

Entry and fusion glycoproteins of elephant endotheliotropic herpesviruses (EEHVs)

Master thesis – Faculty of Veterinary Medicine – Utrecht University



29 – 06 – 2021

By

Eline Koster
e.w.f.koster@students.uu.nl, 5803098
Veterinary Medicine (MSc Student)
Master Companion Animal Health

Supervisor

Tabitha Hoornweg
t.e.hoornweg@uu.nl
Faculty of Veterinary Medicine, UU
Department of Biomolecular Health Sciences
Sections Immunology and Virology

Abstract

Elephant endotheliotropic herpesviruses (EEHV) can cause lethal hemorrhagic disease (EEHV-HD) in juvenile elephants. The rapidly evolving symptoms and lethality make EEHV-HD a threat to the survival of the species. However, research on EEHV has been compromised since efforts to culture the virus failed and EEHV seems to differ significantly from other herpesviruses. Consequently, no sufficient treatment or vaccine are available and diagnostic tests are limited. In this study we aimed to identify important entry and fusion glycoproteins of EEHV. It is known that in all herpesviruses gB and gH/gL induce fusion, sometimes in combination with other glycoproteins. Extrapolating from this knowledge, EEHV gB and gH/gL could be sufficient for entry and fusion *in vitro*, or additional EEHV glycoproteins may be required. To examine this, EEHV gB and gH/gL wildtype constructs were produced and transfected into HeLa cells. No clear cell-cell fusion was seen when transfecting wildtype EEHV gB and gH/gL, which was probably due to lack of cell surface staining. Therefore, multiple EEHV gB and gH/gL constructs were developed and transfected, after which cell surface expression of the construct was analyzed. Constructs of gB with a truncation of the full cytoplasmic tail and gH with a truncation of the Yxx ϕ signal (in combination with gL) showed obvious cell surface staining. In a cotransfection with the gB(-CTD) and gH(-YQKL) construct, no obvious cell fusion could be seen. Cell fusion in a cotransfection with gB(-CTD) and gH(wt) could not be induced by trypsin treatment or pH shock. *In silico* selection of EEHV glycoproteins with a potential role in cell entry and/or fusion was performed based on comparison of the characteristics to other herpesvirus glycoproteins which are important in entry and fusion. EEHV glycoproteins E4, E20A, E31, ORF-O, ORF-P, ORF-Q all contained a signal sequence and multiple N-glycosylated sites. These characteristics are analogous to HCMV U128-131 and HHV-6 gQ1/2, suggesting that they could potentially bind to the gH/gL complex and play an important role in fusion. As follow-up experiments, it would be relevant to observe if these glycoproteins interact with gB and gH/gL and then produce these glycoproteins and cotransfect them with gB and gH/gL, to see if cell fusion is enhanced. Another future possibility is to transfect HeLa cells and target cells with the developed constructs – especially gB(-CTD) and gH(-YQKL) – and seed them, to see whether fusion can be observed therein. This work encompasses the first crucial steps towards an EEHV cell fusion assay. Eventually, EEHV fusion assays are important for development of virus neutralization tests, which can be used to measure neutralizing and therefore protective antibody responses against EEHV and potential vaccine candidates.

Keywords: EEHV, herpesviruses, elephant, cell fusion assay, gB, gH/gL

Introduction

Elephant Endotheliotropic Herpes Viruses (EEHV) may cause lethal hemorrhagic infections (EEHV-HD) in the Asian and African elephant, both endangered species according to the IUCN Red List of Threatened species. (1–4) The first reported EEHV-HD case was in 1988, when a young Asian circus elephant died of a hemorrhagic disease of unknown etiology. (1,5) In 1999, Richman et al. published about a novel herpesvirus called “elephant endotheliotropic herpesvirus” or EEHV, detected in elephant cases from the years prior. (6) Since then, dozens of potential and proven cases have been recognized. (1) However, research on EEHV has been difficult, partly since all efforts to culture the virus were unsuccessful. Consequently, there is currently no successful treatment nor a vaccine available for EEHV-HD.

EEHV is classified under the genus Proboscivirus in the family of herpesviruses. To date, eight subtypes have been recognized, varying in main host species and in number of cases associated. (1) The subtypes EEHV1A, EEHV1B, EEHV4 and EEHV5 have been identified in Asian elephants. In African elephants, the presence of subtypes EEHV2, EEHV3, EEHV6 and EEHV7 has been confirmed. (1,7) Research has shown that most mature Asian elephants in zoos have been exposed to EEHV and consequently are anticipated to be latently and asymptotically infected with one or more EEHV subtypes. (1,3)

Yet, in young elephants between one and nine years of age a (presumably) primary EEHV infection may cause EEHV-HD. Over the last 30 years, EEHV-HD was found to be the most frequently occurring cause of death in captive Asian elephants between the age of 1 and 9 years old in western zoos. (1) Up until recently, 303 captive elephant calves were born in total over the last 30 years. Overall, 96 of these calves died before reaching the age of 9 (32% of the total). Of the 96 deceased calves, 54 calves have died of EEHV-HD (56% of the deceased calves) (pers. comm. W. Schaftenaar). Accordingly, 18% of all 303 captive elephant calves died of EEHV-HD. In addition to the incidence in captive Asian elephants, EEHV-HD is also known to occur in wild Asian elephants, making the virus a threat to the survival of the species. (1,8,9) In African elephants, EEHV-HD cases are increasingly observed as well. (1,3)

Symptoms of EEHV-HD are initially non-specific and consist of lethargy and oedema. However, the disease can rapidly evolve into heart failure and a severe shortage in oxygen. (1) For diagnosis of EEHV, serology and PCR are available. For most EEHV subtypes, specific PCRs were developed. (1) Treatment of EEHV-HD is frequently non-sufficient and no vaccine is available yet. Since EEHV-HD is a significant threat to young Asian elephants and no sufficient treatment is available, an EEHV vaccine would be a significant contribution to the conservation of elephants. In order to develop an evidence-based vaccine, the most suitable target proteins for vaccine development need to be identified.

All known herpesviruses use multiple glycoproteins for attachment, entry and fusion to a host cell. (10,11) Glycoproteins gB, gH and gL are conserved in all herpesviruses and contribute to virus-cell attachment, entry and fusion. Glycoproteins gH and gL form a heterodimer (gH/gL), which is thought to activate the fusogen gB. (11) Previous studies have shown that members of the herpesvirus family often use several other proteins for entry and fusion in addition to gB and gH/gL, which is specific to each genus/species. (10,12)

Attachment, entry and fusion of herpesviruses

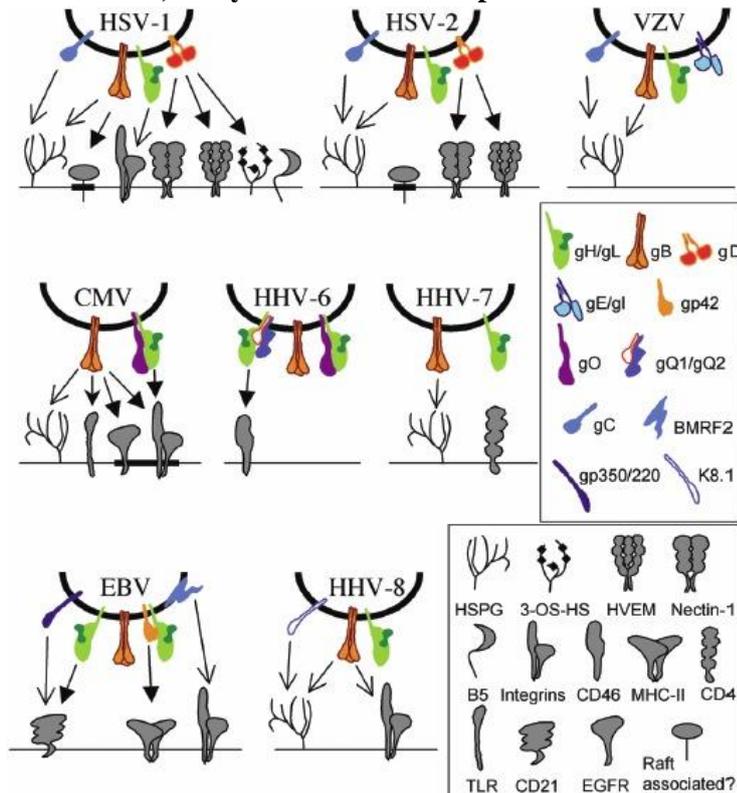


Figure 1: From 'Entry of Herpesviruses into cells: the enigma variations' (12). This figure shows the three subfamilies of herpesviruses; alpha herpesviruses (HSV-1, HSV-2, VZV), beta herpesviruses (CMV, HHV-6, HHV-7) and gamma herpesviruses (EBV, HHV-8). For each genus, the known entry and fusion glycoproteins and their receptors are depicted.

In addition to gB and gH/gL, alpha herpesviruses may use several glycoproteins for entry and fusion of which some are essential and others dispensable (Fig. 1). The initial aspecific attachment of alpha herpesviruses to host cells is generally mediated by gC, which binds to heparan sulfate and proteoglycans. In the absence of gC, which is not an essential glycoprotein, gB provides this function. (12)

For HSV-1 and HSV-2, gB, gH/gL and gD are essential and sufficient for cell entry and fusion. (12) HSV gD binds to receptors of the nectin family, herpesvirus entry mediators (HVEM) and/or to heparan sulfate (HS). Subsequent to the interaction of herpesvirus glycoproteins with a specific entry receptor, interaction of glycoproteins with fusion receptors occurs and the virus enters target cells. It has been hypothesized that HSV gD – after binding to an entry receptor and undergoing multiple conformational changes – binds to the regulatory complex gH/gL. Subsequently, gH/gL is able to activate gB, which results in membrane fusion. (12,13)

The alpha herpesvirus Varicella-Zoster Virus (VZV) lacks a homologue to HSV gD. VZV does possess a gE/gI complex that is used in virus-cell interaction. (14–16) For VZV fusion, it has been reported that VZV gB and gE coexpression results in cell fusion, in contrast to the expression of gB or gE alone. (17) VZV gB and gH/gL are also sufficient for inducing cell fusion. (12) Therefore, it was hypothesized that VZV gB and an additional glycoprotein are required for VZV entry and fusion, with both the gH/gL complex and the gE/gI complex as candidates for the function of additional glycoprotein to facilitate fusion. (12)

In beta herpesviruses, gB and gH/gL are sufficient for cell entry and fusion in some viral species, but in other species gH/gL is known to form complexes with either gQ, gO or UL128/UL130/UL131. Initial attachment is mediated by gB, which binds to proteoglycans of the host cell. (18) Subsequently, gB can bind to epidermal growth factor receptors (EGFR) and to various integrins as well, facilitating entry into the host cell. In addition, gH is known to interact with integrin $\alpha V\beta 3$. (12) HCMV gM is – in addition to gB – also known to mediate initial attachment by binding to proteoglycans. HCMV gM forms a heterodimer with gN (gM/gN) and both glycoproteins are thought to be essential for virus replication. (24,25) Also, neutralizing antibody responses against gM and gN have been described. (26,27) A study by Isaacson et al. has shown that HCMV gB is required for cell entry, but not for virion attachment, since virions without gB were similarly capable of binding to the cell surface as compared to virions containing gB. They proposed that gM is the envelope protein that is largely responsible for virion attachment to proteoglycans. (18) However, gM/gN does not seem to be required for cell fusion of HCMV. (28)

In HCMV, the gH/gL complex is associated with gO and UL128/UL130/UL131. Research has shown that cell entry of HCMV into fibroblasts depends on the trimeric complex of gH/gL/gO. A study by Vanarsdall et al. shows that the gH/gL/gO trimer promotes entry into fibroblasts, but not into epithelial cells. (19) Furthermore, HCMV seems to use a pentameric complex of gH/gL/UL128/UL130/UL131 to enter epithelial and endothelial cells. Zhou et al. have published that the pentamer and the trimer occur more frequently on the virus surface than the complex of just gH/gL. (20) In addition, it has been published that the pentamer is the main target of neutralizing antibodies to HCMV infection in epithelial/endothelial cells. (21,22) It has also been proposed that gO competes with UL128-131 to bind to the gH/gL complex, since the binding sites for these glycoproteins may overlap. (23) In conclusion, depending on the cell type HCMV may use the pentamer gH/gL/UL128/130/131 or the trimer gH/gL/gO for cell entry and fusion, in addition to HCMV gB.

In HHV-6, gO has been suggested to play a role in fusion as well, forming a trimer with gH/gL. (29) However, it has been shown that the absence of HHV-6B gO does not influence fusion activity. In contrast, the absence of HHV6B gB, gH, gL, gQ1 and gQ2 does affect fusion activity, since no significant fusion was found without one or more of these glycoproteins. (30) This suggests that gO in HHV-6B is not required for cell fusion. In a study by Tang et al., it was demonstrated that in HHV-6A, all four glycoproteins were required for binding of the gH/gL/gQ1/gQ2 to the CD46 receptor (gB was not included in this study). (31) The gH/gL/gQ1/gQ2 complex is described as a viral ligand that is able to bind to a cellular entry receptor. (32) The exact function of gO and the gH/gL/gO complex in both HHV-6A and HHV-6B remains to be elucidated.

HHV-7 uses gB for initial attachment to proteoglycans on the cell surface. Like in other herpesviruses, gB is activated by the gH/gL complex and is thought to play an important role in fusion. No other glycoproteins are known to be required for HHV-7 entry and fusion. (12) However, it has been proposed that the HHV-7 U47 gene (which is a positional homolog of CMV gO) associates with gH. (33)

In gamma viruses, other additional proteins are known to have a role in entry and fusion. Attachment to the cell surface in EBV is mediated by the glycoprotein complex gp350/220. This complex binds to complement receptor 2 (CR2) instead of proteoglycans. (12) Additionally, EBV uses the BMRF2 protein to bind to integrins during infection. (12) BMRF2 promotes the cell-to-cell spreading of the virus by increasing intercellular contacts. BMRF2 is heavily glycosylated by O-linked oligosaccharides and contains a RGD motif, which is likely to be the main binding site for integrins. (34,35) It has been proposed that EBV BMRF2 is critical for EBV infection and entry in oral epithelial cells, but not in B lymphocytes. (34) While the binding factors gp350/220 and BMRF2 are thought to play a key role in EBV entry into host cells, these glycoproteins are not thought to be essential for entry in fusion in all cell types. However, they are considered as targets of interest for development of a vaccine against EBV, and are therefore relevant to mention.

Cell entry and fusion of EBV depends on complexes of gH/gL and gH/gL/gp42 to trigger the fusogen gB. In B-cells with human leukocyte antigen (HLA) class II present on their surface, cell entry of EBV depends upon the trimer gH/gL/gp42 and gB. However, the gp42 binding site blocks the binding site for integrins, causing gp42 to inhibit epithelial cell entry. Therefore, in cells lacking HLA class II such as epithelial cells, EBV uses a gH/gL complex without gp42. Thus, for infection of both types of cells, EBV requires both gH/gL and gH/gL/gp42 complexes to create a triggering signal for fusogen gB. (36,37)

In HHV-8, the attachment is presumably mediated by K8.1, a surface protein positionally colinear to EBV gp350/220. It was therefore suggested that HHV-8 K8.1 could play an important role in attachment and entry. (38) HHV-8 K8.1 and gB both independently bind to proteoglycans for attachment. (12) For membrane fusion of HHV-8, gB and the gH/gL complex are required. The K8.1 glycoprotein seems to be dispensable for cell entry and fusion. No other glycoproteins are known to play a crucial role in HHV-8 fusion. (12)

Fusion assays

Extrapolating from the knowledge on herpesvirus entry and fusion, it is possible that for EEHV entry and fusion glycoproteins gB and gH/gL are sufficient. Another possibility is that EEHV uses one or several other glycoproteins – in addition to gB and gH/gL – for cell entry and fusion as well. To explore the first possibility, we aim to develop a fusion assay based on EEHV glycoproteins gB and gH/gL to analyze whether these glycoproteins are sufficient for EEHV entry and fusion. Therefore, cell-cell fusion assays or virus-cell fusion assays with pseudotyped viruses will be developed. Eventually, these assays are important for the development of virus neutralization tests. With virus neutralization tests, we aim to measure neutralizing antibodies against EEHV. Multiple studies have shown that in human herpesviruses such as HSV, HCMV and EBV, only a small fraction of neutralizing antibodies can protect against viral infection. These nABs may lie at the basis of antibody-based treatments against EEHV-HD. In addition, virus neutralization tests are important for assessing the antibody responses against future EEHV vaccine-candidates. With virus neutralization tests, it could be determined how much of the antibody response against the vaccine is actually neutralizing and therefore protective. Therefore, virus neutralization tests based on gB and gH/gL fusion assays would be important in measuring nABs against EEHV. For the development of EEHV gB and gH/gL fusion assays, we aimed to design gB and gH/gL constructs that are efficiently expressed on the cell surface, since this is required for binding to target cells and cell fusion.

