Half of the cells was treated with 5 µg PI-PLC (lower panel, +PIPLC), the other half was used as control (top panel, -PIPLC). After PI-PLC treatment, samples were incubated with (B, B') or without (A, A') gB-specific monoclonal antibodies, followed by incubation with FITC-conjugated goat anti-mouse IgG. After staining, cells were subjected to flow cytometry analysis. Cell numbers and the intensity of surface staining are indicated on the vertical and horizontal axes respectively. Box "d" represents the peak-shift after PIPLC treatment.
7.3.5 Conformational studies of recombinant gB derivatives.

In order to determine the potential function of recombinant forms of gB, their conformational status, including their ability to form oligomers and their reactivity with individual gB-specific monoclonal antibodies were analyzed. Previously, we found that like gB homologues in HSV, pseudorabies virus (PRV), and human cytomegalovirus (HCMV), BHV-1 gB forms dimers (Britt and Vugler 1992; Claesson-Welsh and Spear 1986; Highlander et al., 1991; Whealy et al., 1990; Chapter 6.0). Using a pulse-chase study, we found that newly synthesized BHV-1 gB precursor can rapidly become dimerized and then transported to the Golgi (Chapter 6.0). In the present study, all the samples were labeled for 8 h rather than pulse-labeled. In authentic gB (Fig. 7-6 A), the precursor was present as monomeric (fractions 11 and 12) and dimeric forms (fractions 7 and 8), whereas mature gB predominantly consisted of dimers. All the recombinant gB derivatives also formed oligomers (Fig. 7-6 B).

Previously, a panel of gB-specific monoclonal antibodies has been established and characterized (van Drunen Littel-van den Hurk et al., 1984, 1985, 1986, 1989, 1990). Among these antibodies, some can only recognize epitopes following proper glycosylation or folding, and can be used to verify the proper conformation of the recombinant gB
Fig. 7-6. Sucrose gradient sedimentation study of BHV-1 gB and truncated gB derivatives. BHV-1 infected MDBK cells (A) or heat-shocked recombinant gB expressing cells (B) were labeled with 50 μCi/ml [³⁵S]-Met/Cys for 8 h, then lysed in 0.3 ml lysis buffer. The cell lysates were layered onto 5 ml continuous 5-20% (W/V) sucrose gradients prepared in lysis buffer and centrifuged for 16 h at 240,000 g (45,000 rpm). After centrifugation, 15 fractions were collected from the bottom (fraction 1 is the first bottom fraction). All fractions were subjected to a immunoprecipitation assay and the precipitates were separated on a SDS-7.5% polyacrylamide gel. Protein mass markers were spun in parallel tube which included ferritin (440 kDa), catalase (250 kDa), and hexokinase (100 kDa). Fractions containing the protein markers are indicated with arrows. On the right are the molecular mass markers on the gel (M.W., kDa).
Fig. 7-7. Immunoprecipitation of truncated gB derivatives with individual gB-specific monoclonal antibodies. The wt BHV-1-infected MDBK cells (gB) and the truncated gB expressing cells were radiolabeled. Samples of cell lysate and medium were combined and immunoprecipitated with individual gB-specific monoclonal antibodies. The antibody designations and their corresponding epitopes are indicated on top. On the left is the sample from different gB derivatives.
derivatives. An immunoprecipitation assay was designed to compare the reactivities of these gB products to individual monoclonal antibodies (Fig. 7-7). We found that recombinant gB derivatives could not react with a monoclonal antibody, 1B10, which recognizes epitope I on authentic gB. This epitope was previously shown to be located between residues 744 and 763 (Fitzpatrick et al., 1990a), a segment which is present in all our recombinant gB derivatives. A possible explanation is that in recombinant forms of gB, the conformation around epitope I has been changed, thereby preventing interaction with the 1B10 antibody.

7.3.6 Cell fusion in recombinant gB expressing cells.

In this study, four recombinant MDBK cell lines were established to express different gB derivatives under the control of the same promoter. Among the four recombinant gB expressing cell lines, gBt and gBtM expressing cell lines were indistinguishable from the parental MDBK cells (Fig. 7-8 A, B, C). The cell line which expresses gBtDAF showed a morphology slightly different from normal MDBK cells in that the cells appeared detached and elongated. This phenotype may be caused by accumulation of recombinant gB in the membrane, although no cell fusion was observed (Fig. 7-8 D). A significant observation for the gBtMA expressing cell line was that the expression of gBtMA caused cell fusion (Fig. 7
Fig. 7-8. Morphology of recombinant gB expressing MDBK cells. Cells were incubated overnight at 37°C after heat induction, then stained with Diff-Quik stain (Dade Diagnostic Inc., Aguada, Puerto Rico) and photographed (amplification is 300x). A: normal MDBK cells. B: gBt expressing cells. C: gBtM expressing cells. D: gBtDAF expressing cells. E: gBtMA expressing cells.
The recombinant gBtMA on the membrane of expressing MDBK cells resulted in polykaryocyte or syncytium formation.

