8-22-2014

Examination of herpesvirus entry glycoprotein interactions using proximity biotinylation

Michelle Lajko
DePaul University, MICHELLE.LAJKO@GMAIL.COM

Recommended Citation
Lajko, Michelle, "Examination of herpesvirus entry glycoprotein interactions using proximity biotinylation" (2014). College of Science and Health Theses and Dissertations. 78.
https://via.library.depaul.edu/csh_etd/78

This Thesis is brought to you for free and open access by the College of Science and Health at Via Sapientiae. It has been accepted for inclusion in College of Science and Health Theses and Dissertations by an authorized administrator of Via Sapientiae. For more information, please contact wsulliv6@depaul.edu, c.mcclure@depaul.edu.
Examination of herpesvirus entry glycoprotein interactions using proximity biotinylation

A Thesis

Presented in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

August 2014

By: Michelle Lajko
Advisor: Dr. Sarah Connolly

Department of Biological Sciences
College of Science and Health
DePaul University
Chicago, IL
Acknowledgements

I would like to thank my advisor and mentor, Dr. Sarah Connolly for taking a graduate student on in a stressful first year. She supported me through my Master’s encouraging me to become a better critical thinker and therefore a better scientist. I also want to thank the other lab members: Alexander F. Haddad and Carolyn A. Robinson. Without their continued support, it would have taken me much longer to finish this project. I appreciate the support and guidance that I received from my committee members, Dr. Phillip Funk and Dr. Eric Norstrom. I appreciate the many plasmids, vectors, antibodies, and other reagents that were kindly provided to us from Dr. Richard Longnecker’s lab at Northwestern University. I would also like to thank the chemistry department for allowing us to use their tissue culture hood. In addition, I appreciate Dr. Caitlin Karver’s lab and Margaret Kawarski for the use of their plate reader.

I want to thank the following people for their support and guidance: Dr. Tim Sparkes, Dr. Jason Bystrainsky, and Dr. Margaret Silliker.

Finally, I would like to acknowledge the support, guidance, and friendships that I gained from the other biology graduate students—Elsa Anderson, Kelly Bice, Seth Contreras, Mary Kasparian, Dave Osiecki, Ciaran Shaughnessy, and Tiju Theccanat. In addition, I would like to thank Kyle Haselton for his advice throughout my project especially the help with the proposal presentation and the thesis seminar.

This work was supported by the Department of Biological Sciences and the Department of Health Sciences at DePaul University as well as the DePaul University Research Council Grant 600814. Travel was funded by the College of Science and Health and the Graduate Research Funding Committee.
Abstract

Herpesvirus entry into cells begins when viral membrane glycoproteins bind to cellular receptors. This receptor binding elicits a cascade of interactions among conserved viral membrane glycoproteins that triggers virus-cell membrane fusion. Details of the viral protein interactions are not well understood because they are likely transient and/or low affinity.

Proximity biotinylation (ProB) is a promising technique for dissecting the glycoprotein interactions required for entry. In ProB, one protein is linked to a bacterial biotin ligase and a second protein is linked to a target 15 amino acid acceptor peptide (AP). If the proteins interact, the ligase on the first protein catalyzes site-specific biotinylation of the AP on the second protein. Evidence of interaction can be detected by western blotting with streptavidin to visualize biotinylated proteins. Labeling is covalent and occurs within live cells, thus weak and transient interactions can be detected. The short length of AP reduces the risk of altering the native conformation of the glycoproteins.

Sensitivity and specificity are concerns for all interaction assays. To establish optimal conditions for ProB, the biotin ligase and AP were linked to herpes simplex virus gD and Epstein Barr virus gB. These oligomeric herpesvirus proteins do not interact functionally and assaying for a heterotypic interaction between them serves as a negative control to demonstrate specificity of the ProB assay. Homotypic interactions between their monomers serve as a positive control to demonstrate sensitivity. The positive controls show strong biotinylation, indicating that proximity of viral membrane proteins can be detected. Unexpectedly, the negative controls also showed biotinylation, albeit at a lower level. These results demonstrate the special circumstances that must be considered when examining interactions among proteins that are constrained within the two-dimensional boundaries of a membrane. The lab is currently investigating ways to limit the heterotypic interaction and designing constructs to further test ProB validity for membrane-restricted protein interactions.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Ham’s</td>
<td>Ham’s F12 nutrient mixture</td>
</tr>
<tr>
<td>ProB</td>
<td>Proximity Biotinylation</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS+</td>
<td>Phosphate buffered saline with magnesium chloride and calcium chloride</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline with Tween-20</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>BME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular Fluorescence Complementation</td>
</tr>
<tr>
<td>cELISA</td>
<td>Cell-based enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
</tbody>
</table>
# Table of Contents

## I. Review of Literature

A. The *Herpesviridae* Family ............................................................................. 1
B. Epidemiology .................................................................................................. 2
C. HSV1 Entry Glycoproteins .............................................................................. 3
D. EBV Entry Glycoproteins ............................................................................... 8
E. Glycoprotein Interactions ................................................................................ 9
F. Proximity Biotinylation .................................................................................. 10
G. Project Overview ............................................................................................ 12

## II. Materials and Methods

A. Cells, antibodies, and plasmids ...................................................................... 14
B. Transfection ..................................................................................................... 14
C. Western blot for total cell protein expression ................................................. 15
D. Cell-based enzyme-linked Immunosorbent assay (cELISA) ......................... 15
E. Western blot for proximity biotinylation ......................................................... 16
F. Depletion of biotin from medium .................................................................... 16
G. Proximity biotinylation with immunoprecipitation ........................................ 17
H. Competition proximity biotinylation assay .................................................... 17
I. Competition assay with gH ............................................................................ 18
J. Syncytium formation assay ............................................................................ 18
K. Densitometry .................................................................................................. 18

## III. Results

A. Positive and negative ProB controls ............................................................. 19
B. Expression of HSV1 and EBV constructs ...................................................... 19
C. Initial ProB trial .............................................................................................. 22
D. ProB assay optimization with lower amounts of DNA, shorter biotin exposure ........................................................................................................................................................................................................................................... 26

E. ProB assay optimization using FLAG and HA epitopes .................................................. 26

F. ProB with depletion of free biotin .................................................................................. 29

G. ProB with immunoprecipitation .................................................................................... 32

H. gD construct function in fusion .................................................................................. 32

I. ProB competition assay ............................................................................................... 35

J. ProB HSV1 gH/gL competition assay ........................................................................... 36

IV. Discussion .................................................................................................................. 39

A. Proximity biotinylation potential ................................................................................ 41

B. Future work ................................................................................................................ 43

C. Other protein-protein interaction methods similar to ProB ........................................ 44

V. Appendix: Cloning BirA and APm onto the cytoplasmic tails for HSV1 gB, HSV1 gH, and PIV5 F ................................................................................................................................. 46

VI. References .................................................................................................................. 53
List of Illustrations

Figure 1. Interactions that mediate HSV entry ................................................................. 4
Figure 2. Proximity biotinylation ......................................................................................... 13
Figure 3. Proximity biotinylation constructs ................................................................. 20
Figure 4. Expression of HSV1 constructs ........................................................................ 21
Figure 5. Expression of EBV constructs ........................................................................ 23
Figure 6. Initial trial of proximity biotinylation ....................................................... 25
Figure 7. Proximity biotinylation trial with less DNA and shorter biotin exposure ........ 27
Figure 8. Detection of construct expression using HA and FLAG tags ....................... 28
Figure 9. Proximity biotinylation using HA and FLAG tags .................................. 30
Figure 10. Proximity biotinylation after biotin-depletion of the medium ...................... 31
Figure 11. Proximity biotinylation with immunoprecipitation .................................... 33
Figure 12. Syncytium formation with gD constructs BirA and APm ......................... 34
Figure 13. Competition proximity biotinylation assay ......................................... 37
Figure 14. Competition proximity biotinylation assay with HSV1 gH/gL ............... 38
Figure 15. Depiction of the effect of enzyme concentration on biotinylation ............. 42

List of Tables

Table 1. Primer name and corresponding sequences for HSV1 gB, gH, and PIV5

F protein.............................................................................................................................. 48

Table 2. Optimal PCR conditions to amplify 5’ and 3’ fragments ............................. 49

Table 3. Optimal PCR conditions to amplify fragments of glycoprotein with tag .... 50

Table 4. Confirmation of ligation of the PCR fragments into pSG5 vector ............... 51
I. Review of Literature

A. The *Herpesviridae* Family

Herpesviruses cause a lifelong infection of a large variety of animal hosts, and the average person carries multiple herpesviruses (36, 70). Herpesviruses are double-stranded linear DNA viruses with genomes ranging from 120 to 230 kb in length (60). The herpesvirus genome is encased in a proteinaceous capsid that is enclosed within a lipid envelope (60). Embedded in this envelope are approximately a dozen glycoproteins that are responsible for interactions with the host cell (60). The space between the capsid and the envelope contains proteins called tegument that are essential to the establishment of infection after entry into the cell, for example transcription factors (60).