***In silico* selection**

It would be valuable to know whether there are more proteins (in addition to gB and gH/gL) that contribute to EEHV virus-cell entry and fusion. This knowledge could be used in development, improvement and optimization of diagnostic tests, fusion assays and eventually in vaccine development. This thesis will describe the *in silico* selection of EEHV glycoproteins that may be important in cell attachment, entry and fusion. The characteristics of EEHV glycoproteins will be compared to the characteristics of known herpesvirus glycoproteins important in cell entry and fusion. In this manner, we hope to select EEHV glycoproteins may play a role in EEHV cell entry and fusion.

Material and methods

Induction of mutations and truncations in gB and gH

Codon optimized full length sequences of EEHV1A gB and gH were ordered at GenScript and cloned into a pFRT vector, essentially as described by Hoornweg et al. (Fig. 2) For both proteins, the native signal sequence was exchanged for a Guassia luciferase (Gluc) signal sequence. The gL-6xHis expression plasmid was previously described. (3)

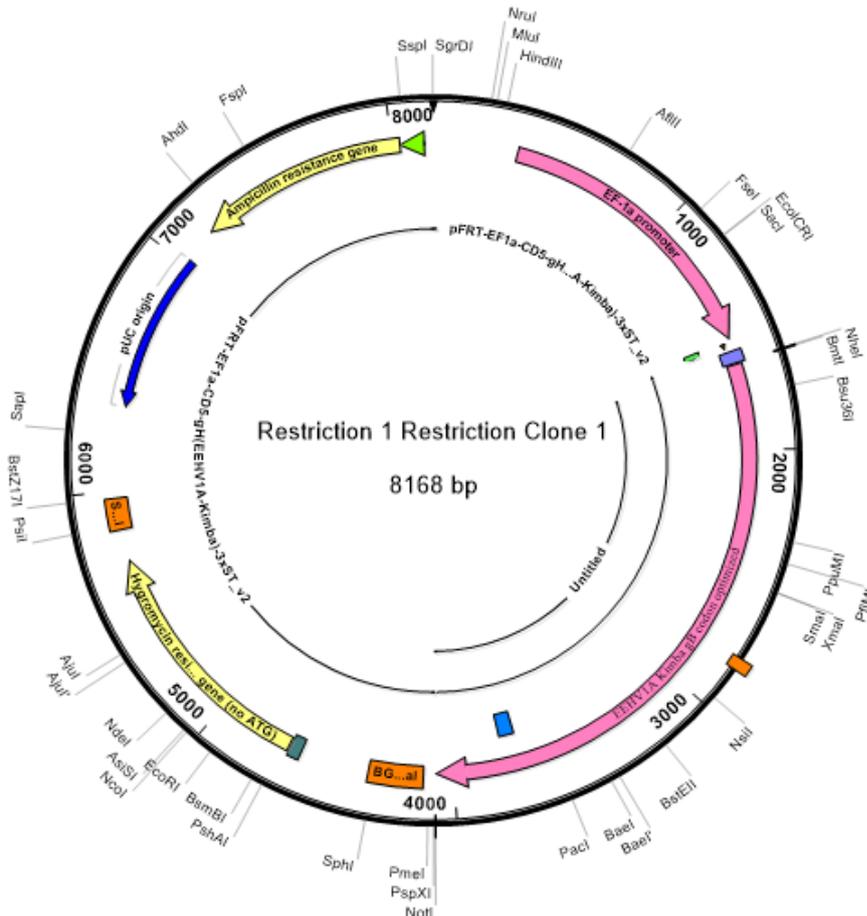


Figure 2: Schematic representation of EEHV1A gB(wt) in pFRT expression plasmid.

Mutations and truncations of constructs were induced by mutagenesis PCRs. Primers were designed using NEBaseChanger by selecting the sequence, mutagenesis type (deletion, insertion or point mutation), mutagenesis region and desired sequence. These primers (forward and reverse; both 1 μ l) were added to a PCR mix with the original construct (plasmid 10 ng/ μ l; 1 μ l added), Q5 buffer (5 μ l), Q5 enhancer (5 μ l), dNTP (10 mM; 1 μ l) and Q5[®] High-Fidelity DNA Polymerase (0,5 μ l) (filled up with MilliQ to a total of 25 μ l). As negative controls, PCR reactions with just primer and just plasmid added were performed. The PCR mix was ran in a thermal cycler, according to the following protocol:

Initial denaturation	95°C	3 min	
Denaturation	95°C	30 sec	┐
Annealing	Gradient °C	30 sec	┌ 25 cycli
Extension	72°C	3 min	└
Final extension	72°C	5 min	
	4°C	∞	

The exact annealing temperatures depended on the primers and were indicated by NEBaseChanger for each specific primer. Of the PCR product, 5 μ l was put on agarose gel to check whether the product has the expected size. Subsequently, a KLD reaction was performed by making a mastermix of 10x T4 ligase buffer (Fermentas), T4 ligase (Fermentas), T4 kinase (NEB), dpn1 (NEB),

PEG4000 (50%) and MilliQ water and then adding 1 µl PCR product to 9 µl mastermix. This KLD reaction was incubated for 60 minutes at room temperature.

For transformation, 5 µl of the KLD product was added to 50 µl of competent *E. coli* XL10 bacteria. After 30 minutes of incubation on ice, the bacteria were heat-shocked at 42 °C for 90 seconds and then again put on ice for 3 minutes. Then, 600 µl of sterile LB medium and the cells were put in an incubator (37 °C, shaking). After 40 minutes, 50-100 µl of the medium containing the transformed bacteria was plated out on a LB agar plate and incubated overnight at 37 °C. The next day, colonies were picked for overnight culture in LB + Amp, after which plasmids were purified.

For plasmid DNA purification, a miniprep was performed using Nucleospin Plasmid Easypure (Machery-Nagel). To ensure that the plasmids harbored the correct mutations, a small sample of the miniprep was sent to MacroGen Europe b.v. for sequencing. Sequences were analyzed with DNASTar Lasergene (Seqman Ultra). (39) Plasmid DNA of correct clones was increased by performing a midiprep, using Nucleobond Xtra Midi EF (Machery-Nagel).

Transfections

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C, 5% CO₂. Cells were passaged every 3-5 days (when grown to >80% confluency) using Trypsin-EDTA solution in a 1:10 ratio (1 ml cell solution added to 9 ml DMEM+FBS+P/S).

HeLa cells were plated in 96-well plates one day before the transfection, to obtain approximately 80% confluency at transfection. Cells were transfected with FuGENE® HD Transfection Reagent (Promega). For the transfection, 100 ng plasmid (per well) was mixed with 300 nl FuGENE® HD Transfection Reagent (in a 3:1 ratio to the plasmid) in a total volume of 5 µl Opti-MEM (Gibco). Transfection mix was incubated for 15 minutes before being added to the cells. 24 or 48 hours post transfection, cells were washed one time with PBS0 and fixated with either 4% PFA (20 minutes at room temperature) or ice-cold methanol (5 minutes at room temperature). In the optional trypsin treatment, three different trypsin dilutions (1:1000, 1:2000, 1:4000; diluted in OptiMEM) were used and were all added for one hour after 24 or 48 hours of transfection but before fixation. The optional treatment with an acid shock was done with citrate buffer (pH 5.00). Before fixation, cells were incubated for 3 minutes with citrate buffer and, after two washing steps, incubated with OptiMEM for another 3 hours.

After fixation, cells were washed once with PBS0. If PFA-fixated cells needed to be permeabilized, cells were treated with 1% TritonX100 in PBS0 for 10 minutes at room temperature. Then, the cells were washed three times with PBST and blocked with blocking buffer with BSA for one hour at room temperature. After one hour, the blocking buffer was removed and the primary antibody was added. For the fluorescent staining of EEHV1A gB, chicken yolk anti-gB antibodies (diluted 1:250) were used as primary antibody for staining. To stain the EEHV1A gH/gL complex, mouse anti-His antibodies (Takara Bio, diluted 1:1000) were used as primary antibodies, since a His-tag is incorporated in the gL construct. The cells were then incubated for one hour on the rocker. Then, cells were washed three times with PBST and the secondary antibody and DAPI staining were added to the cells. To be able to image gB and gH/gL transfected cells, these proteins were stained. For gB staining goat anti-chicken AF 488 antibodies (Thermo Fisher Scientific, diluted 1:400) were used as secondary antibody, added in combination with 4',6-diamidino-2-phenylindole (DAPI; personal stock, diluted 1:1000) to stain the cell nuclei. For gH/gL staining, goat anti-mouse AF 488/ AF 568 antibodies (diluted 1:400) were used as secondary antibodies. The cells were incubated for one hour on the rocker and after, the cells were washed three times with PBST on the rocker (with an interval of 5 minutes between each wash). The cells were kept in 1% PFA (diluted in PBS0) at 4 C until use. Imaging was performed with a fluorescence microscope (Evos). Images were analyzed and edited with FIJI (Fiji Is Just ImageJ).

***In silico* selection of proteins**

For *in silico* selection of proteins that may be involved in EEHV entry and fusion, multiple programs were used. Genbank was used for obtaining the DNA sequence of subtype EEHV1A (genome Kimba; Genbank: KC618527.1). (40) The online Prediction Servers of DTU Bioinformatics were used for running predictions on herpesvirus sequences. (41) Proteins that are involved in EEHV entry and/or fusion should be expressed on the membrane surface, either anchored to the viral envelope by a transmembrane domain or complexed to other glycoproteins with a transmembrane anchor. With the usage of the following Prediction Servers, it was investigated whether:

- glycoproteins contain a signal sequence. Signal peptides are able to translocate proteins to for instance the membrane and can therefore be found in many membrane proteins. If an EEHV glycoprotein is important in cell attachment, entry and/or fusion, it is expected that the glycoprotein contains a signal sequence in order to be located on the membrane surface. Prediction Servers SignalP and TargetP were used for the prediction of a signal sequence and cleavage site. (42,43)
- glycoproteins are anchored to the viral membrane, and in what conformation. In order to find transmembrane helices in proteins, the TMHMM Prediction Server was used, which predicts the amount and location of transmembrane helices. (44)
- glycoproteins contain N-linked glycolysation sites, which are important for the structure and function of many proteins. More specifically, N-linked glycolysation directs trafficking of glycoproteins. For potential functional EEHV glycoproteins, it is expected that there multiple glycolysation sites present, since these glycoproteins need to be transported to the plasma membrane. Prediction server NetNglyc was used to detect N-glycosylated sites. (45)

Subsequently, the EEHV1A glycoprotein characteristics derived from the Prediction Servers were compared to the characteristics of other herpesvirus glycoproteins which are important in entry and fusion. Via these comparisons, we hope to identify candidate EEHV proteins involved in entry and/or fusion.

Results

EEHV fusion assays

To explore whether expression of EEHV1A gB and gH/gL is sufficient to drive cell-cell fusion, we aimed to develop fusion assays with EEHV gB and gH/gL to see whether cell fusion can be induced.

(Cell surface) expression of gB

Wildtype gB is efficiently expressed, but not located on plasma membrane

First, we assessed intracellular and plasma membrane expression of the wildtype gB protein. Clear staining of this protein was observed in permeabilized cells, confirming expression of gB(wt). (fig. 3) However, no staining above background levels was visible in unpermeabilized cells, indicating that the protein was located intracellularly instead of primarily on the plasma membrane.

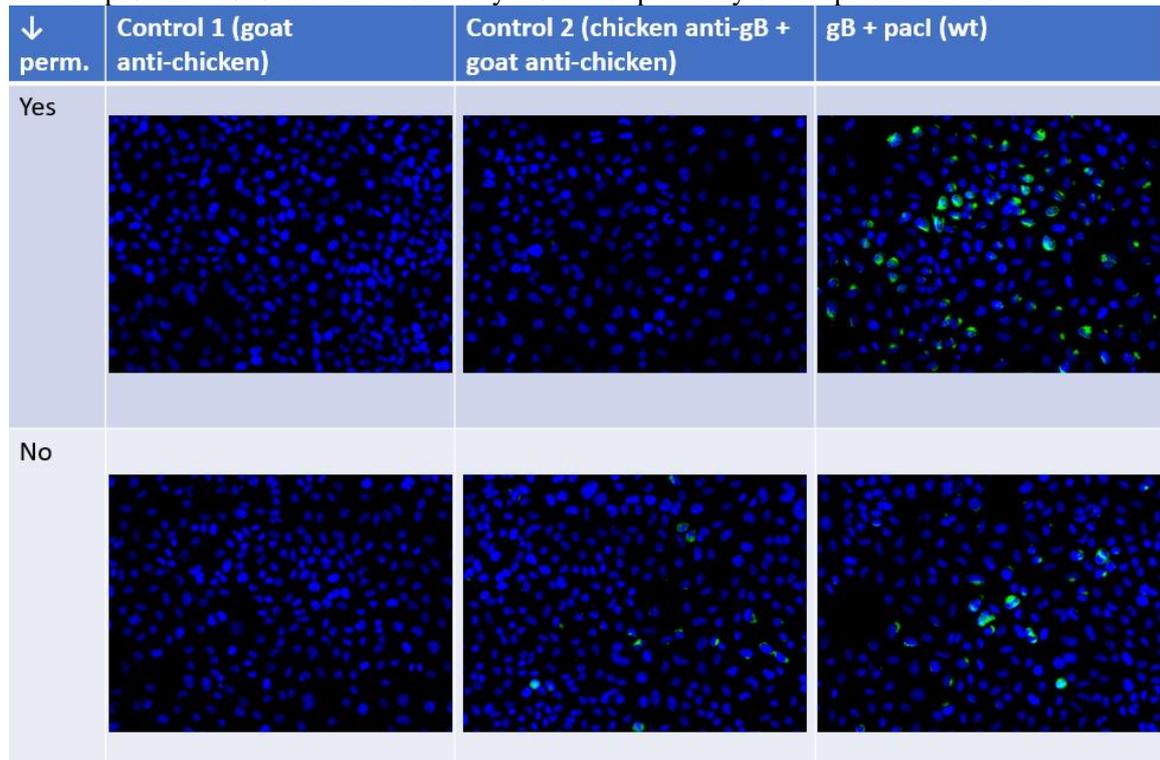


Figure 3: Fluorescence microscopy of HeLa cell transfection with construct EEHV1A gB(wt) (100 ng/well). In control 1, only the second antibody (goat anti-chicken) was added. In control 2, the first (chicken anti-gB yolk) and second antibody (goat anti-chicken) were both added. After a 24 hour transfection, the HeLa cells were PFA fixated (both permeabilized (upper row) and non-permeabilized (lower row)) and stained according to protocol. Microscopy was performed with a fluorescence microscope at 20x objective. The images were edited in the same way with FIJI/ImageJ.