7.4 DISCUSSION

In this section, we describe the expression and characterization of different gB derivatives in MDBK cells, which results in the identification of the fusogenic domain in BHV-1 gB.

Fusion domains have been identified in different viruses. These domains share common properties in that they are always located on the membrane anchored subunit, which is hydrophobic and relatively rich in glycine and alanine residues (Blobel et al., 1992; White 1990, 1992). To search for hydrophobic fragments was the first step in our study in order to find the fusion domain on BHV-1 gB. BHV-1 gB undergoes proteolytic cleavage during its maturation process, which excludes the possibility that the fusion domain is located in the gBb subunit. Therefore, we searched for the hydrophobic domains in the gBc region, the membrane associated subunit (Fig. 7-9). We found that there appears to be no obvious hydrophobic domains before the transmembrane region at window settings of 11 or 19 amino acids. The only hydrophobic peak "x" represents a fragment that has no glycine or alanine residues, and thus may not be the potential fusion peptide. Previously, It was suggested that
Fig. 7–9. Normalized consensus hydrophobicity scale of BHV-1 gBc. The sequence of gBc was analyzed by program AASCALE in PC/GENE software to calculate Eisenberg hydrophobicity in a moving window of 11 amino acids (A) or 19 amino acids (B). Box "TMA" represents the potential transmembrane region. "X" indicates the highest position outside the transmembrane region.
gB homologues in herpesviruses do not contain a hydrophobic fusion peptide in the ectodomain (Pereira 1994), which is supported by sequence analysis. However, gB homologues in herpesviruses are found to be essential for membrane fusion (Pereira 1994; Spear 1993). Therefore, we focused on the long transmembrane region in our search for the potential fusogenic domain.

The long potential transmembrane anchor region has been found in gB of all herpesviruses, and it was suggested that this fragment might span the membrane three times as continuous, antiparallel alpha-helices (Pellet et al., 1985). However, recent studies on HSV-1 gB showed that among the three potential membrane spanning segments, segment 3, and probably segment 2 as well, functions as the membrane anchor (Gilbert et al., 1994; Kasile et al., 1993). A corresponding segment in HCMV gB was also demonstrated to be the real anchor (Reschke et al., 1995). Our studies with BHV-1 gB support the fact that segment 3 has a membrane anchor function, whereas segments 1-2 can provide a weak retention signal for protein anchoring (Chapter 6.0; Fig. 7-3, 7-4).

By sequence alignment of potential gB transmembrane regions from some herpesviruses (Fig. 7-10), we found a conservative pattern in these sequences and in their hydrophobicities as calculated by Eisenberg’s method (1984). Among these potential transmembrane helices, segment 3 has the highest potential to be the transmembrane domain because
their hydrophobicities are close to the average value of 0.70±0.09 (White 1990). However, the alanine and glycine concentrations in segment 3 of herpesvirus transmembrane regions are about 40%, which is higher than average (16±8%) (White 1990). We believe that the difference is caused by the undefined window of the transmembrane domains.