There are eight human herpesviruses that cause a variety of diseases, ranging from cold sores to cancers. The *Herpesviridae* family is divided into three subfamilies—alpha, beta, and gammaherpesviruses. This project focuses on the human alphaherpesvirus herpes simplex virus 1 and the human gammaherpesvirus Epstein-Barr virus. Alphaherpesviruses are characterized by their ability to establish lifelong latent infections in the peripheral nervous system of a host. An infection is defined as latent when the virus is present but dormant and producing no overt symptoms. Alphaherpesviruses have a short growth cycle and rapid spread. The human viruses within the alphaherpesvirus subfamily include herpes simplex virus 1 (HSV1), herpes simplex virus 2 (HSV2), and varicella-zoster virus (VZV), the causative agents of cold sores, genital lesions, and chickenpox respectively. Gammaherpesviruses are characterized by the viral growth in epithelial and B- or T- lymphocytes. The human viruses within the gammaherpesvirus class are Epstein-Barr virus (EBV) (51) and human herpesvirus 8 (HHV-8). EBV causes infectious mononucleosis, Burkitt’s lymphoma, and nasopharyngeal carcinoma, where as HHV-8 causes Kaposi’s sarcoma.
Although the typical course of herpesvirus infection results in modest symptoms, serious and potentially life-threatening complications can occur. Gammaherpesvirus infections may be asymptomatic, but they may also result in the cancers mentioned above. Complications from alphaherpesvirus infection include herpes encephalitis, herpes meningitis, ocular herpes, gingivastomatitis, shingles, and postherpetic neuralgia. The severity of these complications highlights the importance of understanding all aspects of the virus life cycle, including virus entry into cells, replication and assembly within cells, release from cells, spread through tissues and interactions with the immune system. This thesis will focus on HSV1 and the interactions of envelope glycoproteins with the host cell during entry, the first step of viral infection. The entry step is an attractive target for antiviral intervention because if the virus is prevented from entering the cell, the infection is never established.

Herpesviruses use a conserved set of machinery to enter into cells. The glycoproteins for HSV1 and HSV2 are so similar that they are functionally interchangeable. Learning about HSV1 is directly applicable to HSV2 and other enveloped viruses, such as parainfluenzaviruses, which also encode for a receptor binding and fusion of separate glycoproteins.

B. Epidemiology

HSV1 and HSV2 cause genital herpes, however HSV2 is the most common cause. In 2003, more than 500 million people worldwide were infected with HSV2 and more women than men were infected (44). In the 1970s and 1980s, over 30% of women diagnosed with genital herpes were infected with HSV1 (69). HSV1 is now responsible for 40% of new cases of genital herpes (17). In a study tracking primary HSV infection in women for 20 months, 3.7% of the women (127 out of 3438 participants) were infected with HSV1 while only 1.6% were infected with HSV2 (9). Infection rates differ greatly between racial groups. From the study tracking women for 20 months, HSV2 infections were found to vary significantly among racial groups: 74% black, 40% Hispanic, and 23% white (9). Other studies have found similar results. HSV2
seroprevalence was found in 21.9% of the overall population (22). The individuals with HSV2 infections also varied among racial groups: 45.9% black, 22.3% Hispanic, and 17.6% white (22). Overall, HSV1 and HSV2 are increasing in prevalence.

C. HSV1 Entry Glycoproteins

HSV may enter cells through two pathways, depending on cell type. HSV1 virions are able to infect lymphocytes, epithelial cells, fibroblasts, and neurons. There are two entry pathways both requiring viral glycoproteins to induce fusion: fusion can occur at the cell surface at a neutral pH or in the endosome in a low pH-dependent manner. (48, 52). Many aspects of herpes simplex virus entry into cells are understood, but the overall sequence of events and interactions among entry proteins is disputed. For HSV1, a small set of glycoproteins in the viral membrane envelope (gB, gD, gH, and gL) are both necessary and sufficient for fusion of the virus with the host cell (56, 68) (Fig.1). Glycoproteins gD, gB, gH, and gL are the minimum viral requirement needed for entry into the cell and viruses deleted for any of these glycoproteins fail to enter cells (56, 68). The nature of the interaction between these proteins has yet to be defined, including the order of interactions and the site of interactions.

In the first step of entry, the virus tethers to heparan sulfate proteoglycans on the host cell surface via the viral glycoproteins gB and gC. Although the gB and gC interaction with heparan sulfate enhance the efficiency of virus entry, these interactions are not required for entry (30, 41). Removal of heparan sulfate binding capacity does not inactivate the virus. Binding to heparan sulfate does not trigger the conformational changes in the viral proteins that are required to cause fusion of the viral envelope with the cellular membrane.

Fusion is triggered by the binding of gD to a specific cellular receptor(66). The two widely studied receptors that bind to gD include nectin-1 and herpesvirus entry mediator (HVEM). Nectin-1 is thought to be a critical herpesvirus receptor, and it is expressed on neurons; neurons are a key cell type for HSV1 infection as this is the cell type in which HSV1 establishes
Figure 1. Interactions that mediate HSV entry. HSV entry into a cell requires four essential glycoproteins found on the viral envelope: gD, gH, gL, and gB. These glycoproteins both are necessary and sufficient for the fusion of the viral lipid envelope with the cell membrane. In the current model, virus entry is initiated when gD, which exists as a dimer, binds to a specific receptor on the membrane of the host cell. The most common receptors are nectin-1 and herpes virus entry mediator (HVEM). Receptor-binding results in conformational changes in gD that have been observed using crystallography. Due to structural similarity between gB and other viral fusion proteins, gB is believed to be responsible for physically inserting into the host cell membrane and then refolding to execute membrane fusion. gH and gL form a heterodimer, and the role gH/gL is less clear. gH/gL and gD alone are insufficient for fusion and gH/gL does not resemble any known fusion protein. Thus, gH/gL likely plays a role in transmitting a signal to gB to trigger the fusion event. How gD interacts with gH/gL and/or gB to trigger fusion of the virus with the target cell after receptor binding is poorly understood. The interactions among the glycoproteins are hard to capture, presumably because they are either low affinity or transient interactions.
Transgenic mouse models of HSV1 infection have demonstrated that deletion of nectin-1 greatly inhibits virus infection and spread through the host (35, 37, 65). The nectin family of molecules function in intercellular adhesion. HVEM, formerly known as HveA, is a member of the tumor necrosis factor receptor family and is expressed on a variety of tissues, including immune cells (50). Transgenic mouse models of HSV1 infection have suggested that HVEM is a dispensable receptor because HSV1 infection in the absence of HVEM proceeds at near-wild type manner (35, 37, 65). The reason why HSV1 retains the ability to use HVEM as a receptor is unclear. In fact, a point mutation in gD is sufficient to eliminate HVEM binding without preventing nectin-1 usage (39). The retention of HVEM binding by gD suggests that HVEM contributes to the virus life cycle in some manner, perhaps in an as-of-yet undefined immune evasion strategy. A third receptor that gD can bind is a 3-O-sulfated heparan sulfate, a type of heparan sulfate (61). The contribution of 3-O-sulfated heparan sulfate to HSV1 infection of an animal remains unclear.

HSV1 gD has 369 amino acid residues and forms a dimer (39). gD is unique to alphaherpesviruses and other families of herpesviruses use other receptor binding proteins. For example, EBV uses the receptor binding protein, gp42 that communicates with gH/gL and gB for entry into B cells. The crystal structure of gD has been determined alone and in complex with the receptors HVEM or nectin-1 (11, 18, 39). Glycoprotein gD has no sequence homology to other proteins, however it has a V-like immunoglobulin fold at its core from residues 56 to 184 (11). Immunoglobulin-like folds are found on many proteins in the immune system and proteins involved in cell-cell recognition and adhesion in nervous system. The residues on gD that bind HVEM are located entirely on an N-terminal extension that is supported by the core of the gD molecule (11). The binding site of nectin-1 is distinct from but overlaps with the binding site of HVEM. Nectin-1 binds to residues within the gD core as well as residues near the gD N-terminus (18). Specifically, the gD residue tyrosine 38 is critical for nectin-1 binding (18). The gD C-terminus is a flexible structural element that is able to interfere with receptor binding (39). It has
been suggested that the C-terminus of gD normally occludes the receptor-binding site and that upon receptor binding, this gD C-terminus is displaced. This movement of the gD C-terminus may be the signal that triggers the next step of entry. The protein(s) that gD interacts with after receptor binding has not been fully established.