To enhance cell surface expression of the gB protein, multiple truncated and mutated gB constructs were developed. The developed constructs included truncated gB construct without the YTAL signal sequence (gB(-YTAL)), gB with a mutation in the YTAL signal sequence (gB(FTAL)), gB minus 20, 30 and 50 amino acids of the cytoplasmic tail (gB(-20AA), gB(-30AA), gB(-50AA)) and gB minus the whole cytoplasmic domain (gB(-CTD)). Also, two gB constructs with the CTD and TMD of VSV-G were designed (with a difference of five amino acids before the transmembrane domain). It has been shown that fusion of HCMV gB was enhanced as a result of the exchange a VSV-G transmembrane domain and cytoplasmic tail. (46) All developed gB constructs are schematically depicted in Fig. 4.

Glycoprotein B

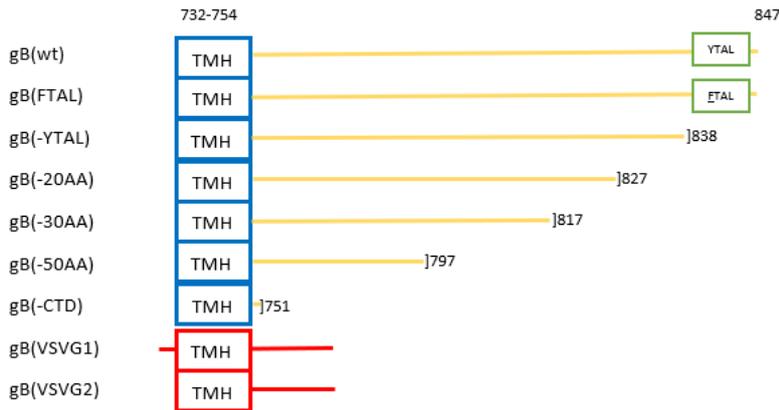


Figure 4: Schematic overview of designed gB constructs.

Deletion of the complete gB CTD leads to cell surface expression of gB

Cells were transfected with the newly produced constructs and fixated with 100% methanol (permeabilized) as well as PFA (non-permeabilized). All constructs except for the gB(-YTAL) construct were well produced in the permeabilized condition.

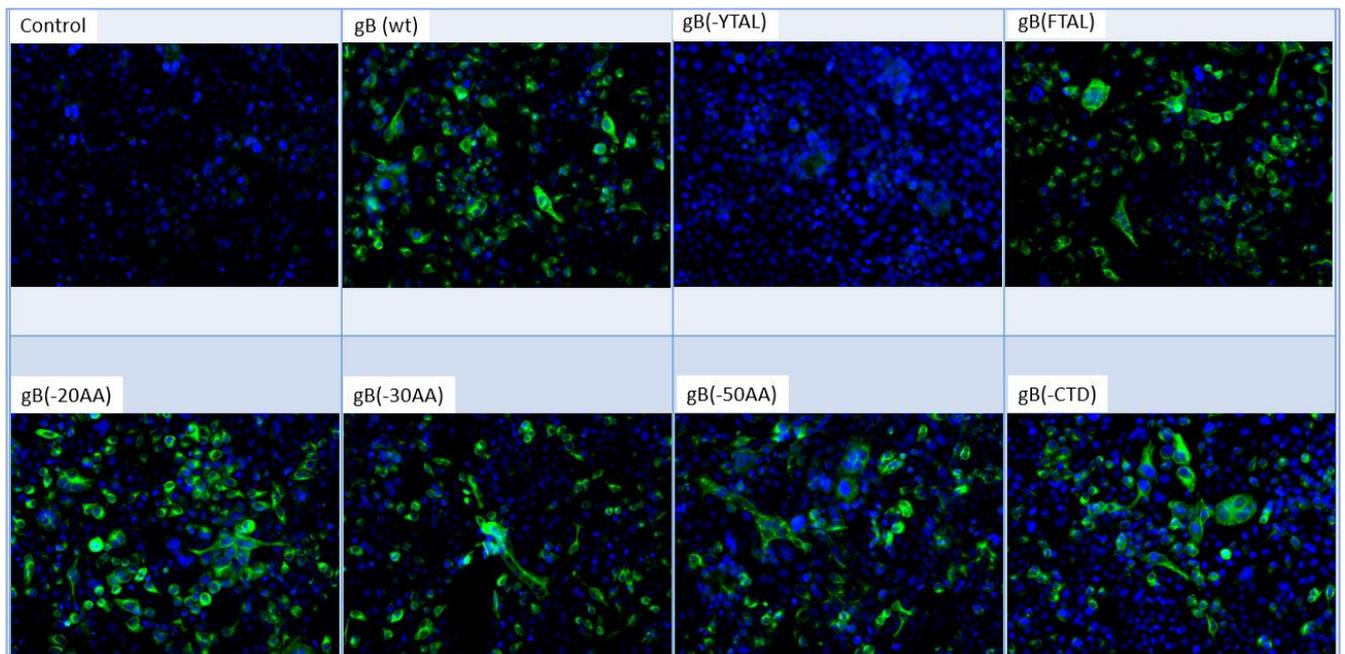


Figure 5: Fluorescence microscopy of HeLa cell transfection with constructs gB(wt), gB(-YTAL), gB(-20AA), gB(-30AA), gB(-50AA), gB(-CTD), gB(FTAL) (100 ng/well). GFP was transfected to determine the fusion rate of HeLa cells (100 ng/well). As control staining, only gH was added (100 ng/well). After a 48 hour transfection, the HeLa cells were fixated under permeabilizing conditions and stained according to protocol. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

Fig. 6 shows HeLa cells transfected with the same gB constructs, but fixated under non-permeabilizing conditions. In the wells with gB(-20AA), gB(-30AA) and gB(-CTD), it was clear that there was gB staining present besides the background staining. The wells transfected with the gB(-CTD) construct showed gB staining at the cell surface most obviously. In the wells with the gB(wt), gB(-

YTAL), gB(-50AA) and gB(FTAL) constructs, gB staining was difficult to distinguish from the background staining. (fig. 6)

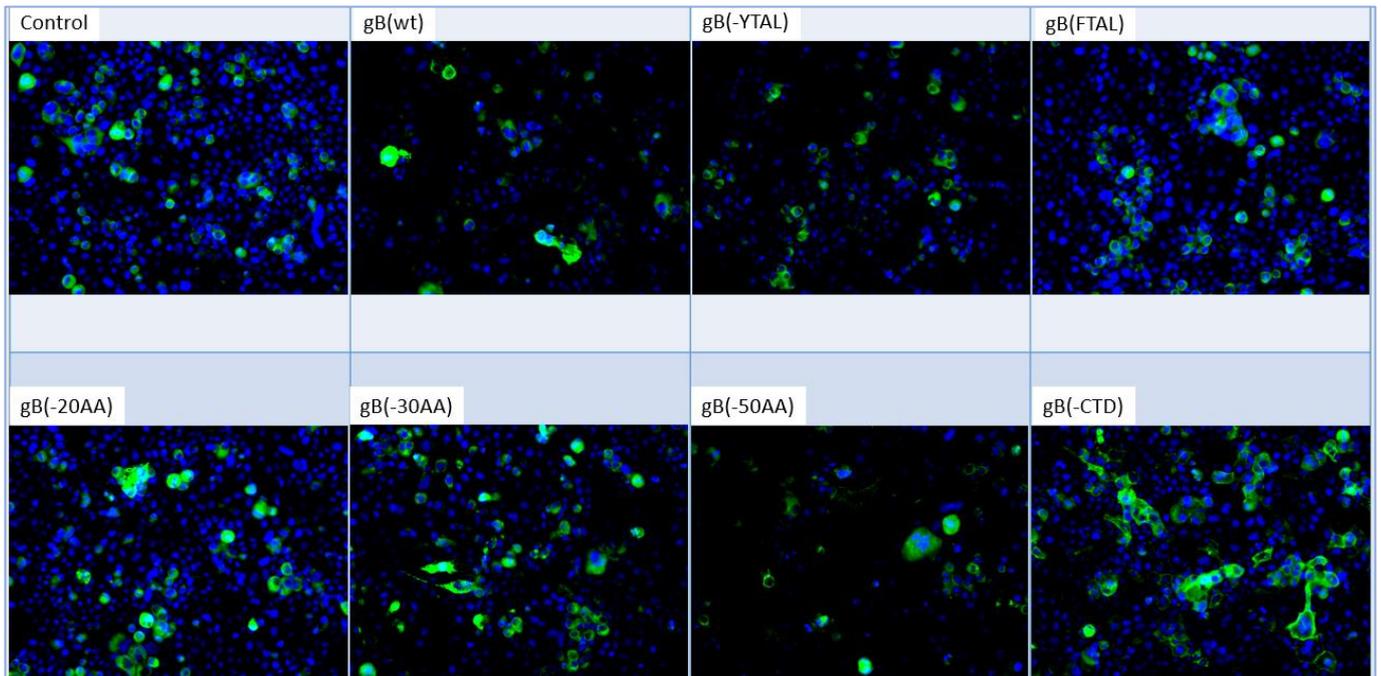


Figure 6: Fluorescence microscopy of HeLa cell transfection with constructs gB(wt), gB(-YTAL), gB(-20AA), gB(-30AA), gB(-50AA), gB(-CTD), gB(FTAL) (100 ng/well). As control staining, only gH was added (100 ng/well). After a 48 hour transfection, the HeLa cells were fixated under permeabilizing conditions and stained according to protocol. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

Exchange of the gB TMD/CTD region for the VSV-G TMD/CTD shows no beneficial effect on plasma membrane expression over the gB-CTD construct

Fig. 7 shows a transfection with gB(-CTD), gB(VSV-G1) and gB(VSV-G2). The gB(VSV-G1) and gB(VSV-G2) constructs were produced in the cells, but did not show more membrane surface staining than the gB(-CTD) construct. Overall, it was observed that exchange of the gB TMD/CTD region for the VSV-G TMD/CTD shows no beneficial effect on plasma membrane expression over the gB(-CTD) construct.

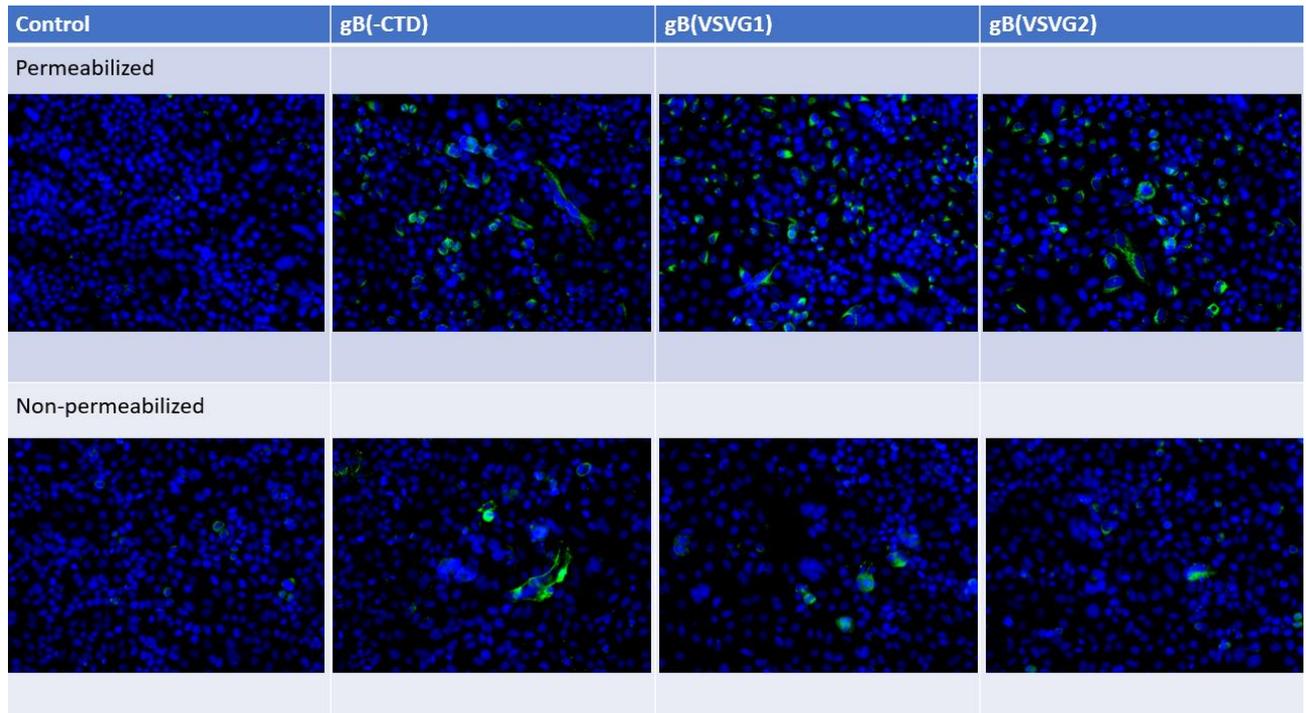


Figure 7: Fluorescence microscopy of HeLa cell transfection with constructs gB(-CTD), gB(VSV-G1) and gB(VSV-G2) (100 ng/well). As control staining, only gL was added (100 ng/well). After a 48 hour transfection, the HeLa cells were fixated and permeabilized (upper row) or non-permeabilized (lower row) and stained according to protocol. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

(Cell surface) expression of gH

All gH constructs were efficiently expressed, but only the deletion of the Yxx ϕ signal led to cell surface expression of gH

In addition to the designed gB constructs, we also designed multiple gH constructs. Three gH constructs were designed and compared: gH(wt), gH(-YQKL) and gH(FQKL). The gH(-YQKL) and gH(FQKL) constructs were developed in addition to gH(wt) since mutations in the YXX ϕ signal could enhance membrane expression of the construct. Fig. 8 shows an overview of all designed mutated/truncated gB and gH constructs.

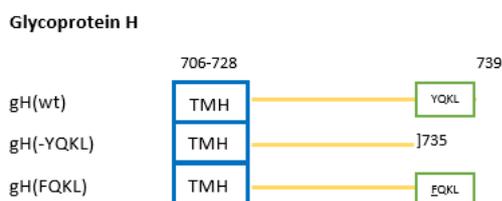


Figure 8: Schematic overview of designed gH constructs.

Under permeabilized conditions, cells showed clear staining above background control. (Fig. 9) It cannot be concluded with certainty that gH was produced, since gL could also be soluble and not bound to gH. Therefore, only gL production could be assessed with certainty in the permeabilized condition. In the non-permeabilized wells, the production of gH could be assessed, since gL can only be membrane-bound through the gH/gL complex.