From Fig. 7-10, we find that the hydrophobicities for segment 2 are about 0.6, which corresponds perfectly with the average hydrophobicity of membrane fusion proteins found in other viruses (0.61±0.09), while the glycine and alanine contents are also comparable with the average in fusion peptides (36±7%) (White 1990). Previously, Reschke et al. (1995) noticed that the region in front of the membrane anchor of herpesviruses is relatively rich in glycine residues which are distributed in a special manner similar to fusion domains of other viral proteins. They also raised the possibility that this portion of the transmembrane region might be involved in fusion. The conservation of serine and glycine in segments 1 and 2 is not required for membrane anchoring, suggesting that they are involved in other important function. Based on the large number of hydrophobic residues and its high Gly/Ala content, segment 2 have the greatest potential to be the fusion peptide. A previous study on HCMV gB supports the fact that the fusion domain may be located in the C-terminal subunit around segment 2 (Tugizov
Fig. 7-10. Sequence alignment and hydrophobicity values of three segments in the herpesvirus gB transmembrane region.
Sequences of gB from selected herpesviruses were aligned by using CLUSTAL (version 1.20) in PC/GENE. S1, S2, and S3 represent three continuous segments in the transmembrane region. S3 is the closest to the cytoplasmic tail of gB. For each segment, the hydrophobicity value (H.I.) was calculated according to Eisenberg’s method (1984), and the local concentration of glycine and alanine (Gly/Ala) was also addressed. In the conservation description, the "." shows that a position is well conserved, and the "*" shows that a position is perfectly conserved. The name of the virus and the corresponding residue number are indicated on the left. BHV-2: bovine herpesvirus type 2. EHV-1: equine herpesvirus type 1. HSV-2: herpes simplex virus type 2. VZV: Varicella-zoster virus. EBV: Epstein-Barr virus.
<table>
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<tr>
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<th>H.I. Ala/Gly (%)</th>
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<tr>
<td><strong>S1</strong></td>
<td></td>
</tr>
<tr>
<td>BHV-1 (760-779)</td>
<td>GNMAI MRGLA NFFQG LGAVG</td>
</tr>
<tr>
<td>HSV-1 (726-745)</td>
<td>ANAAAM FAGLG AFFEG MGDLG</td>
</tr>
<tr>
<td>PRV (750-769)</td>
<td>HNVVL LGLAI NFFQG LGDVG</td>
</tr>
<tr>
<td>BHV-2 (745-764)</td>
<td>PNAAI FAGLH SFFEG LGDVG</td>
</tr>
<tr>
<td>EHV-1 (802-821)</td>
<td>NTAVI MQGIA SFFKG LGKVQ</td>
</tr>
<tr>
<td>HSV-2 (724-743)</td>
<td>ANAAAM FAGLCL AFFEG MGDLG</td>
</tr>
<tr>
<td>VZV (676-695)</td>
<td>STGTAI MQGMA QFFQG LGTAG</td>
</tr>
<tr>
<td>EBV (685-704)</td>
<td>GRNQF VDGGLG ELMDLG SVSAG</td>
</tr>
<tr>
<td>HCMV (703-722)</td>
<td>PLPPY LKGLD DLMSG LGAAG</td>
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<td><strong>Conservation</strong></td>
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| **S2** |                |
| BHV-1 (785-805) | VVLGA AGAAL STVSG IASFI A | 0.66 43 |
| HSV-1 (751-771) | VVMGI VGGVV SAVSG VSSFM S | 0.62 24 |
| PRV (775-795)   | VVLGA TGA VI SAVGG MVSSL S | 0.66 33 |
| BHV-2 (770-790) | VVLGV VGGVV ATVSG VSSFL S | 0.65 24 |
| EHV-1 (827-847) | LVLGA AGAVV STVSG IASFL N | 0.60 33 |
| HSV-2 (749-769) | VVMGV VGGVV SAVSG VSSFM S | 0.60 24 |
| VZV (701-721)   | VVLGA TGALL STVHG FTFTFL S | 0.55 24 |
| EBV (720-730)   | LVSTV GGLFS SLVSG FISFF R | 0.54 14 |
| HCMV (728-748)  | AIGAV GGAPA SVVEG VATFL K | 0.55 42 |
| **Conservation** |                       | . . * . . . . . . . * |

| **S3** |                |
| BHV-1 (808-828) | FGALA TGLLV LAGLV AAFLA Y | 0.78 43 |
| HSV-1 (774-794) | FGALA VGLLV LAGLA AAFFA F | 0.85 48 |
| PRV (798-818)   | FGALA IGLLV LAGLV AAFLA Y | 0.84 43 |
| BHV-2 (793-813) | FGALA IGLLV LGGLV AAFFA F | 0.88 43 |
| EHV-1 (850-870) | FGGLA IGLLV IAGLV AAFFA Y | 0.85 43 |
| HSV-2 (772-792) | FGALA VGLLV LAGLV AAFFA F | 0.87 43 |
| VZV (724-744)   | FGALA VGLLV LAGLV AAFFA Y | 0.83 43 |
| EBV (733-753)   | FGGML ILVLL AGVVI LVISL T | 0.88 19 |
| HCMV (751-771)  | FGAFT IILVA IAVVI ITYLI Y | 0.89 19 |
| **Conservation** |                       | **... . . . . . . . .**

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et al., 1995). They expressed different gB derivatives of HCMV, and found that a deletion in domain D1 (residues 411-447) does not affect syncytium formation, whereas deletions across the C-terminal domain result in dramatic inhibition of fusion.