gB is a 904 residue protein that is conserved among all herpesviruses and exists as a trimer (29). The crystal structure of HSV1 gB revealed a striking structural similarity to a rhabdovirus fusion protein, despite the fact that gB has no sequence homology to this protein (29, 59). The ectodomain of EBV gB has also been characterized (7). This structural similarity to a known fusion protein strongly suggests that gB is also a fusion protein. Fusion proteins are responsible for physically fusing the viral membrane with the cellular membrane. They are expressed initially in a prefusion conformation. Upon triggering, they insert hydrophobic residues (called fusion loops or fusion peptides) into a target membrane and then refold into a postfusion conformation, dragging the viral membrane and cellular membrane together to force membrane fusion. The crystal structure of gB reveals putative fusion loops and these hydrophobic loops are critical for fusion (29). gB represents a class III fusion protein, characterized by the presence of fusion loops and a coiled-coil domain (16, 29). Mutations in a C-terminal region of the gB ectodomain that contacts the coiled-coil domain inhibit the ability of gB to execute fusion, suggesting that residues in this C-terminal region may provide a driving force for gB refolding into its postfusion conformation (16). gB is reported to be required for HSV1 nuclear egress, the process wherein the capsids leave the nucleus after being loaded with genome. The requirement of gB for nuclear egress may indicate that gB mediates fusion of an initial virion envelope (comprised of the inner nuclear membrane) with outer nuclear membrane. When the fusion loops of gB were mutated, the virus was unable to exit the perinuclear space (71). Unexpectedly, gB and gH were reported to have redundant functions in nuclear egress, a redundancy that does not occur during virus entry (71).
The glycoproteins gH and gL are found in a heterodimer (32). gL is required for correct folding, surface expression, and function of gH, and thus the two proteins are treated as one functional unit, referred to as gH/gL (63). Prior to the solution of the crystal structure of gB, gH/gL was thought to be a viral fusion protein. This idea stemmed from four reports. First, as mentioned above, gH was reported to function in nuclear egress. Second, some stretches in gH resemble hydrophobic and helical peptides that are present in known fusion proteins (23, 26). Third, gD and gH/gL were reported to be sufficient for hemifusion in the absence of gB (64). Hemifusion is an intermediate step in fusion wherein the outer leaflets of the two lipid bilayers mix prior to the formation of a fusion pore. Fourth, gH/gL from the alphaherpesvirus VZV was reported to be sufficient alone for cell-to-cell fusion (19). The general consensus in the field now is that gH/gL is not a fusion protein (13). More recent studies report that both VZV gH/gL and gB are required for fusion (45) and that gH/gL is insufficient for hemifusion (34).

The crystal structure of the gH/gL complex from HSV2 were determined recently (13). There are crystal structures of EBV gH/gL and a partial structure of pseudorabies gH as well (6, 46). The sequences of HSV1 gH and gL are highly homologous to those of HSV2 gH and gL, so the structure of HSV1 gH/gL is likely very similar to that of HSV2 gH/gL. HSV2 gH/gL has a boot shaped structure. The glycoprotein gH has three domains (H1-H3). H1 is an N-terminal domain that binds to gL; H2 is a central helical domain; and H3 contains a C-terminal β-sandwich domain (13). The H3 domain sequence is highly conserved among herpesviruses and may play a functionally important role. gH is required for the proper folding of gL since gL does not have a stable core itself. Glycoprotein gL has four cysteines that form two disulfide bonds that are conserved among other herpesviruses (13). gH has been shown to bind αvβ3 intergrins molecules at a Arg-Gly-Asp (RGD) motif (55). Other viruses use intergrins as entry receptors, including HHV8. This may not be the case for HSV since mutations in the RGD motif did not limit infection in vitro (55). Intergrin-gH interactions may play a role in entry in other cell types in vivo. gH/gL is proposed to transmit activating signals to gB, allowing gB execute membrane
fusion (63). The function of gH/gL is still unknown since it does not function as the fusion protein yet is necessary for HSV fusion.

D. EBV entry glycoproteins

EBV and HSV1 share three conserved essential entry glycoproteins—gB, gH, and gL. HSV1 requires gD to bind to a cellular receptor to trigger fusion; however, EBV does not encode a gD. Entry of EBV, like HSV, is cell type dependent; EBV can infect B cells, epithelial cells, and monocytes (43). Entry into B cells occurs through viral glycoproteins interacting with a cellular receptor either on the cell surface or in the endosome in a pH-dependent fashion (14, 47). For B cell entry, glycoprotein 42 (gp42) replaces the function of HSV1 gD and binds to the cellular receptor human leukocyte antigen class II (HLA class II) (42). gp42 is cleaved near the N-terminus but remains attached to the virion due to binding to gH/gL (57). Conformational changes in gp42 and/or gH/gL trigger gB to insert fusion loops into the B cell. gB undergoes a conformational change causing fusion between the virion membrane and B cell membrane.

EBV entry into epithelial cells occurs differently. gp42 is not required for epithelial cells entry, and gp42 inhibits entry into epithelial cells. EBV entry into epithelial cells requires only gH/gL and gB. gH/gL is responsible for attachment to the epithelial cells. Some evidence shows that gH/gL binds to αv integrins on epithelial cells(49, 72, 73). After receptor-binding, gH/gL is able to interact with gB, causing gB to insert its fusion loops into the epithelial cell membrane (12). gB undergoes a conformational change causing fusion of the two membranes.

EBV has evolved a mechanism to alter tropism of the virus from epithelium to B cells and back to epithelium in a host. The viruses made in B cells have low amount of gp42 since the 3-part complex (gHgLgp42) is degraded in the endoplasmic reticulum if the complex interacts with HLA class II (10). The viruses made in epithelial cells have high amounts of gp42 since these cells do not have MHC class II molecules (10). The virus from B cells is more infectious to epithelial cells; while, the virus from epithelial cells is more infectious to B cells.
E. Glycoprotein Interactions

The glycoproteins gD, gB, and gH/gL are required for HSV1 entry and their interactions are an active area of research. Using chemical cross-linkers, heterodimers of gB-gC, gC-gD, and gD-gB have been detected (27, 28). Co-immunoprecipitation of the glycoproteins is challenging because the interactions are likely transient and/or low affinity and the proteins may disassociate during a pull-down assay. The glycoproteins exist in native membrane-anchored states on the virion envelope and the detergents required for a pull-down assay may perturb the normal interactions. One research group has reported using a pull-down assay to detect all of the glycoproteins interacting with all of the other glycoproteins (25).

Given the limitations of the pull-down assay, a new method emerged to study glycoprotein interactions—bimolecular fluorescence complementation (BiFC). BiFC allows for direct visualization of protein interactions in a living cell. One candidate protein is fused with the N-terminal portion of a fluorescent protein and another candidate protein is fused with the C-terminal portion of the fluorescent protein. The interaction between two candidate proteins drives the formation of a functional fluorescent complex as the two fragments of the fluorescent protein are brought into proximity (31). Visualization of physical interactions is possible in the normal environment of a living mammalian cell. BiFC has known complications. Use of a fluorescent tag can drive proteins to the wrong cellular compartment or cause the proteins to misfold (67). In addition, false positives are a concern. The two portions of the fluorescent protein have intrinsic affinity for one another, and this affinity can contribute spuriously to interactions between proteins (15, 31). The fluorescent halves do not come apart after interacting with one another. False negatives are also possible since the fluorescent protein fragments can sterically block protein-protein interactions (31). Another concern is that BiFC is characterized through microscopy, which can be more subjective than western blotting.
BiFC has been used to study glycoprotein interactions in HSV1. Initially, two research groups using BiFC reported that gD is able to interact with gB in the absence of gH/gL (2). The inverse was also true; gD was able to interact with gH/gL in absence of gB (2). Most notably, gH/gL was able to interact with gB, but only in the presence of gD, suggesting that gD binding to receptor triggered a gH/gL-gB interaction (2, 4). These BiFC studies supported a proposed model in which gD binding to receptor causes a conformational change in gD that activates gH/gL to interact with gB which then executes membrane fusion (3, 16) (Fig. 1).

This proposed model stands as the generally accepted model of herpesvirus entry however further studies using BiFC have clouded the model. After further study, one of the research groups above used BiFC to demonstrate that gB can bind to gH/gL in the absence of gD (4, 5). Later studies used BiFC to probe the sites of glycoprotein interactions using mutant glycoproteins and monoclonal antibodies that block virus entry (1). These studies found that gH/gL failed to interact with a gB carrying mutations in its fusion loops. This result led the researchers to propose that gB inserts its fusion loops into the host cell membrane prior to an interaction with gH/gL, a suggestion that leaves the signal that triggers gB to insert its fusion loops into the host cell membrane unaccounted for. In a study on paramyxoviruses, BiFC resulted in fluorescence when the receptor-binding protein was co-expressed with its fusion protein but also when it was co-expressed with unrelated BiFC labeled constructs, showing BiFC may be driving the interactions (15). These conflicting results highlight concerns with the BiFC technique and the leave questions regarding the sequence of interactions among HSV1 glycoproteins.

**F. Proximity Biotinylation**

With the false positive and false negative concerns of BIFC, the interactions between the HSV1 proteins and the sequence of events are unclear. Another new technique, proximity biotinylation, can be used to characterize the protein-protein interactions. Proximity biotinylation uses an enzyme and substrate pair to detect candidate protein interactions. One candidate protein
is tagged with the *Escherichia coli* biotin ligase enzyme (BirA), and another candidate protein is tagged with a 15 amino acid acceptor peptide substrate (AP). If BirA and AP come into close proximity, BirA will covalently ligate biotin to the AP (Fig. 2). The interaction can be observed by microscopy using fluorescently labeled streptavidin or by western blot using streptavidin to visualize the biotinylated proteins. Multiple versions of the AP target peptide with differing affinities for BirA are available, and these options allow for optimization of signal over background noise (21, 67).