To determine which constructs are expressed on the membrane surface, the cells were fixated under non-permeabilizing conditions. Under these conditions, all constructs appeared to show staining

above background control, however only the gH(-YQKL) construct showed distinctive staining on the cell membrane. (Fig. 9)

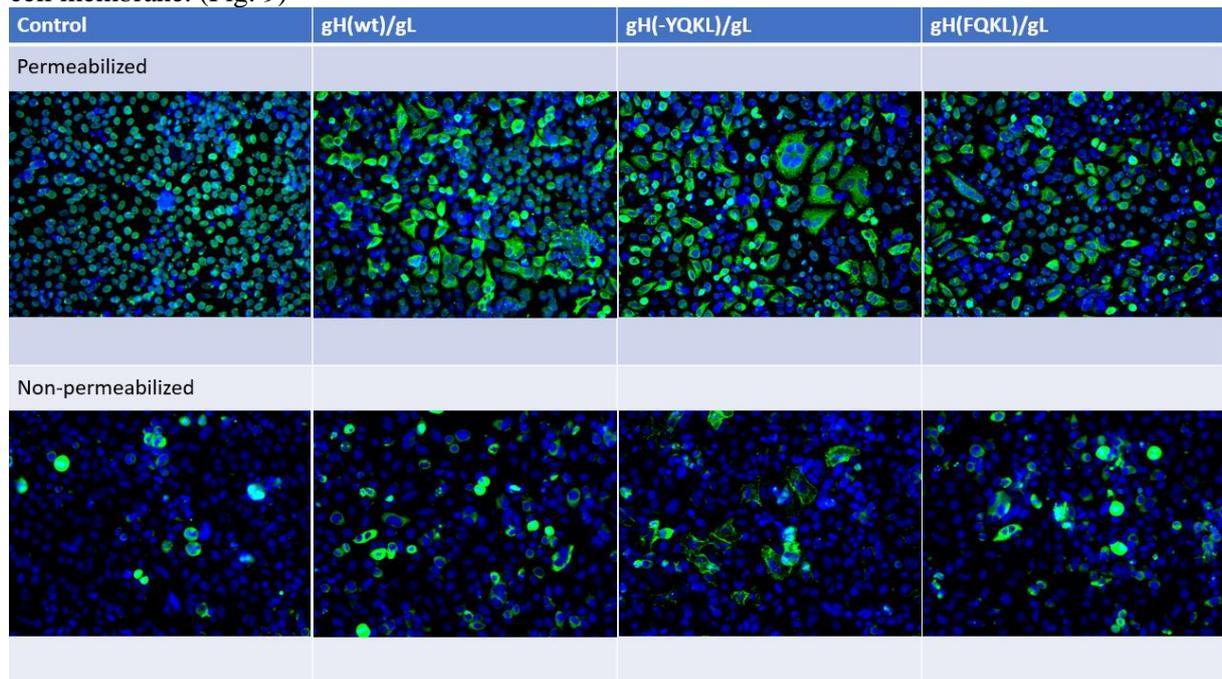


Figure 9: Fluorescence microscopy of HeLa cell transfection with constructs gH(wt), gH(-YQKL) and gH(FQKL) (80 ng/well) and gL (20 ng/well). As a control to determine the amount of background staining, only gH(wt) (without gL) was transfected. After a 48 hour transfection, the HeLa cells were fixated under permeabilized (upper row) or non-permeabilized (lower row) conditions and stained with anti-His (targeting gL) according to protocol. Because gL by itself would not be able to be expressed on the cell surface, cell surface staining of gL indicates that a gH/gL complex was formed and gH is expressed on the cell surface as well. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

Fusion upon single and co-expression of gB and gH constructs

Next, attempts were made to induce cell fusion with the produced constructs. In cells transfected with the gB constructs, syncytia could be found in wells with the constructs gB(FTAL), gB(-20AA), gB(-30AA), gB(-50AA) and gB(-CTD). Especially in the wells with the gB(-50AA) and gB(-CTD) constructs, multiple multinuclear cells could be seen. (Fig. 5)

To see if the presence of gH/gL complex enhanced cell fusion in gB-transfected cells, gB was cotransfected in combination with gH/gL (fixated in permeabilized condition). In this experiment gH(wt) was used, since at the time of this experiment it was not clear yet that the gH(-YQKL) construct was expressed more efficiently on the cell surface. All gB constructs were included to see if the presence of gH/gL could induce cell fusion in combination with the particular gB constructs. (Fig. 10)

In wells with gB(wt) + gH/gL, slightly more syncytia seemed to have formed compared to the transfection of gB solely. The cellular production of the gB(-YTAL) and gB(-20AA) constructs in this transfection was low. The wells with gB(-30AA), gB(-50AA), gB(-CTD) and gB(FTAL) showed a similar amount of syncytia when cotransfected with gH/gL compared to the transfections with no gH/gL transfected. These results have been visually assessed and therefore no major conclusions on syncytia formation can be drawn. (Fig. 10)

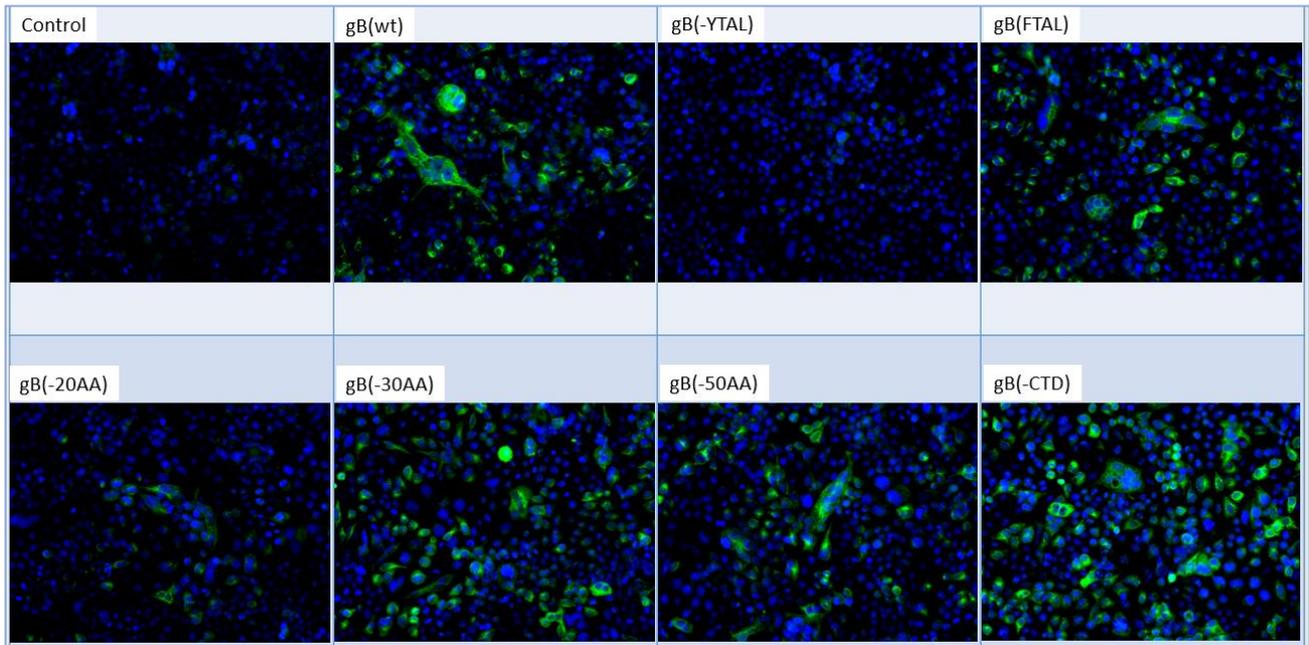


Figure 10: Fluorescence microscopy of HeLa cell transfection with constructs gB(wt), gB(-YTAL), gB(-20AA), gB(-30AA), gB(-50AA), gB(-CTD), gB(FTAL) (all 50 ng/well) and gH/gL (respectively 40 and 10 ng/well). As control staining, only gH was added (100 ng/well). After a 48 hour transfection, the HeLa cells were fixated under permeabilizing conditions and stained according to protocol. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

The gB(-CTD) construct (that showed the most cell surface staining in earlier transfections) was cotransfected with all designed gH constructs and gL, to see whether cotransfection could enhance cell surface staining or cell fusion. A green fluorescent staining was used to show gB staining and a red fluorescent staining was used to show gH/gL staining (binding to the His-tag of gL). In wells under permeabilized conditions, it could be seen that the gB(-CTD) construct was produced well upon single and co-transfection. The gL construct was produced efficiently as well, however the production of the gH construct could not be assessed with certainty under permeabilized conditions (since staining could also indicate soluble gL). (Fig. 11)

Upon co-transfection of gB(-CTD) and gH(-YQKL), both constructs seemed to reach the cell surface. All cotransfected gB + gH/gL wells showed some large syncytia and the syncytia in these images seemed to be formed by double-transfected cells. (showing both gB and gH/gL staining). (Fig. 12-14) However, it cannot be reported with certainty that fusion was gB and gH/gL induced or not, since HeLa cells also appear to show spontaneous formation of syncytia.

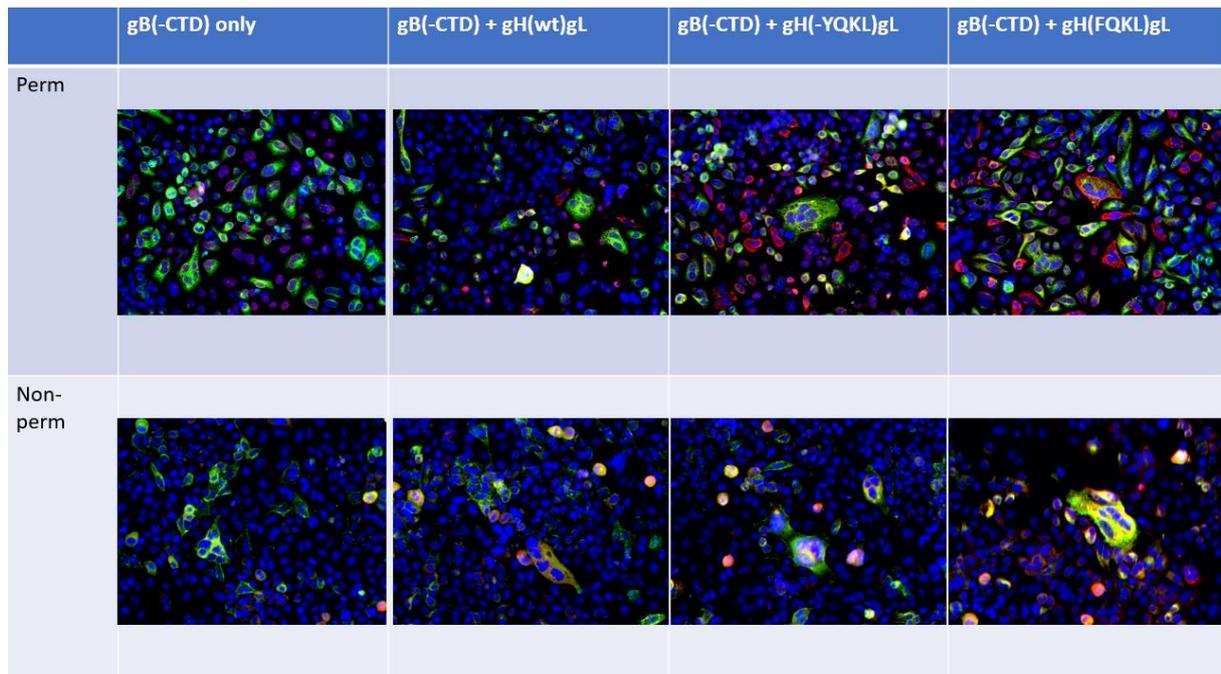


Figure 11: Fluorescence microscopy of HeLa cell transfection with construct gB(-CTD) (100 ng/well when solely transfected, in combination with gH/gL 50 ng/well) and gH/gL (respectively 40 and 10 ng/well). The constructs gH(wt), gH(-YQKL) and gH(FQKL) were all tested in combination with gB(-CTD). After a 48 hour transfection, the HeLa cells were methanol (upper row) or PFA (lower row) fixated and stained according to protocol. Anti-gB green fluorescent staining and anti-His red fluorescent staining (binding to gL and therefore to the gH/gL complex) were used. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

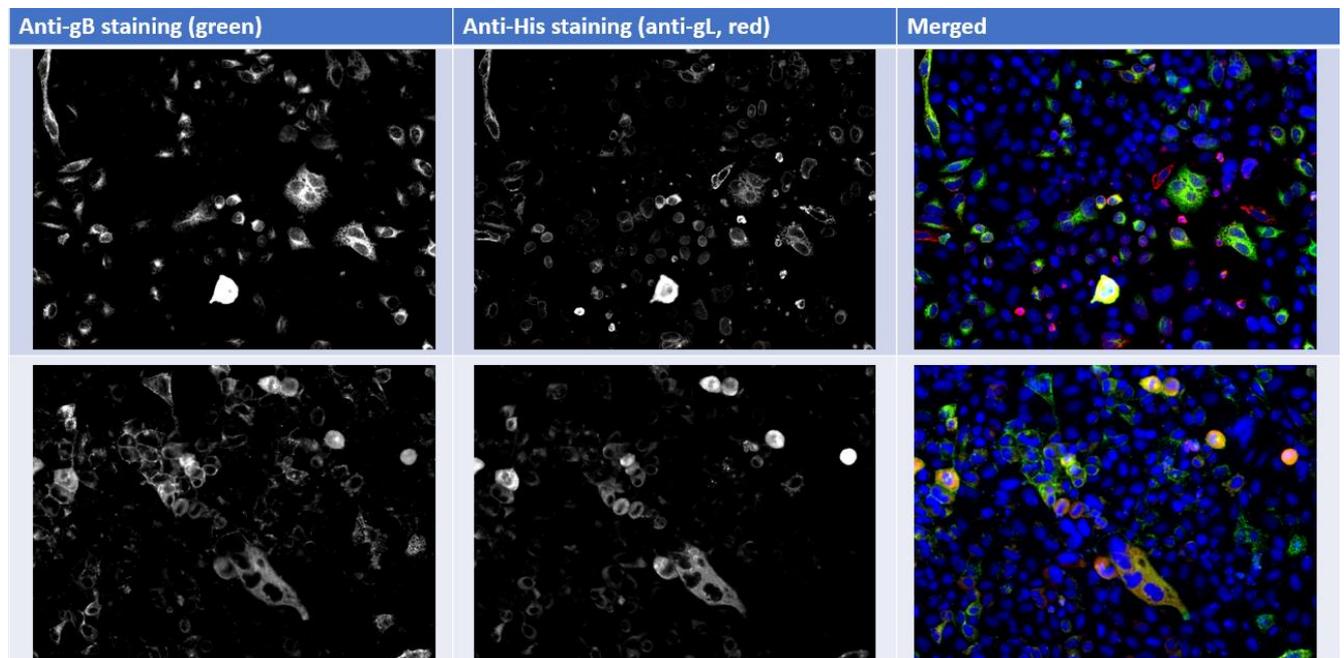


Figure 12: Fluorescence microscopy of HeLa cell transfection with construct gB(-CTD) (50 ng/well) and gH(wt)gL (respectively 40 and 10 ng/well). After a 48 hour transfection, the HeLa cells were methanol (upper row) or PFA (lower row) fixated and stained according to protocol. Anti-gB green fluorescent staining and anti-His red fluorescent staining (binding to gL and therefore to the gH/gL complex) were used. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

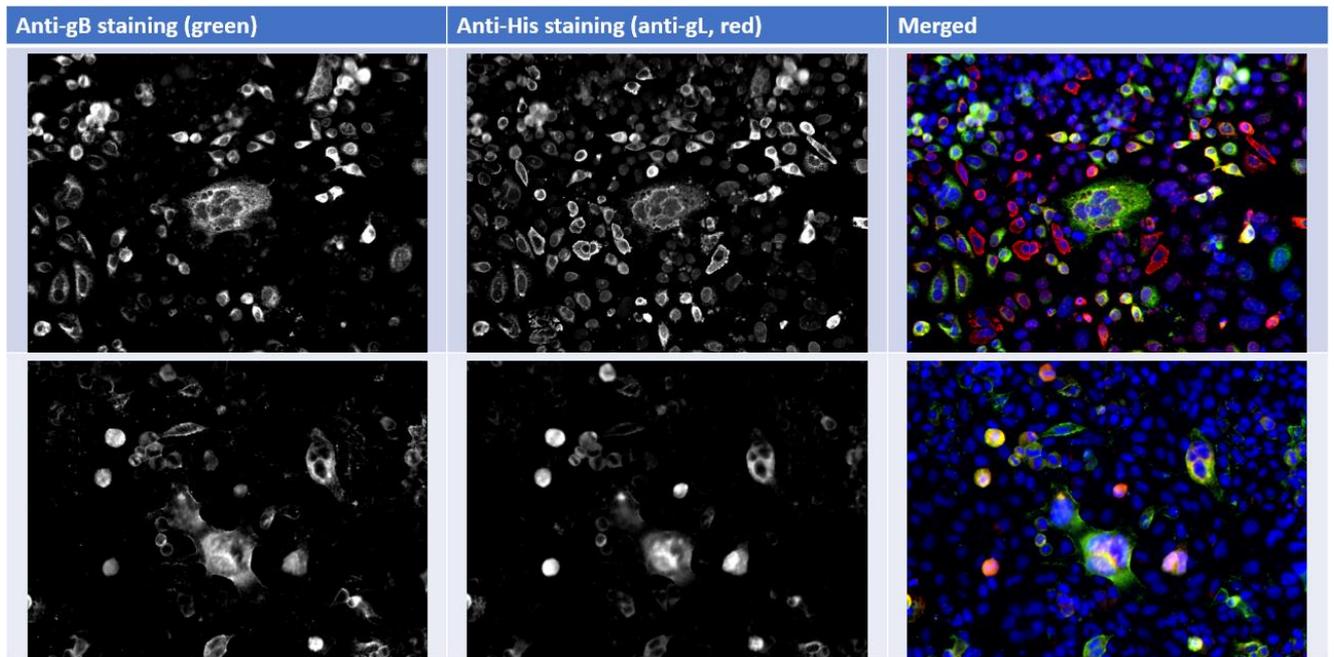


Figure 13: Fluorescence microscopy of HeLa cell transfection with construct gB(-CTD) (50 ng/well) and gH(-YQKL)/gL (respectively 40 and 10 ng/well). After a 48 hour transfection, the HeLa cells were methanol (upper row) or PFA (lower row) fixated and stained according to protocol. Anti-gB green fluorescent staining and anti-His red fluorescent staining (binding to gL and therefore to the gH/gL complex) were used. Microscopy was performed with a fluorescence microscope at 20x. Images were analyzed in the same way with FIJI/ImageJ.