In order to demonstrate that segment 2 is the potential fusion peptide rather than the membrane anchor, we expressed gBtM and gBtMA based on the hypothesis that they may use segment 2 (hydrophobicity 0.66) or 3 (hydrophobicity 0.78) for membrane anchoring respectively. In the present study, we included the expression of gBt, a previously truncated gB, as the control to study the newly generated recombinant gB derivatives. Recombinant gBt was found to be as efficiently transported and glycosylated as authentic gB, and was completely secreted into the medium. Since gBt does not have the membrane anchor, it definitely can not have fusogenic properties and the corresponding expressing cells show no morphological change in comparison to parental MDBK cells. After we provided segments 1 and 2 as the potential anchor, gBtM showed a slightly stronger membrane retention than gBt (Fig. 7-3, 7-4). This result indicates that without segment 3 and the cytoplasmic region, segments 1 and 2 may play a weak role as the membrane anchor for gBtM. However, in gBtM cells, even when we examined the cells at different time points, no cell fusion was observed (Fig. 7-8), indicating that the ectodomain of BHV-1 gB has no fusogenic activity.
This is consistent with our sequence analysis and previous assumptions made by Pereira (1994). In gBtMA, segment 3 is included at the carboxy terminus. This segment has been demonstrated to be the real anchor in HSV-1 and HCMV gBs (Gilbert et al., 1994; Rasile et al., 1993; Reschke et al., 1995). We found that the recombinant gBtMA exhibits a delayed transport to the Golgi, and that the protein can be located on the nuclear membrane whereas the mature one moves to the cell membrane (Fig. 7-2, 7-3, 7-4). However, without the cytoplasmic domain, anchorage of gBtMA on the cell surface is unstable, and the mature protein is secreted into the medium (Fig. 7-4). This phenomenon has not been previously reported. Even with the unstable anchorage of the gBtMA on the cell membrane, or the retention of precursors in the nuclear membrane and ER, expression of gBtMA was still able to cause cell fusion (Fig. 7-8). Previously, the precursor form of gB has been shown to have fusogenic properties (van Drunen Littel-van den Hurk et al., 1992), which supports our finding that cell fusion occurs in the precursor-rich gBtMA cells. When we compare gBtM and gBtMA expressing cells, both show surface staining (Fig. 7-3), indicating that segments 1-2 and segment 3 may independently play a role as the membrane anchor. However, the anchored gBtM does not cause fusion, whereas gBtMA does, indicating that there is no fusogenic domain from residues 506 to 784 when segments 1-2 is used as anchor. These results support our conclusion that segment 2
may be the fusion peptide when segment 3 is used as the anchor.

To further support our suggestion that the fusion of gBtMA cells is caused by the fusogenic property of recombinant gBtMA rather than an unspecific phenomenon because of the protein anchorage, we expressed gBtDAF by fusing the carboxy tail of hDAF to the C-terminus of gBt. This construct provided a signal for the addition of a GPI-based membrane anchor (Medof et al., 1986). In this way, the chimeric protein is targeted to the surface of cells (Caras et al., 1987; Liang et al., 1995) or virions (Liang et al., 1993). The partial release of membrane-associated proteins may be caused by phospholipase cleavage (Caras and Weddell 1989; Liang et al., 1995). The resultant gBtDAF was successfully targeted to the cell membrane like other GPI anchored membrane proteins (Fig. 7-3, 7-4, 7-5). It was previously demonstrated that the GPI anchor associates only with the outer leaflet of the membrane lipid bilayer. This enables the protein to project into the extracellular space, however, the anchored fusion protein does not mediate the complete fusion (Kemble et al., 1994; Ragheb and Anderson 1994; Weiss and White 1993). The GPI anchored gBtDAF was found to have strong membrane retention in gBtDAF expressing cells. The overexpression and accumulation of recombinant gBtDAF on the cell membrane resulted in relatively poor growth behaviour with minor changes in morphology. These
observations support that the fusion in gBtMA cells is caused by specific activity introduced by gBtMA expression.

A popular working mechanism for fusion is that the membrane-anchored fusion peptide can be modelled as an amphipathic alpha helix with most of the bulky hydrophobic residues on one face, such that the fusion protein can interact simultaneously and hydrophobically with both the viral and the target membrane (Blobel et al., 1992; White 1992). These amphipathic alpha-helix structures are also well conserved in segment 2 of gB in all herpesviruses (Fig. 7-11). This provides another line of evidence to support its function as the fusion domain in gB. In Fig. 7-11, we evaluate sequences of segment 2 from gB of different herpesviruses as potential fusion domains. Although they all appear to be amphipathic structures, the accurate window of the fusion domains needs to be determined.