Proximity biotinylation is a promising technique for many reasons. Labeling can be done in live cells. Cells can be transfected with different combinations of viral glycoproteins in the presence and absence of receptor. The interaction between biotin and streptavidin is high affinity and highly sensitive. Endogenous mammalian biotin ligase does not recognize the AP target peptide. A low false positive rate across a wide range of protein expression levels has been reported (21). Since the AP is only 15 amino acids compared to the large BiFC fluorescent tags, it is less likely to alter the glycoprotein function. BirA and the AP are able to move apart after an interaction so proteins are free to move in the membrane, making it an ideal technique for transmembrane protein interactions. Another advantage to ProB is that the biotin is covalently linked to the AP tag permanently tagging the protein with biotin. Since detection of biotinylated proteins is done on the cell lysates through western blotting, the interaction between proteins can be clearly identified. ProB has the potential to be a useful protein-protein interaction technique without the need for microscopy, the ability to use western blotting, no time delay, or the use of a fluorophore. This technique is worth optimizing to use for transient, transmembrane interactions. ProB has potential to characterize herpesvirus glycoproteins interactions due to these lucrative advantages.
G. Project Overview

The goal of this thesis project is to optimize a proximity biotinylation assay and determine whether it can be used to study the herpesvirus entry glycoprotein interactions. One aim is to determine if biotinylation is detectable through the use of positive and negative controls. The other aim is to determine the optimal conditions of ProB assay. Interactions between the subunits of known oligomers serve as the positive controls. Coexpression of HSV gD constructs linked to BirA and AP is expected to result in biotinylation because gD oligomerizes (homotypic interaction). Similarly, coexpression of EBV gB linked to BirA and APm serves as a positive control (homotypic interaction). Coexpression of proteins that are not expected to interact serve as negative controls (heterotypic interactions). HSV gD and EBV gB do not functionally interact and thus coexpression of HSV gD and EBV gB constructs linked to BirA or AP is not expected to result in biotinylation. By establishing positive and negative controls, ProB could be used to detect the glycoprotein interactions between HSV gB, gD, and gH/gL before and after gD binds a cellular receptor, and before and after fusion occurs.
Figure 2. Proximity biotinylation. Proximity biotinylation (ProB) is a technique wherein an enzyme and substrate pair is used to determine if an interaction occurs. One target protein is tagged with *Escherichia coli* biotin ligase enzyme (BirA) and the other protein is tagged with its substrate, a 15 amino acid acceptor peptide (AP). If an interaction occurs between the two target proteins, BirA will covalently ligate biotin to the AP. Streptavidin, which has a high affinity for biotin, can then be used to visualize the biotinylated target proteins by western blot or fluorescence microscopy.
II. Methods

A. Cells, antibodies, and plasmids. Chinese hamster ovary (CHO-K1) cells lack HSV-1 receptors and were grown in Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (58) at 37°C in 5% CO₂. CHO-nectin1 is a cell line derived from CHO-K1 cells that expresses the HSV1 receptor nectin-1. They were grown in Ham’s F-12 medium supplemented with 10% FBS, 1% PS, and 250 µg/mL G418. Trypsin-EDTA was used to detach these adherent cells. The antibodies used include anti-HSV1 gD polyclonal antibody (PAb) R7 (33) anti-HSV1 gD 340-356 PAb (20), anti-EBV gB monoclonal antibody (MAb) CL55 (73) anti-EBV gB PAb (8), anti-hemagglutinin (HA) tag MAb 12CA5 (53), anti-FLAG M2 MAb (Sigma F9291), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG PAb (Pierce), and HRP-conjugated goat anti-rabbit IgG PAb HRP (Pierce). HRP-conjugated streptavidin (Pierce) was used to detect biotin. Wildtype HSV glycoproteins were expressed using the pCAGGS vector (54). HSV1 gD-BirA, gD-AP, gD-APm EBV gB wildtype (24), EBV gB767 (24), EBV gB816 (24), gB-BirA, gB-AP, gB-APm were expressed using the pSG5 vector (Agilent). For the syncytium formation assay the following plasmids were used: pPEP98 (pCAGGS-HSV1 gB), pPEP99 (pCAGGS-HSV1 gD), pPEP100 (pCAGGS-HSV1 gH), and pPEP101 (pCAGGS-HSV1 gL).

B. Transfection. CHO-K1 cells were transfected in Opti-MEM (Gibco) medium using Lipofectamine 2000 (Invitrogen) according to the manufactures directions. Cells were grown in a 24 well dish overnight and transfected with various combinations of expression vectors in Opti-MEM for 3-5 hours. The transfection mixture was removed and the cells supplemented with Ham F-12 media for an overnight incubation at 37°C.
C. Western blot for total cell protein expression. CHO-K1 cells were transfected as described above. After an overnight incubation, cells were rinsed with PBS+ (phosphate-buffered saline containing 0.5 mM Mg\(^{2+}\), 0.9 mM Ca\(^{2+}\)), detached with trypsin-EDTA and lysed using a RIPA buffer (radioimmunoprecipitation assay buffer: 1% NaDOC, 1% TX100, 0.1% SDS, 10 mM Tris-Cl pH 7.4) with a mammalian protease inhibitor cocktail (M250, Amresco). Cell lysates were centrifuged at 14,000 g at 4°C for 5 minutes. The supernatants were collected, added to sample buffer with sodium dodecyl sulphate (SDS) and 2-mercaptoethanol, and boiled for 5 min. Samples were loaded on a 12% precast polyacrylamide gel (Bio-Rad) and separated by SDS-PAGE.

Proteins were transferred to 0.2 µm nitrocellulose membrane (Bio-Rad) at 150V for 1.5 h. Blots were blocked with 5% dry milk for 30 minutes at room temperature (RT) or overnight at 4°C. Blots were then incubated for 2h at RT with anti-HSV1 gD PAb peptide 340-356 antibody or anti-EBV gB PAb antibody diluted in PBS with 0.3% tween (PBS-T) and 5% milk. Blots were washed three times (5 minutes/wash) with PBS-T and goat anti-rabbit-HRP was added at a 1:7,000 dilution in PBS-T with 5% milk for 1 hour, rocking at RT. After three PBS-T washes, the membranes were developed using enhanced chemiluminescence (ECL) substrate (Pierce) and detected with an HD2 imager (ProteinSimple).

D. Cell-based enzyme-linked immunosorbent assay (cELISA). To determine the protein expression on the surface of the cells. CHO-K1 cells were seeded (6 x 10^4 cells/well) in a 96-well plate and triplicate wells were transfected with 110 ng/well of DNA using 0.35 µL Lipofectamine 2000/well (50 µL/well, total volume). The cells were incubated overnight at 37°C. The wells were blocked for 30 minutes at RT with PBS-BSA (PBS+ with 3% [wt/vol] bovine serum albumin). Cells were incubated with R7 diluted 1:2,500 or CL55 diluted 1:10,000 in PBS-BSA on ice for 1 hour. The wells were rinsed three times with PBS+ and fixed in PBS+ with 2% formaldehyde and 0.2% glutaraldehyde. Cells were rinsed and incubated with goat anti-rabbit-
HRP at 1:5,000 dilution or goat anti-mouse-HRP at 1:2,000 dilution for 1 hour at RT. Expression was determined by adding TMB microwell peroxidase substrate (Fisher) and measuring the absorbance at 670 nm on Biotek Cytation 3 Cell Imaging Multi-Mode Reader.

**E. Western blot for proximity biotinylation.** CHO-K1 cells seeded overnight in a 24 well plate (3 x 10^5 cells/well) were transfected as described above. After the overnight transfection, the cells were pulsed with 20 µM biotin at 37°C. Cells lysates were collected and subjected to SDS-PAGE, as described above, using duplicate gels. Samples were transferred to nitrocellulose as above and membranes were blocked with PBS with 1% BSA for 30 minutes at RT or overnight at 4°C. BSA was used to block the membranes because milk inhibits the biotin-streptavidin interaction. One membrane was incubated with streptavidin-HRP in PBS-T with 1% BSA for 1h and washed three times with PBS-T. The duplicate membrane was incubated for 2 hours at RT with either EBV gB PAb (diluted 1:1,000 in PBS-T with 1% BSA) or 12CA5 (diluted 1: 2,000) in PBS-T with 1% BSA) to detect the HA-tagged AP constructs. This membrane was washed and incubated with either goat anti-rabbit-HRP (1:10,000 dilution) or goat anti-mouse-HRP (1:7,000 dilution) in PBS-T with 1% BSA. Both blots were developed using ECL as described above. Then the duplicate blot was stripped using two 7 minute washes in a mild stripping buffer (15g glycine, 1g SDS, 10 mL Tween-20 per liter adjusted to pH 2.2) followed by two 10 minute rinses in PBS and two 5 minutes rinses in Tris-buffered saline with 0.1 % Tween-20. Signal loss was confirmed by imaging the blot using ECL. This blot then was re-probed for 2 hours at RT using either anti-gD 340-356 PAb (diluted 1: 2,000 in PBS-T with 1% BSA) or anti-FLAG MAb (diluted 1: 7,000 in PBS-T with 1% BSA) to detect the FLAG-tagged BirA constructs. The blot then was washed, incubated with goat anti-mouse-HRP and developed as above.

**F. Depletion of biotin from medium.** Ham’s F12 supplemented with 10% FBS and 1%PS was depleted of free biotin by adding of 50 µg/mL of streptavidin (New England Biolabs 721). CHO-
K1 cells were transfected as described above, but the transfection mix was removed after 3-5 hours and biotin-depleted media was added for the overnight incubation.