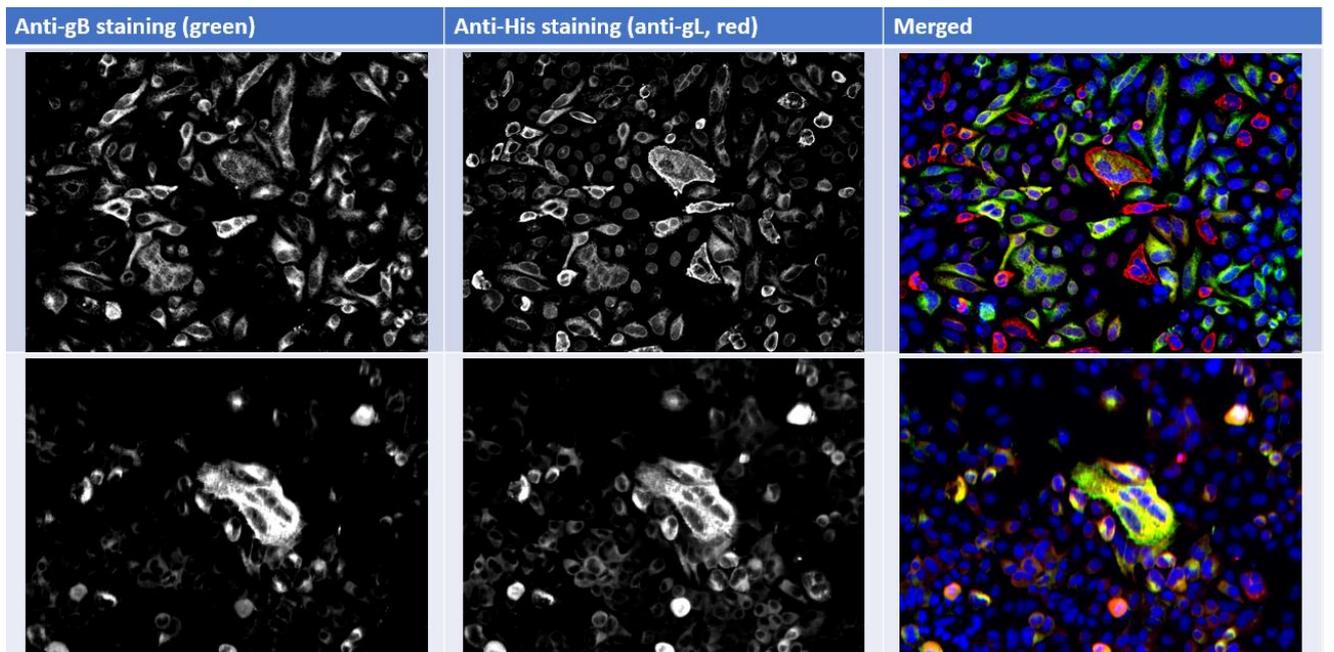


Figure 14: Fluorescence microscopy of HeLa cell transfection with construct gB(-CTD) (50 ng/well) and gH(FQKL)/gL (respectively 40 and 10 ng/well). After a 48 hour transfection, the HeLa cells were methanol (upper row) or PFA (lower row) fixated and stained according to protocol. Anti-gB green fluorescent staining and anti-His red fluorescent staining (binding to gL and therefore to the gH/gL complex) were used. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

Fusion induction

Furthermore, attempts have been made to induce EEHV gB + gH/gL fusion using an acid shock or trypsin treatment, since these treatments are known to induce cell fusion. In this experiment the gH(wt) construct was used instead of the optimal membrane expressed gH(-YQKL) construct since at the time it was not clear yet that gH(-YQKL) showed better cell surface staining. In all conditions, around the same numbers of syncytia could be found. The cells showed no enhanced fusion after the acid shock, trypsin treatment or both. (Fig. 15)

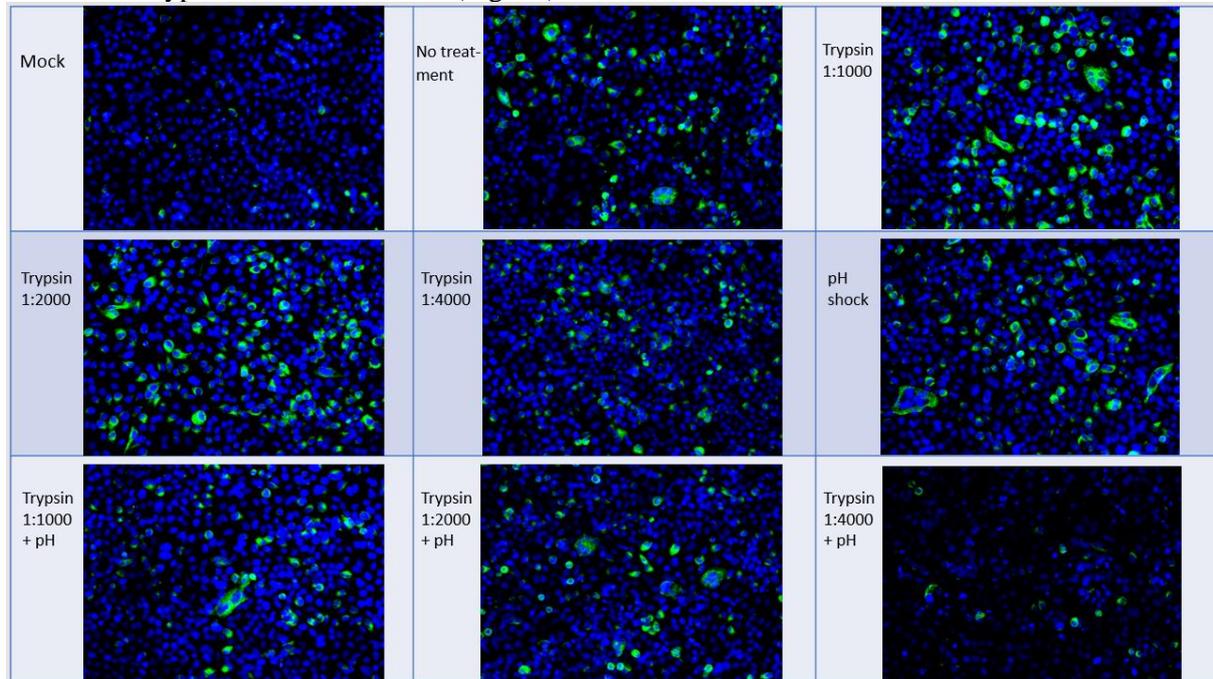


Figure 15: Fluorescence microscopy of a transfection of HeLa cells with construct gB(-CTD) (100 ng/well). After 48 hours of transfection, cells were treated with trypsin (Sigma), an acid shock or with both. After the extra treatment, all cells were PFA fixated and stained according to protocol. Microscopy was performed with a fluorescence microscope at 20x objective. Images were analyzed in the same way with FIJI/ImageJ.

In silico selection of potential EEHV fusion glycoproteins

Characteristics of glycoproteins essential for virus entry and fusion of the human alpha-, beta-, and gamma herpesviruses were studied and compared with characteristics of predicted glycoproteins of EEHV for the purpose of identifying EEHV glycoproteins with a putative role in entry and/or fusion.

Alpha herpesviruses

Glycoprotein	Length (amino acids)	Signal peptide	Signal sequence	TargetP	Transmembrane helices	Where	N-glycosylation
HSV1 gB	904	++	1-30	Signal	++	3 (12-34, 748-770, 777-799)	++
HSV1 gH	838	++	1-18	Signal	++	1 (805-827)	++
HSV1 gL	224	++	1-19	Signal	-	-	+
HSV1 gC	511	++	1-24	Signal	++	1 (478-500)	++
HSV1 gD	394	++	1-25	Signal	++	1 (342-364)	+
HSV2 gB	904	++	1-22	Signal	++	3 (7-29, 745-767, 774-796)	++
HSV2 gH	838	++	1-18	Signal	++	1 (805-827)	++
HSV2 gL	224	++	1-19	Signal	-	-	+
HSV2 gC	480	+	1-27	Signal	++	2 (7-29, 447-469)	++
HSV2 gD	393	+	1-25	Signal	++	1 (341-363)	++
VZV gB	931	-	-	Other	++	2 (46-68, 789-811)	++
VZV gH	841	++	1-17	Signal	++	1 (804-826)	++
VZV gL	159	+	1-21	Signal	-	-	+
VZV gC	560	++	1-21	Signal	++	1 (532-554)	++
VZV gE	623	-	-	Other	++	2 (7-24, 538-560)	+
VZV gI	354	++	1-20	Signal	++	1 (273-295)	++

Table 1: Alpha herpesvirus glycoproteins important in cell attachment, entry and fusion. Information on glycoproteins was assembled through Genbank and Prediction Servers (SignalP, TargetP, TMHMM and NetNglyc). (41) The herpesvirus strains that were used in this study include HSV-1 (strain KOS; Genbank: JQ780693.1), HSV-2 (strain HG52; Genbank: NC_001798.2) and VZV (strain Dumas; Genbank: X04370.1). (47–49)

Table 1 shows the results of the in silico analyses of important alpha herpesvirus fusion proteins. All proteins contained either a signal sequence and/or a transmembrane helix and were predicted to be N-glycosylated. The only protein that did not contain a transmembrane helix was gL, which is anchored to the viral surface via its interaction with gH. Notably, the gB protein of both HSV-1 and -2 was predicted to contain 3 transmembrane domains, while the actual protein is known to contain only 1 transmembrane helix at pos. 771-801. (50) The first predicted transmembrane helix overlapped with the gB signal sequence and is likely cleaved from the mature protein. Some transmembrane helices may have a similar effect as signal sequences, especially when they are located similarly. The second transmembrane helix included the membrane proximal region, which contains a relatively high percentage hydrophobic amino acids. The third largely overlapped with the actual transmembrane helix. For VZV gB, gE and HSV-2 gC additional transmembrane helix regions were predicted as well, which again overlapped with the signal peptide.

Bèta herpesviruses

Glycoprotein	Length (amino acids)	Signal peptide	Signal sequence	TargetP	Transmembrane helices	Location of transmembrane helices	N-glycosylation
HCMV gB	907	++	1-20	Signal	++	3 (5-24, 725-744, 751-773)	++
HCMV gH	743	++	1-29	Signal	++	1 (719-737)	++
HCMV gL	278	++	1-30	Signal	++	1 (7-29)	+
HCMV gO	472	-	-	Other	++	1 (7-29)	++
HCMV UL128	171	++	1-27	Signal	-	-	-
HCMV UL130	214	++	1-25	Signal	-	-	+
HCMV UL131	129	++	1-20	Signal	-	-	+
HHV-6 gB	830	++	1-22	Signal	++	1 (688-710)	++
HHV-6 gH	694	++	1-16	Signal	++	1 (668-690)	++
HHV-6 gL	250	++	1-18	Signal	-	-	+
HHV-6 gO)	738	++	1-20	Signal	++	1 (7-24)	++
HHV-6 gQ (gp82-105)	616	++	1-24	Signal	-	-	++
HHV-7 gB	822	++	1-22	Signal	++	2 (654-676, 683-705)	++
HHV-7 gH	690	-	1-16	Signal	++	1 (665-687)	++
HHV-7 gL	246	++	1-19	Signal	-	-	++
HHV-7 gO (gene U47)	313	-	-	Other	++	1 (7-29)	++

Table 2: Bèta herpesvirus glycoproteins important in cell attachment, entry and fusion. Information on these glycoproteins was assembled through Genbank and Prediction Servers (SignalP, TargetP, TMHMM and NetNglyc). (41) The herpesvirus strains that were used in this study include HCMV (strain Merlin; Genbank: NC_006273.2), HHV-6B (strain Z29; Genbank: NC_000898.1) and HHV-7 (strain RK; Genbank: NC_001716.2). (51–53)

The gB proteins from HCMV, HHV-6 and HHV-7 all contained a signal sequence, which was expected. In HHV-6 gB one transmembrane helix was predicted, which correlates with the knowledge on gB structure. (11,54,55) In HHV-7 gB two transmembrane helices were predicted, where the first overlapped largely with the membrane proximal region and the second overlapped with the actual transmembrane helix. In HCMV gB three transmembrane helices were predicted, of which – like HSV-1 and -2 gB – the first largely overlapped with the signal sequence, the second overlapped with the membrane proximal region and the third overlapped with the actual transmembrane helix. (56)

HHV-6 gL and HHV-7 gL contained a signal sequence but no transmembrane helix, which is expected since gL is anchored to the viral surface via its interaction with gH. In HCMV gL a transmembrane helix was predicted, but the transmembrane helix overlapped with the gL signal sequence and is therefore likely to be cleaved off from the protein. (57)

The glycoprotein gO is shared within all beta herpesviruses, although the characteristics according to the Prediction Servers vary. In HCMV gO and HHV-7 gO no signal sequence was detected, in contrast to HHV-6 gO. All beta herpesvirus gO glycoproteins did contain N-glycosylated sites and one transmembrane helix with a very similar location. However, it is known that HCMV gO contains a signal sequence which is cleaved. (58) The predicted transmembrane helixes are therefore possibly signal sequences that are cleaved off, since gO is presumably soluble. All components of the HCMV UL128-131 complex did include a signal sequence. In none of the components a transmembrane helix was detected and N-glycosylation sites were only present in UL130 and UL131.