In this section, based on the sequence analysis and experimental evidence, we propose that segment 2 of gB is the fusion peptide in herpesviruses. Previously, gB has been well accepted as the fusion protein. There is evidence that gB-specific antibodies possess post-adsorption neutralization activity which may block fusion (Dubuisson et al., 1992; Highlander et al., 1988; Navarro et al., 1992). In addition, expression of gB in transfected cells can induce syncytium formation (Ali et al., 1987; Fitzpatrick et al., 1988, 1990; Tugizov et al., 1994, 1995; van Drunen Littel-van den Hurk et
Fig. 7-11. Potential fusion peptides of selected herpesviruses. The sequences of segment 2 of selected herpesvirus gBs are plotted in the helix format, with the hydrophobic face encircled. The bottom residue represents the first residue in segment 2. The hydrophobic residues are in bold and italic.
al., 1992). Mutations in the cytoplasmic region have been found to produce a syncytium phenotype (Baghian et al., 1993; Bzik et al., 1984; Cai et al., 1987, 1988; Gage et al., 1993), and mutations in the extracellular domain of gB can increase the rate of penetration of virus into cells (Bzik et al., 1984; Highlander et al., 1989) or block entry (Cai et al., 1987). Based on the location of the fusion peptide in gB, we believe that all these factors may indirectly affect the fusion domain by influencing the functional conformation of gB. It has been suggested that communication between the external and cytoplasmic domains may be required for gB-mediated membrane fusion events (Gage et al., 1993). This supports the suggestion that cytoplasmic regions provide a conformational signal to the fusion domain. Mapping of the fusion related epitopes by monoclonal antibodies may also be explained as they may interfere with the conformation of the fusion domain. This may result in neutralization because of the defect in fusion (Fuller et al., 1985, 1987, 1989; Highlander et al., 1987, 1988; Kuhn et al., 1990). Previously, one monoclonal antibody of BHV-1 gB, 1B10, has been found to have the highest inhibition for gB-induced fusion (van Drunen Littel-van den Hurk et al., 1992). The epitope for this antibody is located between residues 744-763 (Fitzpatrick et al., 1990a), which is very close to segment 2, supporting the hypothesis that it functions by blocking gB fusion. However, the fusion can also be inhibited by
monoclonal antibodies located in the N-terminal subunit, for example, the epitope IV between residues 68-119 (van Drunen Littel-van den Hurk et al., 1992). These antibodies do not directly react with the fusion domain. However, they may block the conformation required for fusion. Similar observations in HSV-1 have been reported by Navarro et al. (1993), in that among the three domains in HSV-1 gB which are involved in fusion, two of them, D1 and D5a, correspond to the BHV-1 gB epitopes IV and I, respectively.

The fusion process in herpesviruses is believed to be a complex process. Monoclonal antibodies against different viral glycoproteins appear to have similar post-adsorption neutralizing activity which is actually caused by blocking fusion (Fuller et al., 1985, 1987, 1989; Highlander et al., 1987, 1988; Kuhn et al., 1990). This diversity in proteins involved in neutralization indicates the complexity of the fusion process. As gB plays a central role in fusion, the other proteins may modulate the gB fusion activity in a complex way. Any change in this complex cooperation may result in syncytium-inducing mutations. In addition to gB, mutation-related syncytium phenotypes have been found in gK, which may be the fusion inhibitor (Hutchinson et al., 1992, 1993, 1995, 1995a), the UL20 gene, a membrane protein (Baines et al., 1991; MacLean et al., 1991), and the UL24 gene (Jacobson et al., 1988; Sanders et al., 1982). In support of this contention, BHV-1 gD was previously suggested to
modulate other virus-cell interaction(s) involved in productive virus penetration, most likely gB's fusogenic activity (Liang et al., 1995).

Based on the sequence analysis, all herpesvirus gB homologues have a very conserved proline between segments 2 and 3, which is probably very important in regulating the position of segment 2. Proline has been shown to be critical in the formation of the tertiary structure of proteins (Nall 1989). It has been found near the centre of the internal fusion peptides to kink the amphipathic α helix (White 1992). In herpesvirus gB, the close location of the fusion domain and the transmembrane anchor may suggest an unique mechanism for the exposure of the fusion domain. During viral infection, interactions between viral proteins and cellular receptors may establish the appropriate condition that exposes the fusion domain of gB. It is likely that after these interactions, the proline may help the fusion domain to position itself. At this time, we have no evidence for this assumption.