**G. Proximity biotinylation with immunoprecipitation.** CHO-K1 cells were seeded in a 12 well plate (6 x 10^5 cells/well) and transfected as described with 440 ng of DNA/construct (880 ng/well). After an overnight incubation, the cells were pulsed with 20 µM biotin for 5 minutes at 37°C and cell lysates were collected as above using 400 µL/well of RIPA buffer. The lysates were split, with 75 µL of each lysate reserved for immunoprecipitation (IP) (36). The rest of the cell lysates were analyzed by SDS-PAGE and western blot as described above.

To IP the AP constructs, 5µL of 12CA5 MAb was added to 75µL of lysates for 2h at 4°C on a rotator. 15µL of a 50% protein A/G+ bead slurry (Santa Cruz Biotechnology) was added for 1h at 4°C on the rotator. After pelleting at 14,000 rpm for 1 minute at 4°C, the beads were washed with 1 mL RIPA buffer 5 times, with 30 seconds of centrifugation at 4°C after each wash. SDS-sample buffer at 2X with 2-mercaptoethanol was added to each pellet of beads. The samples were boiled for 5 minutes and centrifuged at 14,000g for 1 minute. The IP samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with streptavidin-HRP as described above.

**H. Competition proximity biotinylation assay.** CHO-K1 cells seeded overnight in a 24 well plate (3 x 10^5 cells/well) were transfected with 450 ng/µL DNA per well, including 100 ng per AP- or BirA-linked construct and 250 per competitor construct. Cells were transfected in Opti-MEM (Gibco) medium using Lipofectamine 2000 (Invitrogen) according to the manufactures directions. After 3-5 hours, the transfection mixture was removed and the cells were supplemented with Ham’s F-12 media at 37°C. After an overnight incubation, the cells were pulsed with 20 µM biotin at 37°C for 5 minutes. Cells lysates were collected and subjected to SDS-PAGE, as described above, using duplicate gels. The western blots were performed as
described above, with one blot probed using streptavidin-HRP and the other blot probed with anti-HA antibody and then re-probed with anti-FLAG antibody.

I. Competition assay with gH. CHO-K1 cells seeded overnight in a 12 well plate (6 x 10^5 cells/well) were transfected with 880 ng DNA per well (340 ng per construct, 100 ng HSV1 gH, 100 ng HSV1 gL), as described above. After an overnight incubation, the cells were pulsed with 20 μM biotin at 37°C for 5 minutes. Western blots were performed using cell lysates as described above, with one blot probed using streptavidin-HRP and another probed with anti-HA antibody followed by stripping and reprobing using anti-FLAG antibody.

J. Syncytium formation assay. CHO-nectin1 cells were seeded (6 x 10^5 cells/well) overnight in 24-well plates and then transfected with plasmids encoding HSV1 gB (pPEP98), gH (pPEP100), gL (pPEP101), and either gD (pPEP99), gD-BiRA or gD-APm. Each well received 900 ng DNA total, including 300 ng gB plasmid and 200 ng for each of the rest of the plasmids. The cells were transfected in Opti-Mem (Gibco) medium for 3-5 hours using Lipofectamine 2000 (Invitrogen) according to the manufactures directions. The transfection mixture was replaced with Ham F-12 supplemented with 10% FBS and PS and cells were incubated overnight at 37°C. Cells were fixed with methanol, stained with Giemsa (GibcoBRL), rinsed with water, and scored for syncytium formation by microscopy.

K. Densitometry. To quantify the western blot bands the backgrounds of the blot were subtracted from the band. Densitometry values were determined using AlphaView Software from the FluorChem HD2 imager from ProteinSimple. Values are provided under each blot.
III. Results

A. Positive and negative ProB controls. HSV and EBV entry glycoproteins form multimers and thus detection of proximity between monomers of a glycoprotein can be used a positive control for the ProB assay. The entry glycoproteins of EBV cannot be substituted for those of HSV and vice versa. Since glycoproteins from the two viruses do not functionally interact, they are not expected to physically interact, and thus they provide a negative control for the ProB assay. HSV1 gB and EBV gB share 29% identity and 43% similarity (7). Although the crystal structures of HSV1 and EBV gB demonstrate a common fold for these homologous proteins, their extensive sequence variations should preclude interaction of gB with heterotypic viral glycoproteins (7, 29). EBV does not utilize a glycoprotein gD. When EBV enters B cells, it uses gp42; however, gp42 is not required for entry into epithelial cells. There is no functional reason that EBV gB should interact with HSV1 gD.

To validate ProB as a viable method to detect interactions between the HSV1 glycoproteins necessary for entry, ProB constructs were cloned for HSV1gD and EBV gB. The cytoplasmic tails of these proteins were linked to either biotin ligase (BirA) or its target peptide (AP or APm) (Fig. 3). APm is a 3 amino acid deletion, removing a tryptophan, histidine, glutamic acid from the original AP, shown to reduce background signal since it has a lower affinity for BirA (21). The BirA-linked constructs have a FLAG epitope tag, and AP- or APm-linked constructs have a hemagglutinin (HA) epitope tag.

B. Expression of HSV1 and EBV constructs. To determine if HSV1 gD constructs were expressed, plasmids encoding the constructs were transfected into CHO-K1 cells, and proteins from total cell lysates were analyzed by western blot (Fig. 4A). The wildtype HSV1 gD, gD-AP, and gD-APm migrated to 50 kDa, while gD-BirA migrated to 75 kDa (Fig. 4A). The proteins were detected with a polyclonal antibody serum specific for HSV1 gD. HSV1 gD-BirA was
Figure 3. Proximity biotinylation constructs. Schematic representation of HSV1 gD (A) and EBV gB (B) constructs linked with the biotin ligase BirA or the target peptides AP and APm. The BirA, AP, and APm were added to the cytoplasmic tails of the glycoproteins. The dark blue and green boxes represent the transmembrane domains. BirA-linked constructs include a C-terminal FLAG epitope. AP- and APm-linked constructs include a C-terminal HA tag. HSV gD-BirA is expected to migrate to 75 kDa while HSV gD-AP and –APm are expected to migrate to 50 kDa. EBV gB-BirA is expected to migrate to 200 kDa, and EBV gB-AP and –APm are expected to migrate to 140 kDa.
Figure 4. Expression of HSV1 constructs. (A) Western blot of HSV1 gD constructs. Total cell lysates of CHO-K1 cells transfected with the gD constructs or empty vector were analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and probed using a polyclonal antibody serum specific for HSV1 gD. Size markers in kDa are indicated on the left. Densitometry values for the bands (~75 kDa for lane 2, ~50 kDa doublet for lanes 3-5) are below the blot. (B) CELISA of HSV1 gD constructs. CHO-K1 cells were transfected with the gD constructs or empty vector and probed for cell surface expression of gD using a polyclonal antibody serum. HRP-conjugated secondary antibody was added followed by substrate. Absorbance at 670 nm was recorded as a measure of expression.
expressed at lower levels than wildtype gD. Cell surface expression of these gD proteins in CHO-K1 cells was determined by cELISA (Cell-based Enzyme-Linked Immunosorbent Assay) using a gD polyclonal antibody (Fig. 4B). All the HSV1 gD constructs were present on the cell surface, suggesting that they were properly processed. gD-BirA was expressed on the cell surface at near wild-type levels, whereas gD-AP and gD-APm were present on the surface at somewhat lower levels.

The expression of the EBV gB constructs also was detected by western blotting of transfected CHO-K1 cell lysates (Fig. 5A) and cELISA of transfected CHO-K1 cells (Fig. 5B). Wild-type EBV gB is known to be poorly expressed at the cell surface, so two gB mutants (gB767 and gB816) that were previously shown express at higher levels on the cell surface were included as controls. gB767 and gB816 have cytoplasmic tail truncations that remove putative endocytosis motifs. As expected, EBV gB-BirA migrated to 200 kDa, wildtype gB, gB-AP, and gB-APm migrated to 140 kDa, and the truncated gB767 and gB816 migrated more rapidly (Fig. 5A). The gB ProB constructs were expressed at near wild-type levels in the cell lysates whereas the truncation mutants were expressed at lower levels. As previously reported, wild-type gB expression on the surface was barely detectable (Fig. 5B). The ProB constructs were also poorly expressed at similar levels on the cell surface. As expected, the gB truncation mutants were expressed at higher levels on the cell surface. The increase in surface expression for these mutants without a concurrent increase in total cell expression is consistent with previous reports. Surface expression of the ProB mutants at near wild-type levels suggests that the proteins are properly processed.

C. Initial ProB trial. In the initial trial of ProB, CHO-K1 cells were transfected with combinations of HSV1 gD constructs, EBV gB constructs, and/or empty vector. Cells were pulsed with 20 µM biotin for 15 minutes and lysed. Cell lysates were analyzed by western blots. The levels of protein biotinylation were detected using streptavidin-HRP (Fig.6A).
Figure 5. Expression of EBV constructs. (A) Western blot of EBV gB constructs. Total cell lysates of CHO-K1 cells transfected with gB constructs or empty vector were analyzed by SDS-PAGE. gB767 and gB816 are mutant forms of gB that were previously shown to be expressed at high levels on the cell surface. Proteins were transferred to nitrocellulose and probed with a polyclonal antibody serum specific for EBV gB. Size markers in kDa are indicated on the left. Densitometry values for the bands are below the blot. (B) CELISA of EBV gB constructs. CHO-K1 cells were transfected with the gB constructs or empty vector and probed for cell surface expression of gB using a polyclonal antibody serum. HRP-conjugated secondary antibody was added followed by substrate. Absorbance at 670 nm was recorded as a measure of expression.
Coexpression of the homotypic pair HSV1 gD-BirA and HSV1 gD-APm resulted in strong biotinylation of the gD-APm target (~60 kDa, lane 3). Coexpression of the homotypic pair EBV gB-BirA and EBV gB-APm also resulted in strong biotinylation of the gB-APm target (~140 kDa, lane 8). Thus, proximity was detected in the positive controls. Results were similar whether the AP or APm constructs were used (data not shown). APm is shown instead of AP because it has a reported lower affinity for BirA and resulted a reduction in non-specific bands (data not shown).