In the gQ glycoprotein of HHV-6, a signal sequence and multiple N-glycosylation sites were detected, but no transmembrane helixes. It was expected that HCMV gO, HCMV UL128-131 and HHV-6 gQ do not contain a transmembrane helix, since these proteins are proposed to form a complex with gH/gL and held in the viral envelope by the transmembrane helix of gH. (19,23,57–59)

Gamma herpesviruses

Glycoprotein	Length (amino acids)	Signal peptide	Signal sequence	TargetP	Transmembrane helixes	Locations of transmembrane helixes	N-glycosylation
EBV gB	857	++	1-22	Signal	++	1 (733-752)	++
EBV gH	706	++	1-17/18	Signal	++	1 (680-702)	++
EBV gL	137	++	1-22	Signal	++	1 (4-26)	++
EBV GP42	223	+	1-33	Signal	++	1 (7-29)	++
EBV GP350	907	-	-	Other	++	1 (863-885)	++
EBV GP220	715	-	-	Other	++	1 (671-693)	++
EBV BMRF2	357	-	-	Other	++	10 (9-31, 46-65, 70-89, 99-121, 134-152, 157-179, 218-240, 265-287, 299-321, 331-353)	-
HHV-8 gB	845	++	1-26	Signal	++	2 (7-26, 733-752)	++
HHV-8 gH	730	++	1-21/22	Signal	++	1 (704-726)	++
HHV-8 gL	167	++	1-20	Signal	-	-	+

Table 3: Glycoproteins important for cell attachment, entry and fusion in gamma herpesviruses. Information on these glycoproteins was assembled through Genbank and Prediction Servers (SignalP, TargetP, TMHMM and NetNglyc). (41) The gamma herpesvirus strains that were used in this study are EBV (strain B95-8; Genbank: V01555.2) and HHV-8 (strain BCBL1; Genbank: MT936340.1). (60,61)

As expected, in EBV gB and gH a signal sequence, one transmembrane helix and N-glycosylated sites were predicted. In EBV gL a transmembrane helix was predicted, but like in HCMV gL, the transmembrane helix overlapped largely with the signal sequence and is likely to be cleaved off.

In EBV gp42, a signal sequence, one transmembrane helix and N-glycosylation sites were detected by the Prediction Servers. The predicted transmembrane helix of EBV gp42 overlaps with the signal peptide and is therefore likely to be cleaved off. This correlates with the proposition that EBV gp42 is soluble and only bound to the virus particle via gH/gL. (62) Both components of the gp350/220 complex contained one transmembrane helix and multiple N-glycosylation sites, but no signal sequence. In EBV BMRF2, no signal sequence or N-glycosylation sites were detected, but ten transmembrane helixes were found.

EEHV

All known glycoproteins in the EEHV1A strain of Kimba were ran through the same Prediction Servers as all other herpes glycoproteins.

Glycoprotein	Length (amino acids)	Signal peptide	Signal sequence	TargetP	Transmembrane helixes	Location of transmembrane helixes	N-glycosylation
E1	481	+	1-19	Signal	++	7 (214-236, 249-271, 281-303, 315-337, 378- 400, 405-422, 432-454)	++
E4	441	-		Other	++	1 (13-35)	++
E20A	104	~	1-24	Signal	-	-	+
E23 (vOX2-4)	264	-		Other	-	-	++
E24 (vOX2-3)	176	++	1-20	Signal	-	-	++
E25 (vOX2-2)	260	-		Other	++	1 (227-249)	++
E31	224	++	1-19	Signal	-	-	++
gB	850	++	1-42	Signal	++	1 (732-754)	++
gN	96	++	1-23	Signal	++	2 (7-29, 61-83)	-
gO	212	-		Other	-	-	++
gH	739	++	1-29	Signal	++	1 (706-728)	++
gM	362	-		Other	++	8 (13-35, 82-104, 125- 147, 151-173, 201-223, 238-260, 267-287, 297- 319)	+
gL	304	-		Other	-	-	++
ORF-O (E37)	383	++	1-21	Signal	-	-	++
ORF-P (E38)	525	++	1-16	Signal	-	-	++
ORF-Q	326	++	1-17	Signal	-	-	++
E49	137	-		Other	++	1 (114-136)	+
E50 (vIGfam1)	142	-		Other	-	-	++
E51	130	-		Other	-	-	++
E52 (vIGfam2)	160	++	1-19	Signal	-	-	++
E53 (vIGfam2.5)	109	-		Signal	++	2 (13-35, 85-107)	++
E54 (vOX2-1)	295	-		Signal	++	2 (7-29, 257-279)	++
E55 (vIGfam3)	127	-		Signal	++	1 (7-29)	++

Table 4: All known EEHV1A(Kimba) glycoproteins were listed in this table. (40) Information on these glycoproteins was assembled through Genbank and Prediction Servers (SignalP, TargetP, TMHMM and NetNglyc). (41)

EEHV glycoproteins with predicted homologs in other herpesviruses

For EEHV1A gB and gH, the amino acid length, signal sequence, transmembrane helixes and N-linked glycosylation was very similar to gB and gH of other herpesviruses. Remarkably, in EEHV1A gL, neither a signal peptide nor a transmembrane helix was detected, even though in gL proteins of other EEHV subtypes a signal peptide and transmembrane helix were found (data not shown).

Other EEHV glycoproteins with known homologs in other HVs are gO, gM and gN. In gM no signal sequence was detected, but gM does contain multiple transmembrane helixes and some N-glycosylated sites. The first transmembrane helix of gM is likely to behave as a signal sequence. This is supported by a paper by Krzyzaniak et al, in which gM is described with seven transmembrane helixes. Therefore, the first predicted transmembrane helix of EEHV gM could possibly be cleaved of and function as a signal sequence. (25) Glycoprotein gN is a type I membrane protein and does contain a signal sequence. Two transmembrane helixes were detected in gN, but no N-glycosylated sites. However, gN is thought to contain one transmembrane helix. (25) Since the first predicted transmembrane helix overlaps with the signal sequence of EEHV gN, it is likely to be cleaved off.

In gO, nor a signal sequence or transmembrane helixes were detected. gO did contain N-glycosylated sites. Since gO is presumably soluble, it is plausible that gO does not contain a transmembrane helix. It would be expected that EEHV gO contained a signal sequence if it is important in entry and fusion, in order to be transported towards the plasma membrane. However, this was not predicted by the prediction

servers. It is important to note that the size of EEHV gO does not match other herpesvirus gO proteins, which raises the question whether EEHV gO was annotated correctly.

ORF-O, ORF-P, ORF-Q

EEHV glycoproteins ORF-O, ORF-P and ORF-Q are all signal proteins and novel herpesvirus proteins. ORF-P is conserved in EEHV1, EEHV2, EEHV5 and EEHV6 (thus the AT-rich branch) but absent in EEHV3 and EEHV4. ORF-Q is conserved in EEHV1 and EEHV6, absent in EEHV2 and EEHV5 and replaced by ORF-R in EEHV3 and EEHV4. (40)

ORF-O, ORF-P and ORF-Q all contained a signal sequence and multiple N-glycosylated sites, but no transmembrane helices. These characteristics are most similar to HCMV U128-131 and HHV-6 gQ1/2, which also contain a signal sequence and N-glycosylated sites but no transmembrane helices.

Other EEHV glycoproteins

Glycoprotein E1 Glycoprotein E1 is a novel herpesvirus protein common to all EEHVs. In EEHV1A E1, a signal sequence, seven transmembrane helices and multiple N-glycosylation sites were detected. These characteristics do not clearly compare to other herpesvirus fusion glycoproteins.

Glycoprotein E4 Glycoprotein E4 is common to all EEHVs as well. In EEHV1A E4, one transmembrane helix and multiple N-glycosylation sites but no signal sequence was detected. However, the transmembrane helix could possibly behave like a signal sequence, since transmembrane helices with a similar location in other herpesvirus glycoproteins had this function as well. Assuming that the predicted transmembrane helix in EEHV E4 carries out the function of a signal sequence, this glycoprotein shares most characteristics with HCMV U128-131 and HHV-6 gQ1/2.

Glycoprotein E20A Glycoprotein E20A is a type I membrane glycoprotein and common to all EEHVs. The Prediction Server SignalP did not clearly detect a signal sequence, but TargetP did. Some N-glycosylation sites were detected but no transmembrane helices. These characteristics are most similar to HCMV U128-131 and HHV-6 gQ1/2, as these glycoproteins also contain a signal sequence of the same length, some N-glycosylation sites and no transmembrane helices.

Glycoprotein E31 EEHV1A E31 is present only in the AT-rich branch of EEHV subtypes (EEHV1A, EEHV1B, EEHV5). E31 contained a signal sequence and multiple N-glycosylation sites, but no transmembrane helices. These characteristics are most similar to HCMV U128-131 and HHV-6 gQ1/2 as well.

Glycoprotein E49 In EEHV1A E49, no signal sequence but one transmembrane helix and some N-glycosylation sites were detected. This protein does not clearly compare to another herpesvirus glycoprotein.

Glycoprotein E51 EEHV1A E51 is a type II membrane glycoprotein and is highly subtype variable. E51 does neither contain a signal sequence nor an apparent transmembrane helix, but multiple N-glycosylation sites were detected. EEHV1A E51 is not clearly comparable to any of these glycoproteins.

vOX-2 membrane glycoproteins (vOX2-1 (E54), vOX2-2 (E25), vOX2-3 (E24), vOX2-4 (E23))

A separate group of EEHV glycoproteins is the group of EEHV vOX2 glycoproteins. Through the Prediction Servers, a signal sequence was detected in EEHV1A vOX2-3 (glycoprotein E24). vOX2-3 did not contain a transmembrane helix but did contain multiple N-glycosylation sites. These characteristics would correlate most with HCMV U128-131 and HHV-6 gQ1/2.

In EEHV1A vOX2-1 (E54) two transmembrane helices were predicted, of which one is probably cleaved off and carries the function of a signal sequence. EEHV1A E54 also contained N-linked glycosylation sites. Therefore, this protein probably carries most similarities with HSV gD, which also contains one signal sequence, one transmembrane helix located towards the C-terminus of the protein. The HSV gD protein is of larger size than EEHV E54. EEHV E54 is absent in EEHV4. In vOX2-2 (E25) a transmembrane helix was detected, but no signal sequence. In vOX2-4 (E23), no signal sequence nor a transmembrane helix were predicted.

vIGfam glycoproteins

Another group of EEHV glycoproteins is the vIGfam glycoprotein group. Four vIGfam glycoproteins were found in the EEHV1A(Kimba) sequence; E50, E52, E53 and E55. E52 is a type II membrane glycoprotein and is highly subtype variable. No transmembrane helices were detected in E52, but a signal sequence and multiple N-glycosylated sites were found. In E53 and E55, a signal sequence was predicted by the TargetP prediction server (not by SignalP). In E53 two transmembrane helices were detected and in E55 one transmembrane helix was detected (probably within the signal sequence part of the protein) and both glycoproteins contained multiple N-glycosylated sites. In E50, no signal sequence or transmembrane helix was found, but multiple N-glycosylated sites were detected.

Discussion

Towards an EEHV fusion assay

In this study, we were able to design and produce gB and gH/gL constructs that are adequately expressed on the membrane of HeLa cells. The gB(-CTD) construct, in which the cytoplasmic tail of gB was truncated, showed most cell surface staining. Other designed constructs did not show obvious cell surface staining. To investigate whether deletion of the YXX ϕ peptide signal would affect the cell surface expression of EEHV gB, we produced a gB construct with the deletion of the YXX ϕ peptide signal (amino acids YTAL in EEHV1A gB; construct gB(-YTAL)) and a construct with a mutation of the YXX ϕ peptide signal (gB(FTAL)). We produced a construct with a mutation as well as with a deletion of the YXX ϕ peptide signal, because it has been studied that the cell surface expression of HCMV gB is influenced by the phosphorylation of serine (Ser900). In our truncated gB constructs, serine was not present anymore since all truncations removed this amino acid from the construct. Therefore, we produced a gB construct with a one point mutation in the YXX ϕ peptide signal sequence (YTAL \rightarrow FTAL). Consequently, the mutated YXX ϕ peptide signal would not affect localization anymore, but Ser900 would still be present in the cytoplasmic tail and its influence could be studied. (63,64) In a paper by Beitia Ortiz de Zarate et al., multiple mutations in the cytoplasmic tail of HSV-1 gB have been performed to examine the effect of the mutations on intracellular transport and fusion of HSV-1 gB. (65) Mutations of the YXX ϕ peptide signal were shown to effect gB transport; deletion of the whole region of the YXX ϕ peptide signal or substitution of the first glutamate residue with alanine both decreased gB transport from the plasma membrane to the Golgi complex by >95%. Other proteins containing comparable motifs accumulate in the Golgi apparatus as well, mainly due to endocytosis from the plasma membrane to the TGN. (65) However, the deletion of the YXX ϕ peptide signal did not affect expression as expected, since the gB(-YTAL) construct showed no clear cell surface staining. The presence of serine (and disruption of the YXX ϕ peptide signal) in the gB(FTAL) construct did not affect intracellular trafficking as much as expected, since no remarkable differences were observed between the membrane expression of construct gB(FTAL) (with serine) and construct gB(-YTAL) (without serine). The gB(FTAL) construct did seem to show some membrane expression, but not convincingly more as compared to the gB(-YTAL) or the gB(wt) construct.

Furthermore, gB constructs with deletions of 20, 30 and 50 amino acids and the whole cytoplasmic tail were produced, since other peptide regions in these parts may influence gB transport as well. For instance, the charge of some amino acids is proven to affect cellular localization, hence the truncation of an amino acid region could induce a difference in transport and localization. (66) A study by Garcia et al. reported an enhanced expression on the plasma membrane when the full cytoplasmic tail of gB was truncated. (67) Of all designed gB constructs, gB(-CTD) was expressed most adequately on the cell membrane. This might indicate that the amino acid part that was truncated in gB(-CTD) relative to gB(-50AA) carries an amino acid motive that has a function in protein localization, since the gB(-CTD) construct was expressed efficiently on the cell membrane but the gB(-50AA) construct was not.

The cytoplasmic tail domain (CTD) and the trans membrane domain (TMD) of gB were exchanged for the cytoplasmic tail domain and trans membrane domain of VSV-G. In a paper by Reuter et al. it was proposed that cell fusion of HCMV gB was improved due to this exchange. (68) In addition, this exchange could also be important for incorporation of the construct in pseudotyped VSV particles, which is needed for an eventual fusion assay. Two constructs of gB with a VSV-G cytoplasmic tail were designed. In the gB(VSV-G1) construct, the VSV-G domain was added directly after the membrane proximal region of gB. In the gB(VSV-G2) construct, five additional amino acids after the membrane proximal region were kept in the gB construct and after those the VSV-G domain was added. The exchange of the CTD and TMD of gB for the VSV-G CTD and TMD in constructs gB(VSV-G1) and gB(VSV-G2) did not have a clear effect on membrane expression or fusion either, although this effect was described for HCMV gB. (68)

Furthermore, gH(-YQKL) and gH(FQKL) were developed in addition to the original gH(wt) construct. Like the gB protein, gH also contains a YXX ϕ peptide signal (which is YQKL in EEHV gH). Since this signal is known to influence protein transport, the deletion of the YXX ϕ peptide signal in gH(-YQKL) could also enhance cell surface expression. Following the same reasoning as with the design of the gB constructs, we also designed a gH construct with a one point mutation in the YXX ϕ peptide signal (YQKL \rightarrow FQKL). This way the YXX ϕ peptide signal is no longer functioning, but the remainder of the cytoplasmic tail remains intact. Of the designed constructs gH(wt), gH(-YQKL) and gH(FQKL), it was clear that gH(-YQKL) showed cell surface staining, in contrast to gH(wt) and gH(FQKL). A similar cell surface staining was expected of gH(-YQKL) and gH(FQKL), since the YXX ϕ peptide signal should not be functional in both constructs. Possibly, the one point mutation that was made in the gH(FQKL) only partially blocks functioning of the signal.