Previously, we demonstrated that BHV-1 gB has two types of cell binding sites for cellular HS and non-HS receptors (Chapter 4.0). The high-affinity binding to the non-HS receptor is important for productive infection with BHV-1. This high-affinity binding site can be lost in truncated gB derivatives which have no cytoplasmic region (Chapter 6.0), indicating that the cytoplasmic part plays a
role in maintaining the conformation of gB. This result also suggests the importance of interactions between different parts of gB. The functions of gB have been shown to have strict structural requirements (Baghian et al., 1993; Navarro et al., 1992; Whealy et al., 1990), which may include oligomerization and proper folding. In our study, we demonstrated that all our recombinant gB derivatives have a structure similar to authentic gB with exception of the loss of epitope I (Fig. 7-7). Previously, we found this epitope to be important for maintaining the high-affinity binding site of gB which plays a critical role in virus infection (Chapter 6.0). However, the loss of this epitope does not affect the function of the fusion peptide. This result indicates that the fusion of gB in BHV-1 is modulated in a complex manner, whereas in gB expressing cells, a different mechanism may apply.

In conclusion, we found that in BHV-1 gB, the real membrane anchor is segment 3 (residues 808-828), whereas segment 2 is important in fusion. The function and structure of this region is probably conserved in all herpesviruses.
8.0 GENERAL DISCUSSION AND CONCLUSION

The gB glycoprotein homologues constitute the most conservative glycoprotein family in herpesviruses. In alphaherpesviruses, all gB homologues share sequence similarity of about 50% (Table 1-3). The specific conservation of cysteine residues and glycosylation sites suggest that they also share higher structural identity. These structural similarities suggest that they may have similar functions in virus infectivity. Previous studies, mostly in HSV-1, have shown that gB is an essential multifunctional glycoprotein involved in attachment and fusion required for virus entry in both virus-cell infection and cell-to-cell spread. However, the detailed mechanism(s) involved in each specific event of viral entry and spread as well as the functional requirements for each event are still unclear.

Our laboratory has been working on BHV-1, another alphaherpesvirus, as a model to study herpesvirus biology. In my study, the early steps of the entry process of BHV-1 were of primary interest. My objective was to delineate the functions of gB in viral attachment and fusion and to analyze
the specific domains (regions) responsible for these putative functions. In this thesis, several different issues were addressed, including intracellular maturation, involvement in cell-attachment, and involvement in membrane fusion. Regions or domains of BHV-1 gB involved in specific functions were described. We identified the regions which were required for intracellular transport, oligomerization, and heparin-binding. The putative domains of membrane anchoring and membrane fusion were also identified.

In this study, a series of truncated BHV-1 gB derivatives were expressed in stably transfected MDBK cells under the control of the bovine hsp70A gene promoter. All the recombinant gB molecules had the expected molecular weight and proper post-translational modifications, including inter-chain disulfide bond formation, proteolytic cleavage and glycosylation. Their antigenic properties were also similar to authentic gB. Therefore, by characterization of these gB derivatives in transfected cells, it was possible to study gB without constructing various mutant viruses. Furthermore, possible complication from other viral proteins, which could have occurred if mutant viruses had been used, were eliminated. This strategy has been used in other herpesviruses like HSV-1 and HCMV (Cai et al., 1988; Navarro et al., 1993; Tugizov et al., 1995).

The exocytic transport of BHV-1 gB involves several post-translational modifications. After the high mannose-
containing monomeric precursor is synthesized, two monomers may become dimerized to form oligomers, and are then transported to the Golgi (Fig. 6-4). The transmembrane and cytoplasmic regions do not appear to be required for gB oligomerization, whereas the fragment between residues 506 to 763 does (Fig. 6-5). For intracellular transport of BHV-1 gB from the ER to the Golgi, oligomerization is not required. However, a conformational signal provided by the gBb subunit is responsible for directing this exocytosis transport (Fig. 3-5, 3-6). When the precursors are transported to the Golgi, correct glycosylation occurs, producing mature glycoproteins containing complex oligosaccharides (Fig. 6-1, 7-2). After conversion of the high mannose-containing precursor to the mature form of gB, proteolytic cleavage occurs, generating the mature gB products which can be transported out of the cells, or retained in the membrane (Fig. 3-5). This exocytic pathway is similar to that of PRV gB previously described by Whealy et al. (1990).