Unexpectedly, coexpression of the heterotypic pairings also resulted in biotinylation of the APm targets. Coexpression of HSV1 gD-BirA with EBV gB-APm resulted in biotinylation of gB-APm (~140 kDa, lane 4); however, the biotinylation was weaker than that observed for homotypic gB-BirA and gB-APm expression (lane 8). The other heterotypic combination (EBV gB-BirA and HSV1 gD-APm) also showed a weak biotinylation of the gD-APm (~60 kDa, lane 9). The band migrating at ~60 kDa in lanes 4 and 8 is likely a degradation product of biotinylated EBV gB-APm since it is only present in gB-APm containing samples.

gD-BirA, gD-APm, and wildtype gD expression was detected with a polyclonal antibody specific for peptide 340-356 of HSV1 gD. Both the enzyme and the substrate gD constructs were expressed but HSV1 gD-APm is expressed at a higher level compared to HSV1 gD-BirA (Fig. 6C). Expression of EBV gB-BirA, APm, and wildtype was detected with a polyclonal antibody specific for EBV gB (Fig. 6B). Expression of both EBV gB-BirA and gB-APm is detected, however EBV gB-BirA expression in the last two lanes is weak. Interpretation of the heterotypic interaction between gB-BirA and gD-APm (lane 9) is complication by the poor detection of the gB-BirA expression. Subsequent experiments determined that the antibody for EBV gB does not detect gB as well as the antibody used for HSV1 gD, which could explain the lack of detection of the EBV gB glycoproteins on the cell surface. To try to reduce the signal for the heterotypic interaction, the exposure to biotin and amount of DNA transfected was reduced.
Figure 6. Initial trial of proximity biotinylation. CHO-K1 cells were transfected overnight with combinations of HSV1 gD constructs, EBV gB constructs, and/or empty vector. Each sample was transfected with 660 ng DNA total, comprising 220 ng per construct. Cells were pulsed with 20 µM biotin for 15 min and then lysed. Cell lysates were analyzed by SDS-PAGE and proteins were transferred to nitrocellulose. Duplicate blots were generated and size markers in kDa are indicated on the left. (A) Biotinylated proteins were detected by probing with streptavidin-HRP. The arrowhead denotes biotinylated EBV gB and the asterisk denotes a doublet band of biotinylated HSV1 gD. (B) EBV gB expression was detected by probing a duplicate blot with polyclonal serum. (C) The blot from B was stripped and reprobed with polyclonal serum to detect HSV1 gD expression. Densitometry values for the bands of interest are below each blot. For A and C, the upper value represents the larger molecular weight species.
D. ProB assay optimization with lower amounts of DNA, shorter biotin exposure. Since the level of biotinylation was greater than expected in the heterotypic combinations, the amount of DNA transfected was reduced and the cell exposure to biotin was shortened (Fig. 7). The amount of DNA was reduced more than 2-fold from the previous experiment (to 300 ng DNA per well). Cells were exposed to the same concentration of biotin (20 µM), but for 5 minutes rather than 15 minutes. The HSV1 gD homotypic combination showed a strong biotinylation signal (lane 3, ~60 kDa), as did the EBV gB homotypic combination (lane 7, ~140 kDa). The heterotypic pairing of HSV1 gD-BirA and EBV gB-APm resulted in biotinylation of gB-APm (lane 4, ~140 kDa), but quantification of this band is difficult due to the presence of a background band in all of the lanes that migrates at the same molecular weight. This cellular biotinylated protein was present in the previous experiment (Fig. 6), but at lower levels compared to gB. Reducing the amount of DNA transfected complicated interpretation of the blot because the ratio of expression of gB to the background band was reduced. A heterotypic interaction between EBV gB-BirA and HSV1 gD-APm is apparent (lane 8, ~60 kDa). A degradation product of biotinylated gB-APm is apparent again in lanes 4 and 8 at ~60 kDa. The dots in lane 3 are an artifact, most likely streptavidin-HRP precipitate that adhered to the blot. The gD and gB constructs EBV were expressed at levels similar to the previous western blots (data not shown). The EBV polyclonal antibody still was not detecting gB levels well. To better detect gB and gD constructs, a new set of antibodies was used that allows direct comparison of the levels of BirA enzyme and APm substrate.

E. ProB assay optimization using the FLAG and HA epitopes. Since the EBV gB antibody did not detect EBV gB well, antibodies specific for the FLAG and HA epitopes on the proteins were tested. Using the epitope tags to detect the proteins also allowed better comparison of protein levels, since both of the BirA enzymes constructs have FLAG tags, and both of the APm substrate constructs have HA tags. Constructs were co-transfected in this experiment to determine the expected expression levels of the glycoproteins when co-transfected in future experiments (Fig.
Figure 7. Proximity biotinylation trial with less DNA and shorter biotin exposure. CHO-K1 cells were transfected overnight with combinations of HSV1 gD constructs, EBV gB constructs, and/or empty vector. Each sample was transfected with 300 ng DNA total, comprising 100 ng per construct. Cells were pulsed with 20 µM biotin for 5 min and then lysed. Cell lysates were analyzed by SDS-PAGE and proteins were transferred to nitrocellulose. Biotinylated proteins were detected by probing with streptavidin-HRP. The two halves of the blot are from the same exposure. Size markers in kDa are indicated on the left. The arrowhead denotes biotinylated EBV gB and the asterisk denotes a biotinylated HSV1 gD. Densitometry values for the bands of interest are below each blot. The upper value represents the larger molecular weight species.
Figure 8. Detection of constructs expression using HA and FLAG tags. Western blot of APm and BirA constructs. Total cell lysates of CHO-K1 cells transfected with HSV1 gD constructs, EBV gB constructs, and/or empty vector were analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with a monoclonal antibodies specific for the HA tag (A) or the FLAG tag (B). Size markers in kDa are indicated on the left. The arrowhead denotes EBV gB and the asterisks denote HSV1 gD. Densitometry values for the bands of interest are below each blot. For A, the upper value represents the larger molecular weight species.
8). EBV gB-APm was detected at ~140 kDa and HSV1 gD-APm was detected at ~60 kDa (Fig. 8A). EBV gB-BirA was detected at ~150 kDa and HSV1 gD-BirA was detected at ~75 kDa (Fig. 8B). Stronger signals were perceived with the anti-FLAG monoclonal antibody and the anti-HA monoclonal antibody compared to the EBV gB antibody seen in previous blots. Cellular proteins that react with the anti-HA antibody are present in all lanes and migrate to the same molecular weight as gD as well as at ~75 kDa.

A ProB assay was performed as previously, using 375 ng DNA per well and the HA or FLAG epitopes to assess protein expression levels (Fig. 9). Biotinylation is apparent in the homotypic combinations for both HSV1 gD (lane 2) and EBV gB (lane 6). The heterotypic combinations have weaker but detectable biotinylation signals (lanes 3 and 7). All of the APm and BirA constructs were expressed, with the gB constructs again at higher levels than the gD constructs again (Fig. 9B, 9C). The detection of the enzyme and substrate with the HA and FLAG epitopes were useful for comparison of the enzyme levels (gB-BirA and gD-BirA) and substrate levels (gB-APm and gD-APm).