As can be seen in Fig. 3, quite some background was observed upon protein staining. Consequently, different fixation and permeabilization methods were used in this study. With methanol fixation, staining of the constructs could be best distinguished from background staining. However, methanol does not only fixate but permeabilizes cells as well, making methanol fixation unsuitable for assessing solely membrane surface staining. Therefore, cells were fixated with PFA to observe membrane surface staining. However, background staining could be seen in wells that were PFA fixated. Therefore, multiple attempts were made to lower this background staining that occurred with PFA fixation. For example, a purified version of the chicken anti-gB was used, since the unpurified yolk could cause background staining. However, similar amounts of background staining were seen when using the purified chicken anti-gB antibodies. Additionally, fetal calf serum (FCS) was used instead of bovine serum albumin (BSA), since FCS generally contains less undesirable complements and could therefore decrease background staining. No clear difference between usage of FCS or BSA in background staining was observed. The difference in staining between a 24 hours transfection and a 48 hours transfection was observed as well. The cell staining was more apparent and distinctive from background staining in the 48 hours transfection. For that reason, the 48 hours transfection was used in the rest of this study. Lastly, unreacted aldehydes (remaining after fixation) were quenched with glycine. Glycine quenching should block residual aldehydes after PFA fixation, which can be the cause of an increased background staining. However, cells that were quenched with glycine did not show less background fluorescence than cells that were not quenched with glycine. Multiple plasmid:Fugene ratios were tested, but cells transfected with our original ratio 1:3 (100 ng plasmid:300 nl Fugene) showed the most stained cells. Cell staining did not significantly increase when the amounts of construct and Fugene were doubled (1:3 ratio; 200 ng plasmid:600 nl Fugene).

The large syncytia in the cotransfection of gB(-CTD) with the gH constructs + gL contained both gB staining and gH/gL staining when looking at the stainings separately. Especially when cotransfecting gB(-CTD) with gH(-YQKL), obvious cell surface staining of both constructs could be seen. Although syncytia could be seen, it is not certain whether the fusion was induced by gB and gH/gL or not, since HeLa cells may also fuse spontaneously.

In addition, we tried to induce fusion in the transfected wells by treating the cells with trypsin and/or a pH shock, since membrane fusion may be trypsin- or low pH-induced. The trypsin treatment and/or pH shock did not seem to enhance cell fusion. However, we only performed the acid shock and trypsin treatment on wells with gB(CTD) cotransfected with the gH(wt) construct + gL, since at that time it was still unknown that gH(-YQKL) showed more cell surface staining. For cell-cell fusion, gB and gH/gL are both required to be membrane expressed. In the cotransfection with gB(-CTD) and gH(wt)/gL, the gH/gL complex was probably not efficiently expressed on the cell surface. Therefore, it could be the case that obvious cell fusion did not occur because not all required glycoproteins were expressed on the cell surface. Potentially, fusion may be induced when performing the acid shock and trypsin treatment on wells cotransfected with gB(CTD) and gH(-YQKL)/gL, since those two constructs showed most obvious membrane expression.

Now that membrane expressed gB and gH constructs are designed and produced, transfections with other cell types could be performed to determine whether fusion can be induced therein. The next step would be to develop a fusion assay to determine the characteristics of EEHV fusion to host cells. This could either be a cell-cell fusion assay or a fusion assay with pseudotyped VSV- Δ G virions. For a cell-cell fusion assay, cells should be cotransfected with EEHV gB, gH and gL. These cells are then seeded together with target cells, after which fusion could be observed. The target immune cells could be seeded together with transfected HEK cells, since immune cells are notorious for difficult transfection. For a VSV- Δ G fusion assay, VSV- Δ G (vesicular stomatitis virus) virions pseudotyped with EEHV gB and gH/gL should be developed. EEHV gB and gH/gL would be incorporated in the newly produced virions. Subsequently, these virions could be used to infect target cells. These virions carry a reporter gene, making it more obvious to assess whether infection has taken place or not. Constructs gB(-CTD) and gH(-YQKL) should be included in these fusion assays, since these constructs showed the best membrane expression and can therefore be incorporated in the virion.

Eventually, fusion assays could be used for virus neutralization tests, which are important in determining whether (a sufficient amount of) neutralizing antibodies are made against gB and gH/gL. These neutralizing antibodies (nAbs) bind and neutralize certain (neutralizing) epitopes of the virus particle, preventing cell entry and fusion and therefore blocking infection. (57,69,70) Since nAbs against other herpesviruses are primarily targeting gB and gH/gL, it is plausible that during an immune response against EEHV most nAbs target gB and gH/gL as well. Neutralizing antibodies may be used to develop antibody-based treatments against EEHV-HD. In addition, the measurement of neutralizing antibodies is important to assess the immune response against potential vaccine candidates. For an accurate protective immune response, the antibodies obtained through vaccination need to be neutralizing and therefore protective in order to interfere with viral entry and fusion. Accordingly, gB and gH/gL fusion assays are important for the development of virus neutralization tests and thus for the measurement of neutralizing antibodies against EEHV.

In silico selection

In this study we attempted to make predictions on which EEHV glycoproteins are most likely to potentially play a role in entry and fusion, based on the *in silico* selection. Predictions on the characteristics of EEHV glycoproteins and of entry and fusion glycoproteins of other herpesviruses were made and compared. Since the EEHV genus is also proposed to be classified as new subfamily – the delta herpesviruses, it could be the case that none of the known herpesvirus glycoproteins match with the characteristics of EEHV glycoproteins. (1,71)

EEHV gB and gH seemed similar to gB and gH of other herpesviruses in size and characteristic predictions. EEHV gL did not seem to contain a signal sequence. In the gL protein of other herpesviruses and in other EEHV subtypes, a signal sequence was always present. A signal sequence would be expected in the gL protein, but gL could contain a signal sequence that is not recognized by the Prediction Servers. Therefore, the absence of a signal sequence in EEHV1A gL may be due to the fact that the predictions are not always entirely accurate.

Multiple EEHV glycoproteins contained characteristics similar to HCMV UL128-131 and HHV-6 gQ1/2. These known herpesvirus glycoproteins contain a signal sequence and N-glycosylated sites but no transmembrane helix and form a complex with gH/gL to be membrane-bound. In EEHV E4, E20A and E31 similar characteristics were predicted, and could therefore carry out a comparable role in entry and fusion as HCMV UL128-131 and HHV-6 gQ1/2. EEHV1A E31 is present only in the AT-rich brand of EEHV subtypes (EEHV1A, EEHV1B, EEHV5). Since this glycoprotein misses in some EEHV subtypes, it is probably less likely to play an important role in EEHV entry and fusion. ORF-O, ORF-P and ORF-Q contained characteristics similar to HCMV UL128-131 and HHV-6 gQ1/2 as well, and could therefore bind to the gH/gL complex and be membrane bound via the transmembrane helix of gH. Therefore, it would be possible for one or more of these glycoproteins to play a role in EEHV entry and fusion. In addition, ORF-P is absent in EEHV3 and 4 and ORF-Q is absent in EEHV2 and EEHV5 and replaced by ORF-R in EEHV3 and EEHV4. (40) ORF-Q is absent in EEHV2 and EEHV5 so is not required for fusion in these subtypes, but is replaced by ORF-R in other subtypes. ORF-O is conserved in all subtypes. Based on this knowledge, a possible hypothesis could be that ORF-O binds to the gH/gL complex in all subtypes, and that ORF-P, ORF-Q or ORF-R (based on the subtype) bind to ORF-O. The exact role of these protein remains unknown, and more research has to be done to extend knowledge on these glycoproteins, since they are potential candidates to have an important role in EEHV entry and fusion.

Other EEHV glycoproteins seemed less likely to play a role in EEHV entry and fusion. In EEHV gO, a transmembrane helix was not expected, since gO is presumably soluble. In gO of other herpesviruses, transmembrane helices were predicted but overlapped largely with the signal sequence and are likely to be cleaved off. The absence of a signal sequence in EEHV gO could indicate that EEHV gO does not play as big a role in cell fusion as gO in beta herpesviruses. The size of EEHV gO did not correlate with the size of other herpesvirus gO proteins as well. Because EEHV gO shared little similarities with other herpesvirus gO proteins and did not seem to contain a signal sequence, the question was raised whether EEHV gO was annotated correctly. In a paper by Ehlers et al., it was described that EEHV ORF-D was annotated as gO based on the number of cysteine residues and N-glycosylated sites. (25) Since this protein does not seem to share other similarities with other gO proteins, this annotation should be further examined. Glycoprotein gN is a type I membrane protein. One of the transmembrane helices overlapped with the signal sequence, making it likely that this transmembrane helix is cleaved off and behaves like a signal sequence, especially since it is known that gN only contains one transmembrane helix. (58) In EEHV gM, one of the transmembrane helices again seemed to overlap with the signal sequence and was likely to be cleaved off. It is known that gM contains seven transmembrane helices. (25) gM is thought to associate with gN, but an important role in herpesvirus fusion has never been described. (12) Therefore, it would be less likely that the gM/gN complex does play an important role in EEHV fusion. EEHV E1, E49 and E51 did not clearly share characteristics with other herpesvirus entry and fusion glycoproteins, so it is less likely that these proteins are important in EEHV entry and fusion.

OX2 (or CD200) glycoproteins are type I membrane glycoproteins which contain two immunoglobulin domains. (72) These EEHV glycoproteins were significantly homologous to cellular OX2 and viral OX2 (vOX2). (40,73) Although some vOX genes do contain characteristics that match with characteristics of herpesvirus glycoproteins that have a role in entry and fusion, vOX genes may primarily act as an activator of leukocytes to produce inflammatory cytokines. It was demonstrated by Chung et al. that like cellular OX2, HHV-8 vOX2 targets myeloid cells. They also proposed that HHV-8 vOX2 activates multiple leukocytes to produce inflammatory cytokines. These cytokines could potentially stimulate the angiogenic proliferation of HHV-8-infected cells and lesions. (73) This known function of vOX glycoproteins may make it less likely that they play an important role in entry and fusion as well. Most of the vOX glycoproteins did not contain a signal sequence, making an important role in entry and fusion less expected. vOX2-3 (E24) showed similar characteristics to HCMV U128-131 and HHV-6 gQ1/2. A role in entry and fusion can therefore not be entirely ruled out for vOX2-3, but is not expected considering the known function of vOX glycoproteins.

Viral Ig proteins are thought to have a function within the immune response, probably in lymphocyte activation or signaling. (74,75) Furthermore, some research has been done regarding the role of viral Ig proteins in cell adhesion, entry and fusion. However, in herpesviruses a role of viral Ig in entry and fusion has never been described. Therefore, glycoproteins of the EEHV vIg glycoprotein group (E50, E52, E53 and E55) are not very likely to have an important role in EEHV entry and fusion.

It should be noted that the Prediction Servers are not always fully adequate and could miss some characteristics. For instance, some glycoproteins (like EEHV E4, E53 and E55) were predicted to contain a transmembrane helix, but this region sometimes overlapped with the signal sequence and was likely to be cleaved off. Therefore, these transmembrane helices were likely to behave as a signal sequence. As another example, in some proteins such as EEHV E20A, a signal sequence was detected by the TargetP Prediction Server, but not by the SignalP prediction server. These proteins seemed to contain a sequence that is at least similar to a signal sequence, but it remains uncertain why these sequences were not recognized by SignalP.

In conclusion, EEHV contains glycoproteins that carry similarities with known herpesvirus fusion glycoproteins in terms of characteristics. Especially for EEHV glycoproteins E4, E20A, E31, ORF-O, ORF-P and ORF-Q/ORF-R, it would be interesting to study them more thoroughly, since they shared similar characteristics with HCMV UL128-131 and HHV-6 gQ1/2. Therefore, these glycoproteins could carry out a similar role as HCMV UL128-131 and HHV-6 gQ1/2 in entry and fusion. As follow-up experiment, it would be relevant to observe if these glycoproteins interact with gB and gH/gL. Then these glycoproteins could be produced and cotransfected with gB and gH/gL, to see if cell fusion is enhanced. Some EEHV proteins of the vOX or vIg group also contained similar characteristics, but since no important role in entry or fusion has been described for these groups, it is less likely that these proteins play an important role in entry and fusion.

Acknowledgements

Firstly I would like to thank Dr. Tabitha Hoornweg (Faculty of Veterinary Medicine (UU), Department of Biomolecular Health Sciences, Sections Immunology and Virology) for the supervision, feedback and guidance during the internship. In addition, I want to thank Dr. Xander de Haan, Prof. dr. Victor Rutten and Willem Schaftenaar of the EEHV research group for including me in all the meetings and discussing all results. Lastly, I would like to thank Prof. dr. Femke Broere for letting me do my research internship and track within the EEHV research group.

References

1. Long SY, Latimer EM, Hayward GS. Review of Elephant Endotheliotropic Herpesviruses and Acute Hemorrhagic Disease. *ILAR J.* 2016 Feb 24;56(3):283–96.
2. Choudhury A. IUCN Red List of Threatened Species: *Elephas maximus* [Internet]. IUCN Red List of Threatened Species. 2008 [cited 2020 Sep 19]. Available from: <https://www.iucnredlist.org/en>
3. Hoornweg TE, Schaftenaar W, Maurer G, van den Doel PB, Molenaar FM, Chamouard-Galante A, et al. Elephant Endotheliotropic Herpesvirus Is Omnipresent in Elephants in European Zoos and an Asian Elephant Range Country. *Viruses* [Internet]. 2021 Feb 11 [cited 2021 May 15];13(2). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7917619/>
4. Group) JB (IUCN SAES. IUCN Red List of Threatened Species: *Loxodonta africana* [Internet]. IUCN Red List of Threatened Species. 2008 [cited 2020 Sep 19]. Available from: <https://www.iucnredlist.org/en>

5. Ossent P, Guscetti F, Metzler AE, Lang EM, Rübél A, Hauser B. Acute and Fatal Herpesvirus Infection in a Young Asian Elephant (*Elephas maximus*). *Vet Pathol.* 1990 Mar 1;27(2):131–3.
6. Richman LK, Montali RJ, Garber RL, Kennedy MA, Lehnhardt J, Hildebrandt T, et al. Novel Endotheliotropic Herpesviruses Fatal for Asian and African Elephants. *Science.* 1999 Feb 19;283(5405):1171–6.
7. Latimer E, Zong J-C, Heaggans SY, Richman LK, Hayward GS. Detection and Evaluation of Novel Herpesviruses in Routine and Pathological Samples from Asian and African Elephants: Identification of Two New Probosciviruses (EEHV5 and EEHV6) and Two New Gammaherpesviruses (EGHV3B and EGHV5). *Vet Microbiol.* 2011 Jan 10;147(1–2):28–41.
8. Fuery A, Pursell T, Tan J, Peng R, Burbelo PD, Hayward GS, et al. Lethal Hemorrhagic Disease and Clinical Illness Associated with Elephant Endotheliotropic Herpesvirus 1 Are Caused by Primary Infection: Implications for the Detection of Diagnostic Proteins. *J Virol* [Internet]. 2020 Jan 17 [cited 2020 Sep 25];94(3). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7000966/>
9. Hayward GS. Conservation: clarifying the risk from herpesvirus to captive Asian elephants. *Vet Rec.* 2012 Feb 25;170(8):202–3.
10. Eisenberg RJ, Atanasiu D, Cairns TM, Gallagher JR, Krummenacher C, Cohen GH. Herpes Virus Fusion and Entry: A Story with Many Characters. *Viruses.* 2012 May 10;4(5):800–32.
11. Cooper RS, Heldwein EE. Herpesvirus gB: A Finely Tuned Fusion Machine. *Viruses.* 2015 Dec 11;7(12):6552–69.
12. Krummenacher C, Carfi A, Eisenberg RJ, Cohen GH. Entry of Herpesviruses into Cells: The Enigma Variations [Internet]. *Madame Curie Bioscience Database* [Internet]. Landes Bioscience; 2013 [cited 2021 Feb 21]. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK6257/>
13. Cairns TM, Atanasiu D, Saw WT, Lou H, Whitbeck JC, Ditto NT, et al. Localization of the Interaction Site of Herpes Simplex Virus Glycoprotein D (gD) on the Membrane Fusion Regulator, gH/gL. *J Virol* [Internet]. 2020 Sep 29 [cited 2021 May 15];94(20). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7527043/>
14. Reske A, Pollara G, Krummenacher C, Chain BM, Katz DR. Understanding HSV-1 entry glycoproteins. *Rev Med Virol.* 2007;17(3):205–15.
15. Kimura H, Straus SE, Williams RK. Varicella-Zoster Virus Glycoproteins E and I Expressed in Insect Cells Form a Heterodimer That Requires the N-Terminal Domain of Glycoprotein I. *Virology.* 1997 Jul 7;233(2):382–91.
16. Ali MA, Li Q, Fischer ER, Cohen JI. The Insulin Degrading Enzyme Binding Domain of Varicella-Zoster Virus (VZV) Glycoprotein E is Important for Cell-to-Cell Spread and VZV Infectivity, while a Glycoprotein I Binding Domain is Essential for Infection. *Virology.* 2009 Apr 10;386(2):270–9.
17. Maresova L, Pasiéka TJ, Grose C. Varicella-Zoster Virus gB and gE Coexpression, but Not gB or gE Alone, Leads to Abundant Fusion and Syncytium Formation Equivalent to Those from gH and gL Coexpression. *J Virol.* 2001 Oct;75(19):9483–92.