HSV-1 gB has been found to bind HS and the binding of gB to heparin-like structures was shown to be required for gB-mediated cell fusion (WuDunn and Spear 1989; Shieh and Spear 1994). PRV gB was also found to bind heparin in conjunction with gC (Mettenleiter et al., 1990; Sawitzky et al., 1990). In contrast, it was originally reported that BHV-1 gB was not a heparin-binding glycoprotein (Okazaki et al., 1991). However, a recent report by Bryne et al. (1995) and my
study (Fig. 4-2, 5-1, 5-5) demonstrated that gB does bind heparin-Sepharose as well as HS structures on permissive cells. By comparing the binding of different fragments of the gB ectodomain to heparin-Sepharose, it was possible to localize the heparin-binding domain(s) to gBb (Fig. 5-4). This result is in agreement with sequence analysis, which indicates that basic residue clusters are found only on gBb, but not on gBc (Table 5-2).

This study demonstrated that BHV-1 gB can bind to two different cellular receptors. The first receptor is a HS structure, whereas the second non-HS receptor remains unknown. The existence of the dual cell-binding property of gB is supported by different lines of evidence. First, studies on gB binding to MDBK cells in the presence of heparin or heparinase revealed that gB bound to both HS and non-HS structures. Competition binding curves of gB on MDBK cells also indicated that gB binding shows dual binding kinetics (Fig. 4-2, 4-4, 4-5). My results clearly show that high-affinity binding of gB to the non-HS receptor is important for BHV-1 infectivity (Chapter 4.0). A truncated gB, gBt, which has no high-affinity binding site can not block BHV-1 plaque formation (Fig. 4-7). Another truncated form of gB, gBtM, which represents the entire ectodomain of BHV-1 gB, did not contain the high-affinity binding site, indicating that the cytoplasmic tail of gB is essential for maintaining such a site on gB (Fig. 6-10). The cytoplasmic
region of gB is important to the conformation of gB around epitope I, which can be recognized by a gB-specific mAb, 1B10 (Fig. 6-6, 6-7, 7-7). Therefore, the specific conformation around this region appears to be critical for the integrity of the high-affinity binding site of BHV-1 gB. Although we have no evidence to link high-affinity binding with fusion activity, the observation that high-affinity binding of BHV-1 gB requires initial binding to HS resembles what has been discussed for HSV-1 gB, where gB-mediated fusion is triggered by gB binding to heparin-like structures (Shieh and Spear 1994).

Sequence analysis of gB of herpesviruses reveals a long conserved potential transmembrane anchor region on the C-terminal part of the molecule (Fig. 1-2). It was previously suggested that this transmembrane region may span the membrane three times. Recent studies, in HSV-1 and HCMV, showed that among the three segments which potentially span the membrane, segment 3 appears to be critical for retaining the gB molecule in the membrane, with segment 2 providing a minor contribution. In our study, several recombinant BHV-1 gB derivatives containing different segments of the transmembrane region were expressed. It was found that in BHV-1 gB, segment 3 is sufficient for nuclear membrane retention, whereas segment 1 and 2 may contribute slightly to retention of gB in the membrane (Fig. 7-2, 7-3, 7-4). This is consistent to what has been reported in HSV-1 gB. However,
gBtMA, a truncated gB which contains all three segments of the transmembrane region, was mostly retained intracellularly in the precursor form, with only small amounts being transported to the Golgi. After modification to complex oligosaccharides, mature gBtMA was efficiently secreted. This result indicates that without the cytoplasmic tail, membrane-retention of gBtMA is not very stable, with some precursors transported via the exocytic pathway (Fig. 7-2, 7-3, 7-4). In the case of gBtM, segments 1 and 2 might only provide weak retention of the precursors in the nuclear membrane. These unstably retained proteins were transported to the Golgi to undergo glycosylation and were then secreted into the medium (Fig. 6-2, 7-3).

By analyzing the potential long transmembrane region of BHV-1 gB, it was found that segment 2 has some structural properties that correspond to the general structure of viral fusion peptides (Fig. 7-10, 7-11). This contention is supported by the observation that in transfected cell lines expressing different gB derivatives gBtMA can cause cell fusion, whereas gBtM can not. Studies using gBtDAF clearly showed that retention of gB in the membrane is not in itself responsible for cell fusion. This study provides direct evidence that segment 2 is the membrane fusion domain (Fig. 7-8). Sequence analysis of the corresponding segments from different herpesviruses indicates that they are all highly conserved and that their sequences can form amphipathic
alpha-helix structures (Fig. 7-11). This result further supports my contention that this conserved fragment may be the fusion domain in BHV-1.