F. ProB with depletion of free biotin. The cellular protein(s) that react with streptavidin-HRP and migrate to ~140 kDa obscure observation of biotinylated gB-APm. These bands may result from endogenous biotin being detected by the streptavidin-HRP. In attempt to decrease the presence of these background biotinylated species, two sets of ProB samples were compared. One set was treated as before (Fig. 10A) and for a second set, the media was depleted of free endogenous biotin by the addition of streptavidin (Fig. 10B). For this second set, streptavidin was maintained in the media after transfection for the duration of the overnight incubation prior to the biotin pulse. CHO-K1 cells were transfected with 660 ng DNA per well total, including 220 ng per construct. As seen previously, the homotypic combinations yield strong biotinylation signals (Fig. 10A, lanes 2 and 6). The heterotypic combination yields weaker biotinylation signals than the homotypic interactions (Fig. 10A, lanes 3 and 7). Depletion of the free biotin did not
Figure 9. Proximity biotinylation using HA and FLAG tags. CHO-K1 cells were transfected overnight with combinations of HSV1 gD constructs, EBV gB constructs, and/or empty vector. Each sample was transfected with 300 ng DNA total, comprising 150 ng per construct. Cells were pulsed with 20 μM biotin for 5 min and then lysed. Cell lysates were analyzed by SDS-PAGE and proteins were transferred to nitrocellulose. Size markers in kDa are indicated on the left. (A) Biotinylated proteins were detected by probing with streptavidin-HRP. The arrowhead denotes biotinylated EBV gB and the asterisks denote a doublet band of biotinylated HSV1 gD. (B) APm was detected by probing a duplicate blot with an antibody specific for the HA tag. (C) The blot from B was stripped and reprobed with an antibody specific for the FLAG tag, detecting BirA expression. The arrowhead denotes EBV gB and the asterisks denote HSV1 gD. Densitometry values for the bands of interest are below each blot. For A, the upper value represents the larger molecular weight species.
Figure 10. Proximity biotinylation after biotin-depletion of the medium. Duplicate sets of CHO-K1 cells were transfected with combinations of HSV1 gD constructs, EBV gB constructs, and/or empty vector. Each sample was transfected with 660 ng DNA total, comprising 220 ng per construct. One set of samples was treated as in Fig. 5 (A). The medium for the other set (B) was supplemented with 50 µg/mL streptavidin to bind endogenous biotin. After an overnight incubation, all cells were pulsed with 20 µM biotin for 5 min and then lysed. Cell lysates were analyzed by SDS-PAGE and proteins were transferred to nitrocellulose. Biotinylated proteins were detected by probing with streptavidin-HRP. The arrowhead denotes biotinylated EBV gB and the asterisks denote a biotinylated HSV1 gD. Densitometry values for the bands of interest are below each blot. The upper value represents the larger molecular weight species.
affect the results (Fig. 10B) and did not decrease the signal from the background cellular protein migrating at the same size as gB-APm. The biotinylated species is not impacted by depletion of the free biotin in the media.

**G. ProB with immunoprecipitation (36).** To reduce the impact of the non-specific bands in the western blots, the APm constructs were immunoprecipitated prior to loading the gels. The ProB was performed and cell lysates were collected as previously. Then the APm constructs were immunoprecipitated from the lysates using an antibody specific for the HA tag and beads covalently-conjugated to protein A/G. Samples were reordered on the gel for easier comparison of homotypic and heterotypic combinations. The IP eliminated the biotinylated cellular species that previously obscured the gB-APm band. As before, both homotypic and heterotypic interactions are apparent. Biotinylation of gD-APm is similar when it is coexpressed with either homotypic gD-BirA or heterotypic gB-BirA (Fig. 11A, lanes 1 and 2). Given than gB-BirA is expressed at a greater level than gD-BirA (Fig. 11C), this may indicate that biotinylation of the homotypic combination is more specific than the heterotypic combination. Biotinylation of gB-APm is greater when it is coexpressed with the homotypic gB-BirA rather than the heterotypic gD-BirA (Fig. 11A, lanes 4 and 5), however this results may be influenced by the fact that gD-BirA is expressed at lower levels than gB-BirA (Fig. 11C).

**H. gD construct function in fusion.** The HSV1 gD constructs tagged with BirA and APm were tested for their ability to mediate cell-cell fusion. CHO-nectin-1 cells were used since this cell type expresses nectin-1, a herpesvirus receptor expressed on neurons. CHO-nectin-1 cells transfected with wildtype HSV1 gB, gD, gH, and gL formed large syncytia (Fig. 12). When the fusion protein gB was excluded from the transfection, cells did not form syncytia. When cells were transfected with plasmids encoding gB, gH, gL and either gD-APm or gD-BirA, many large
Figure 11. Proximity biotinylation with immunoprecipitation. CHO-K1 cells were transfected overnight with combinations of HSV1 gD constructs, EBV gB constructs, and/or empty vector. Each sample was transfected with 880 ng DNA total, comprising 440 ng per construct. Cells were pulsed with 20 µM biotin for 5 min and then lysed. Lysates were divided for immunoprecipitation (A) or direct loading onto the gel (B and C). (A) The APm constructs were immunoprecipitated from the lysates using an antibody specific for the HA epitope tag and protein A/G beads. Biotinylated proteins were detected by probing with streptavidin-HRP. (B) APm was detected by probing a duplicate blot with an antibody specific for the HA tag. (C) The blot from B was stripped and reprobed with an antibody specific for the FLAG tag, detecting BirA expression. The arrowhead denotes EBV gB and the asterisks denote HSV1 gD. Densitometry values for the bands of interest are below each blot. For A, the upper value represents the larger molecular weight species.
Figure 12. Syncytium formation with gD constructs BirA and APm. CHO-nectin-1 cells were transfected with plasmids encoding HSV1 glycoproteins or gD constructs, and/or empty vector. The results shown are representative of 3 replicates. The images were taken 24h post transfection. Arrows indicate syncytia.
syncytia formed. The results indicated that gD-APm and gD-BirA are functional proteins, further supporting that they are properly folded in the cell.

I. ProB competition assay. To examine specificity of the ProB assay and address whether the affinity of BirA for APm contributes to the biotinylation signal, wildtype protein was added in attempt to outcompete the heterotypic interaction. If the interactions that yield biotinylation are driven by attractions between the glycoprotein ectodomains, excess wild-type protein should be able to compete for the binding and decrease the biotinylation signal. In contrast, if the interactions that yield biotinylation are driven by an inherent attraction between BirA and APm, the addition of excess wildtype gD or gB should not affect the level of APm biotinylation.

For the heterotypic gD-BirA and gB-APm combination, coexpression of wildtype EBV gB resulted in a slight reduction in gB-APm biotinylation (Fig. 13, lane 5 and 6). This reduction suggested that the heterotypic gD-BirA interaction with gB-APm might result from interactions with the gB ectodomain rather than from an inherent affinity between BirA and APm, however this competition was not consistently detected. When the heterotypic combination was reversed (gB-BirA coexpressed with gD-APm), coexpression of wildtype gD did not inhibit biotinylation (lanes 3 and 4). Furthermore, the level of biotinylation for the homotypic gB-BirA and gB-APm combination was not affected by the addition of wildtype gB (lane 7 and 8). The crystal structure of the gB ectodomain (29) demonstrates that the gB ectodomin trimerizes, so wildtype gB was expected to inhibit the interaction between gB-BirA and gB-APm. The inability to detect competition by wild-type gB suggests that the inherent affinity between BirA and APm may contribute to gB-APm biotinylation. The gD homotypic combination failed to show high levels of biotinylation (lanes 9 and 10). This may be due to poor expression of gD-BirA. When the BirA blot was stripped and re-probed with the anti-gD polyclonal antibody, no gD-BirA was detected. It was expected to migrate to 75 kDa. Subsequent experiments failed to show clear competition when the wildtype protein was coexpressed with corresponding combinations.
**J. ProB HSV1 gH/gL competition assay.** On the HSV1 virion, gB and gD exist in combination with gH/gL. To examine whether gH/gL influences the HSV1 gD-EBV gB interaction, HSV gH/gL was added to the ProB assay. This assay is an alteration of the competition assay above. Instead of using wildtype gD or gB to compete the interaction, HSV1 gH and gL were coexpressed with the BirA and APm constructs. In one experiment, coexpression of the gH/gL complex decreased biotinylation for the heterotypic combination of gB-BirA and gD-APm (Fig. 14, lanes 3 and 4). In the same experiment, coexpression of gH/gL did not affect homotypic biotinylation of gD-APm by gD-BirA (lanes 1 and 2). The levels of APm biotinylation were similar with or without gH/gL coexpression. Expression of the BirA constructs was unable to be detected in that experiment and the results were not reproducible.
Figure 13. Competition proximity biotinylation assay. CHO-K1 cells were transfected overnight with combinations of HSV1 gD constructs, EBV gB constructs, and either empty vector or wildtype proteins. Each sample was transfected with 450 ng DNA total, comprising 100 ng per construct or 250 ng per competitor. Cells were pulsed with 20 μM biotin for 5 min and then lysed. Cell lysates were analyzed by SDS-PAGE and proteins were transferred to nitrocellulose. (A) Biotinylated proteins were detected by probing with streptavidin-HRP. (B) A blot detecting BirA expression using an antibody specific for the FLAG tag. (C) HSV1 gD expression using a polyclonal antibody serum specific for HSV1 gD. Size markers in kDa are indicated on the left. The arrowhead denotes biotinylated EBV gB and the asterisks denote a biotinylated HSV1 gD. Densitometry values for the bands of interest are below each blot. For A, the upper value represents the larger molecular weight species.
Figure 14. Competition proximity biotinylation assay with HSV1 gH/gL. CHO-K1 cells were transfected overnight with combinations of HSV1 gD constructs, EBV gB constructs, and either empty vector or HSV1 gH/gL. Each sample was transfected with 880 ng DNA total, comprising 340 ng per construct or 100 ng for gH and gL. Cells were pulsed with 20 µM biotin for 5 min and then lysed. Cell lysates were analyzed by SDS-PAGE and proteins were transferred to nitrocellulose. (A) Biotinylated proteins were detected by probing with streptavidin-HRP. (B) A blot detecting APm expression using an antibody specific for the HA tag. Size markers in kDa are indicated on the left. The asterisk denotes a biotinylated HSV1 gD. Densitometry values for the bands of interest are below each blot. For A, the upper value represents the larger molecular weight species.
IV. Discussion

The HSV1 gD constructs with BirA and APm linked to the cytoplasmic tails were expressed, as demonstrated by western blot and CELISA (Fig. 4). Total cell protein expression of gD-BirA expressed was reduced compared to wild-type gD, but cell surface expression was at wild-type levels, suggesting that the mature protein is properly folded. The double banding pattern apparent in the western blot for both wildtype and mutant gD constructs are likely due to different glycosylation of this glycoprotein. The syncytia assay confirms the proper folding of these gD ProB constructs, since they are functional in fusion (Fig. 12).