18. Isaacson MK, Compton T. Human Cytomegalovirus Glycoprotein B Is Required for Virus Entry and Cell-to-Cell Spread but Not for Virion Attachment, Assembly, or Egress. *J Virol.* 2009 Apr 15;83(8):3891–903.
19. Vanarsdall AL, Chase MC, Johnson DC. Human Cytomegalovirus Glycoprotein gO Complexes with gH/gL, Promoting Interference with Viral Entry into Human Fibroblasts but Not Entry into Epithelial Cells ∇ . *J Virol.* 2011 Nov;85(22):11638–45.
20. Zhou M, Yu Q, Wechsler A, Ryckman BJ. Comparative Analysis of gO Isoforms Reveals that Strains of Human Cytomegalovirus Differ in the Ratio of gH/gL/gO and gH/gL/UL128-131 in the Virion Envelope. *J Virol.* 2013 Sep;87(17):9680–90.
21. Fouts AE, Chan P, Stephan J-P, Vandlen R, Feierbach B. Antibodies against the gH/gL/UL128/UL130/UL131 Complex Comprise the Majority of the Anti-Cytomegalovirus (Anti-CMV) Neutralizing Antibody Response in CMV Hyperimmune Globulin. *J Virol.* 2012 Jul;86(13):7444–7.
22. Freed DC, Tang Q, Tang A, Li F, He X, Huang Z, et al. Pentameric complex of viral glycoprotein H is the primary target for potent neutralization by a human cytomegalovirus vaccine. *Proc Natl Acad Sci U S A.* 2013 Dec 17;110(51):E4997–5005.
23. Ryckman BJ, Rainish BL, Chase MC, Borton JA, Nelson JA, Jarvis MA, et al. Characterization of the Human Cytomegalovirus gH/gL/UL128-131 Complex That Mediates Entry into Epithelial and Endothelial Cells. *J Virol.* 2008 Jan;82(1):60–70.
24. Vanarsdall AL, Johnson DC. Human cytomegalovirus entry into cells. *Curr Opin Virol* [Internet]. 2012 Feb [cited 2021 May 24];2(1). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3880194/>
25. Krzyzaniak M, Mach M, Britt WJ. The Cytoplasmic Tail of Glycoprotein M (gpUL100) Expresses Trafficking Signals Required for Human Cytomegalovirus Assembly and Replication. *J Virol.* 2007 Oct;81(19):10316–28.
26. Shimamura M, Mach M, Britt WJ. Human Cytomegalovirus Infection Elicits a Glycoprotein M (gM)/gN-Specific Virus-Neutralizing Antibody Response. *J Virol.* 2006 May;80(9):4591–600.
27. Mach M, Kropff B, Dal Monte P, Britt W. Complex Formation by Human Cytomegalovirus Glycoproteins M (gpUL100) and N (gpUL73). *J Virol.* 2000 Dec;74(24):11881–92.
28. Vanarsdall AL, Johnson DC. Human cytomegalovirus entry into cells. *Curr Opin Virol* [Internet]. 2012 Feb [cited 2021 Jun 6];2(1). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3880194/>
29. Mori Y, Akkapaiboon P, Yonemoto S, Koike M, Takemoto M, Sadaoka T, et al. Discovery of a Second Form of Tripartite Complex Containing gH-gL of Human Herpesvirus 6 and Observations on CD46. *J Virol.* 2004 May;78(9):4609–16.
30. Tanaka Y, Suenaga T, Matsumoto M, Seya T, Arase H. Herpesvirus 6 Glycoproteins B (gB), gH, gL, and gQ Are Necessary and Sufficient for Cell-to-Cell Fusion. *J Virol.* 2013 Oct;87(19):10900–3.
31. Tang H, Hayashi M, Maeki T, Yamanishi K, Mori Y. Human Herpesvirus 6 Glycoprotein Complex Formation Is Required for Folding and Trafficking of the gH/gL/gQ1/gQ2 Complex and Its Cellular Receptor Binding ∇ . *J Virol.* 2011 Nov;85(21):11121–30.

32. Nishimura M, Mori Y. Chapter Eight - Entry of betaherpesviruses. In: Kielian M, Mettenleiter TC, Roossinck MJ, editors. *Advances in Virus Research* [Internet]. Academic Press; 2019 [cited 2021 Mar 16]. p. 283–312. (Virus Entry; vol. 104). Available from: <https://www.sciencedirect.com/science/article/pii/S0065352719300156>
33. Sadaoka T, Yamanishi K, Mori Y 2006. Human herpesvirus 7 U47 gene products are glycoproteins expressed in virions and associate with glycoprotein H. *J Gen Virol.* 87(3):501–8.
34. Xiao J, Palefsky JM, Herrera R, Berline J, Tugizov SM. The Epstein-Barr Virus BMRF-2 Protein Facilitates Virus Attachment to Oral Epithelial Cells. *Virology.* 2008 Jan 20;370(2):430–42.
35. BMRF2 - Protein BMRF2 - Epstein-Barr virus (strain GD1) (HHV-4) - BMRF2 gene & protein [Internet]. [cited 2021 May 15]. Available from: <https://www.uniprot.org/uniprot/Q3KSU2>
36. Borza CM, Hutt-Fletcher LM. Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. *Nat Med.* 2002 Jun;8(6):594–9.
37. Heldwein EE. gH/gL supercomplexes at early stages of herpesvirus entry. *Curr Opin Virol.* 2016 Jun;18:1–8.
38. Wang F-Z, Akula SM, Pramod NP, Zeng L, Chandran B. Human Herpesvirus 8 Envelope Glycoprotein K8.1A Interaction with the Target Cells Involves Heparan Sulfate. *J Virol.* 2001 Aug;75(16):7517–27.
39. DNASTAR Bioinformatics Software | DNASTAR home_hero_btn [Internet]. DNASTAR. [cited 2021 Jun 26]. Available from: <https://www.dnastar.com/software/>
40. Elephant endotheliotropic herpesvirus 1A isolate Kimba NAP23, complete genome. 2019 Aug 27 [cited 2021 May 16]; Available from: <http://www.ncbi.nlm.nih.gov/nuccore/KC618527.1>
41. CBS Prediction Servers [Internet]. [cited 2021 May 15]. Available from: <http://www.cbs.dtu.dk/services/>
42. SignalP-5.0 [Internet]. [cited 2021 Jun 6]. Available from: <http://www.cbs.dtu.dk/services/SignalP/>
43. TargetP-2.0 [Internet]. [cited 2021 Jun 6]. Available from: <http://www.cbs.dtu.dk/services/TargetP/>
44. TMHMM Server, v. 2.0 [Internet]. [cited 2021 Jun 6]. Available from: <http://www.cbs.dtu.dk/services/TMHMM/>
45. NetNGlyc 1.0 Server [Internet]. [cited 2021 Jun 6]. Available from: <http://www.cbs.dtu.dk/services/NetNGlyc/>
46. Reuter N, Kropff B, Schneiderbanger JK, Alt M, Krawczyk A, Sinzger C, et al. Cell Fusion Induced by a Fusion-Active Form of Human Cytomegalovirus Glycoprotein B (gB) Is Inhibited by Antibodies Directed at Antigenic Domain 5 in the Ectodomain of gB. *J Virol* [Internet]. 2020 Aug 31 [cited 2021 Jun 29];94(18). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7459561/>

47. Human herpesvirus 1 strain KOS, complete genome. 2012 Apr 24 [cited 2021 May 15]; Available from: <http://www.ncbi.nlm.nih.gov/nuccore/JQ780693.1>
48. Human herpesvirus 2 strain HG52, complete genome. 2016 May 16 [cited 2021 May 15]; Available from: http://www.ncbi.nlm.nih.gov/nuccore/NC_001798.2
49. Human herpesvirus 3 (strain Dumas) complete genome. 2005 Apr 18 [cited 2021 May 15]; Available from: <http://www.ncbi.nlm.nih.gov/nuccore/X04370.1>
50. Vollmer B, Pražák V, Vasishtan D, Jefferys EE, Hernandez-Duran A, Vallbracht M, et al. The prefusion structure of herpes simplex virus glycoprotein B. *Sci Adv.* 2020 Sep 1;6(39):eabc1726.
51. Human herpesvirus 5 strain Merlin, complete genome. 2018 Aug 13 [cited 2021 May 15]; Available from: http://www.ncbi.nlm.nih.gov/nuccore/NC_006273.2
52. Human herpesvirus 6B, complete genome. 2018 Aug 13 [cited 2021 May 15]; Available from: http://www.ncbi.nlm.nih.gov/nuccore/NC_000898.1
53. Human herpesvirus 7, complete genome. 2018 Aug 13 [cited 2021 May 15]; Available from: http://www.ncbi.nlm.nih.gov/nuccore/NC_001716.2
54. U39 - Envelope glycoprotein B - Human herpesvirus 6B (strain Z29) (HHV-6 variant B) - U39 gene & protein [Internet]. [cited 2021 Jun 5]. Available from: <https://www.uniprot.org/uniprot/Q9WT25>
55. Mahmoud NF, Jasirwan C, Kanemoto S, Wakata A, Wang B, Hata Y, et al. Cytoplasmic tail domain of glycoprotein B is essential for HHV-6 infection. *Virology.* 2016 Mar 1;490:1–5.
56. Liu Y, Heim KP, Che Y, Chi X, Qiu X, Han S, et al. Prefusion structure of human cytomegalovirus glycoprotein B and structural basis for membrane fusion. *Sci Adv.* 2021 Mar 1;7(10):eabf3178.
57. Ciferri C, Chandramouli S, Leitner A, Donnarumma D, Cianfrocco MA, Gerrein R, et al. Antigenic Characterization of the HCMV gH/gL/gO and Pentamer Cell Entry Complexes Reveals Binding Sites for Potently Neutralizing Human Antibodies. *PLoS Pathog* [Internet]. 2015 Oct 20 [cited 2021 May 19];11(10). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4617720/>
58. Theiler RN, Compton T. Characterization of the Signal Peptide Processing and Membrane Association of Human Cytomegalovirus Glycoprotein O. *J Biol Chem.* 2001 Oct;276(42):39226–31.
59. Ryckman BJ, Chase MC, Johnson DC. HCMV gH/gL/UL128–131 interferes with virus entry into epithelial cells: Evidence for cell type-specific receptors. *Proc Natl Acad Sci U S A.* 2008 Sep 16;105(37):14118–23.
60. Epstein-Barr virus (EBV) genome, strain B95-8. 2016 Jul 26 [cited 2021 May 15]; Available from: <http://www.ncbi.nlm.nih.gov/nuccore/V01555.2>
61. Human gammaherpesvirus 8 strain BCBL1, partial genome. 2021 Jan 12 [cited 2021 May 15]; Available from: <http://www.ncbi.nlm.nih.gov/nuccore/MT936340.1>

62. Rowe CL, Chen J, Jardetzky TS, Longnecker R. Membrane Anchoring of Epstein-Barr Virus gp42 Inhibits Fusion with B Cells Even with Increased Flexibility Allowed by Engineered Spacers. *mBio* [Internet]. 2015 Jan 6 [cited 2021 Jun 5];6(1). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4313908/>
63. Fish KN, Soderberg-Naucler C, Nelson JA. Steady-State Plasma Membrane Expression of Human Cytomegalovirus gB Is Determined by the Phosphorylation State of Ser900. *J Virol*. 1998 Aug;72(8):6657–64.
64. Jarvis MA, Jones TR, Drummond DD, Smith PP, Britt WJ, Nelson JA, et al. Phosphorylation of Human Cytomegalovirus Glycoprotein B (gB) at the Acidic Cluster Casein Kinase 2 Site (Ser900) Is Required for Localization of gB to the trans-Golgi Network and Efficient Virus Replication. *J Virol*. 2004 Jan;78(1):285–93.
65. Zarate IBO de, Kaelin K, Rozenberg F. Effects of Mutations in the Cytoplasmic Domain of Herpes Simplex Virus Type 1 Glycoprotein B on Intracellular Transport and Infectivity. *J Virol*. 2004 Feb 1;78(3):1540–51.
66. Mbaye MN, Hou Q, Basu S, Teheux F, Pucci F, Rooman M. A comprehensive computational study of amino acid interactions in membrane proteins. *Sci Rep*. 2019 Aug 19;9(1):12043.
67. Garcia NJ, Chen J, Longnecker R. Modulation of Epstein-Barr Virus Glycoprotein B (gB) Fusion Activity by the gB Cytoplasmic Tail Domain. *mBio* [Internet]. 2013 Jan 22 [cited 2021 May 15];4(1). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3551549/>
68. Rogalin HB, Heldwein EE. Characterization of Vesicular Stomatitis Virus Pseudotypes Bearing Essential Entry Glycoproteins gB, gD, gH, and gL of Herpes Simplex Virus 1. *J Virol*. 2016 Oct 28;90(22):10321–8.
69. Bootz A, Karbach A, Spindler J, Kropff B, Reuter N, Sticht H, et al. Protective capacity of neutralizing and non-neutralizing antibodies against glycoprotein B of cytomegalovirus. *PLoS Pathog* [Internet]. 2017 Aug 30 [cited 2021 May 19];13(8). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5595347/>
70. Cairns TM, Huang Z-Y, Gallagher JR, Lin Y, Lou H, Whitbeck JC, et al. Patient-Specific Neutralizing Antibody Responses to Herpes Simplex Virus Are Attributed to Epitopes on gD, gB, or Both and Can Be Type Specific. *J Virol*. 2015 Aug 19;89(18):9213–31.
71. Richman LK, Zong J-C, Latimer EM, Lock J, Fleischer RC, Heaggans SY, et al. Elephant Endotheliotropic Herpesviruses EEHV1A, EEHV1B, and EEHV2 from Cases of Hemorrhagic Disease Are Highly Diverged from Other Mammalian Herpesviruses and May Form a New Subfamily. *J Virol*. 2014 Dec;88(23):13523–46.
72. Wright GJ, Jones M, Puklavec MJ, Brown MH, Barclay AN. The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans. *Immunology*. 2001 Feb;102(2):173–9.
73. Chung Y-H, Means RE, Choi J-K, Lee B-S, Jung JU. Kaposi's Sarcoma-Associated Herpesvirus OX2 Glycoprotein Activates Myeloid-Lineage Cells To Induce Inflammatory Cytokine Production. *J Virol*. 2002 May;76(10):4688–98.

74. Farré D, Martínez-Vicente P, Engel P, Angulo A. Immunoglobulin superfamily members encoded by viruses and their multiple roles in immune evasion. *Eur J Immunol*. 2017;47(5):780–96.
75. Tan Y, Schneider T, Leong M, Aravind L, Zhang D. Novel Immunoglobulin Domain Proteins Provide Insights into Evolution and Pathogenesis of SARS-CoV-2-Related Viruses. *mBio* [Internet]. 2020 May 29 [cited 2021 Jun 1];11(3). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7267882/>