To summarize the studies conducted in this thesis, a schematic diagram is shown in Fig. 8-1. Some structural properties are illustrated along the protein sequence, including N-glycosylation sites, basic residue clusters, potential alpha-helix segments, and the antigenic epitopes. The N-terminal subunit, gBb, which contains most of the basic residue clusters, is responsible for the heparin-binding property of gB. gBb also has most of the N-glycosylation sites and the presently identified antigenic epitopes. gBb is the most exposed part of gB and provides the conformational signal for intracellular transport of recombinant gB derivatives. The portion of the molecule between the cleavage site and the transmembrane region may contain the oligomerization domain and thereby directs this important post-translational modification of gB. It is most likely that the conformational structure around epitope I is critical for the integrity of the high-affinity binding site on gB. The cytoplasmic domain appears to be necessary for maintaining such a site and the native conformation of epitope I. Segment 3 is the real membrane anchor, whereas segment 2 is the fusion peptide of gB. Since this fusion domain is located close to the membrane, it can be affected by both intracellular and extracellular modulations. This has been
Fig. 8-1. Structural properties of BHV-1 gB and the putative functional regions. The boxes represent different fragments of gB with the corresponding residue numbers on top. "N" represents site of N-glycosylation. "⊕" represents basic residue clusters. Single lines represent potential helix structures. Double lines represent antigenic epitopes. S: signal sequence; C: cleavage site; TMA: transmembrane anchor region.
supported by previous studies which showed that the mAb which recognizes epitope I can inhibit gB-mediated fusion. Other mAbs which recognize epitopes distinct from this fusogenic domain can also affect fusion, suggesting they may interfere with the conformation of the fusion domain.

To date, fusion peptides have been found in different DNA and RNA viruses (White 1990; Stegmann et al., 1989), but not in herpesviruses. Studies in HSV-1 suggest that fusion is a complex process involving different proteins. Among these proteins, gB may be the fusion protein, whereas others may modulate this complex fusion process. Thus, any change in any protein or glycoprotein involved in this complex process may alter the fusion phenotype, resulting in enhanced or decreased fusion. The fact that gK, a potential fusion-regulating glycoprotein, does not need to be exposed on the cell surface to cause fusion (Hutchinson et al., 1995) suggests that fusion may be modulated at both compartments, intracellularly and extracellularly. This further supports my contention that gB, a type I integral glycoprotein with a fair length of cytoplasmic domain, is a good candidate for a fusion protein. The roles played by other fusion-regulating proteins are to modulate this fusion protein in order to form the conformation required for fusion. Any interference may result in conformational change of this fusion protein, which may affect the fusion process.
**Fig. 8-2.** Hydropathic analysis of HSV-1 gB and the location of fusion-related mutations. The hydropathic index was computed using the SOAP program (version 3.05) in PC/GENE (release 6.70) with an interval of 15 amino acids. The arrows going down indicate the mutations which cause decreased fusion, whereas the arrows going up indicate mutations caused syncytial mutants. TMA: transmembrane region.

It has been documented that different mutations in HSV-1 gB affect fusion properties of the mutant viruses (Fig. 1-3). Fig. 8-2 illustrates the location of these mutations on
gB in association to the hydrophatic score of gB. All the mutations involved in syncytia formation are located in hydrophilic regions of the gB cytoplasmic domain. These regions are possible interaction sites for the intracellular fusion-regulating proteins, for example, gK. Mutations at these sites may affect the intracellular modulation of gB, or directly affect the conformation of the fusion domain located across the membrane. Without the correct intracellular modulations, the gB fusion domain may be exposed and result in enhanced fusion. In contrast, the fusion-related mutations in the gB ectodomain do not follow any specific rule. All the mutations on the gB ectodomain may alter the conformation of the fusion domain, which results in decreased fusion. In order to understand the complex fusion process, it will be necessary to differentiate between the fusion domain per se and the conformational structure of gB involved in fusion. I believe that many different proteins and different mutations on gB can affect the conformation of gB which in turn can modulate the exposure of the fusion domain. In such a way, the fusion process appears to involve gB, gK, gD, gH/gL, and other proteins like products of UL20, UL24, UL25, UL45.
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