The EBV gB constructs with BirA and APm linked to the cytoplasmic tails also were expressed as detected by western blotting (Fig. 5). As seen previously, detection of wild-type EBV on the cell surface is difficult. The gB ProB constructs were detected on the cell surface at the same low levels as wild-type gB. The EBV gB tail mutants (EBV gB767 and gB816) that are known to be expressed at higher levels on the surface of the cell were detected at higher levels by cELISA. The tail mutants do not show higher levels of overall expression in the western blot, suggesting that these proteins localize preferentially to the cell surface. The tail mutants lack internalization signals present in wildtype gB. These results suggest that the gB-BirA and gB-APm constructs exhibit wild-type levels of internalization. Overall, the gB constructs are expressed at similar levels to wild-type gB.

The initial ProB trial showed biotinylation in both homotypic combinations (Fig. 6). The gB homotypic biotinylation was greater than the HSV homotypic biotinylation. Both of the heterotypic combinations also demonstrated biotinylation. Since these transmembrane glycoproteins are tethered to a membrane, they are in close proximity with one another. This proximity may facilitate random collisions that are sufficient to allow for the biotinylation of the AP tag without specific protein-protein binding. Optimizations of the ProB assay conditions are required to differentiate between a specific versus random interaction.
When the amount of DNA and the biotin exposure time were reduced, the homotypic interactions were harder to detect (Fig. 7). Since biotinylation was still seen, the shorter biotin exposure time was sufficient for biotinylation. The protein expression after transfection using 100 ng per construct was too low for reliable detection in this ProB assay.

The HSV gD and EBV gB polyclonal antibodies did not detect the glycoproteins effectively. The ProB constructs were designed with FLAG and HA epitopes and antibodies specific for those tags gave enhanced detection of the proteins (Fig. 8). In addition, use of these epitope tags allowed direct comparison of the levels of gB-BirA versus gD-BirA.

Using the epitope tags to compare enzyme levels during the ProB assay provided a clearer picture. The substrate constructs (APm) were expressed at similar levels, but the enzyme constructs (BirA) were not expressed at similar levels (Figs. 8, 9, 11). gD-BirA is expressed at a lower level than gB-BirA and this complicates the interpretation of the results. Specifically, comparing the gB homotypic interaction with the heterotypic interaction between gD-BirA and gB-APm is difficult, since gD-BirA is expressed at lower levels than gB-BirA. The weaker biotinylation seen with the heterotypic combination (Fig. 11) could result simply from the lower levels of enzyme expressed. This result cannot be interpreted to indicate that the homotypic interaction is stronger than the heterotypic interaction.

A nonspecific band that migrates at the same molecular weight as gB-APm was apparent on many of the western blots probed with streptavidin-HRP. This signal is from endogenous biotinylated proteins, since it is present in samples that did not receive BirA. When the media was depleted of free biotin by the addition of streptavidin, the nonspecific bands remained, indicating that depleting endogenous biotin overnight was insufficient to eliminate endogenous biotinylation (Fig. 10).

To eliminate this confounding nonspecific band, an antibody specific for the HA tag on APm constructs was used to immunoprecipitate these proteins. The IP blot still shows a nonspecific band at a molecular weight similar to gD-APm, but the band that overlapped the gB-
APm was successfully lost in the immunoprecipitation (Fig. 11). As seen in the previous ProB trials, both of the homotypic combinations showed strong biotinylation, however the heterotypic combinations also showed biotinylation. Interpretation of the specificity of the interaction requires consideration of the expression levels of the constructs. The gD-APm and gB-APm constructs are expressed at similar levels, so the effect of different APm concentrations in the samples is minimized. In contrast, gB-BirA is expressed at greater levels than gD-BirA (up to two-fold greater in some cases). Given that BirA is an enzyme, capable of biotinylating several APm substrates, difference in BirA construct expression levels may be amplified (Fig. 15).

The homotypic combination of gD-BirA and gD-APm (Fig. 11, lane 1) results in biotinylation levels similar to the heterotypic combination of gB-BirA and gD-APm (Fig. 11, lane 2). Since gD-BirA is expressed at lower levels than gB-BirA, this result may indicate that this homotypic gD interaction is stronger than the heterotypic interaction (Fig. 15). In contrast, although the homotypic combination of gB-BirA and gB-APm (Fig. 11, lane 4) results in over two-fold greater levels of biotinylation than the heterotypic combination of gD-BirA and gB-APm (Fig. 11, lane 5), the lower expression of the gD-BirA may account for this difference.

The competition assay with HSV1 gH/gL showed that gH/gL may outcompete the heterotypic interaction between HSV1 gD-APm and EBV gB-BirA (Fig. 14). A slight decrease is seen in the heterotypic interaction with gH/gL compared to the heterotypic interaction without (lane 3 and 4). This decrease was difficult to reproduce and more experiments need to be performed to replicate these results.

**A. Proximity biotinylation potential.** ProB has been used previously to detect cytosolic protein interactions, however its use as a method to detect transmembrane protein interactions may be limited. The ProB assay was used successfully to study with the intracellular protein-protein interactions FKBP or FK506 and FKBP-rapamycin binding (FRB) protein, which is regulated by rapamycin (21). Transmembrane proteins have restricted mobility while in the membrane. This
Figure 15. Depiction of the effect of enzyme concentration on biotinylation. (A,B) Homotypic combinations. (C, D) Heterotypic combinations. (A and C) gD-BirA and gB-BirA catalyze similar levels of biotinylation when coexpressed with gD-APm, however gD-BirA is expressed at a lower level than gB-BirA. This may suggest that gD-BirA interacts more frequently with the substrate gD-APm than does gB-BirA. (B and D) gB-APm is biotinylated to a greater extent when coexpressed with homotypic gB-BirA rather than heterotypic gD-BirA, however gB-BirA is expressed at a higher level than gD-BirA. This enhanced expression of gB-BirA may contribute to its ability to biotinylate gB-APm to a greater degree.
may contribute to unexpected non-specific interactions between the HSV1 gD and EBV gB proteins.

As discussed, the expression level of the BirA-linked construct is critical for interpretation of the data. In addition, the functionality of the BirA enzyme linked to each construct may differ. ProB is ideal for proteins with flexibility in their structures or proteins that are able to function with an altered shape. The spacing from the membrane of a BirA and APm pair may affect the frequency of biotinylation.

ProB has the potential to be a useful method to study protein interactions due to the use of an enzyme-substrate pair instead of the use of a fluorophore. The target substrate is a small tag, which has a limited ability to affect the function of the protein whereas the BirA enzyme is about 20 kDa. The detection of the interaction is determined via western blot, making it easier to quantify than microscopy. The major advantage of ProB is the covalent linking of biotin to the AP tag, permanently tagging the protein.

This thesis project was to establish and optimize a protocol for proximity biotinylation. The number of cells used, the antibody dilutions, the selection of antibodies, the amounts of DNA, biotin exposure, endogenous biotin depletion, and immunoprecipitation conditions were optimized for this assay.

B. Future work. The interactions between the HSV1 glycoproteins are an active area of research. Further optimization of the ProB assay to reduce detection of nonspecific interaction may be possible.

HSV1 gD and EBV gB were not expected to physically interact because EBV gB and HSV1 gB cannot functionally replace one another. In addition, they have low sequence homology (~30%) and EBV does not encode a gD homolog. These facts do not preclude the possibility that HSV1 gD and EBV gB interact in a specific but non-functional manner. An alternative negative control could be used to optimize the ProB assay, such as the fusion
glycoprotein F from the paramyxovirus parainfluenza virus 5 (PIV5). F protein is a fusion protein on the surface that PIV5 that triggers membrane fusion. PIV5 F has no sequence homology to HSV1 proteins. This is reasonable negative control since it is a transmembrane protein that forms a trimer and thus could be used to examine both homotypic and heterotypic interactions.

The ProB competition assay results with wildtype EBV gB and HSV1 gD, as well as HSV1 gH/gL, should be repeated to determine if the results are reproducible. Examination of the interaction of HSV1 gH/gL with HSV1 gD and gB can be examined once the ProB specificity concerns are addressed.

BirA and APm interactions can also be visualized through fluorescent microscopy. The procedure is similar except the cells are not lysed but fixed and then stained with streptavidin conjugated to AlexaFluor 568 or another fluorescent molecule. The media depletion of biotin can reduce background biotin signal. Fluorescent microscopy analysis of the HSV1 gD and EBV gB constructs can be beneficial in characterizing if any nonspecific interactions are happening during processing in the endoplasmic reticulum instead of interacting on the cell surface.

C. Other protein-protein interaction methods. Further development in protein interaction methods for herpesvirus proteins is necessary to characterize the interaction of the glycoproteins for entry.

Proximity utilizing biotinylation and mass spectrometry (PUB-MS) employs the same enzyme-substrate method using biotin ligase (BirA) and biotin-acceptor tag (AP). The interactions can be monitored by western or mass spectrometry. The researchers used a strong CMV promoter or a weaker MoMuLV enhancer to have AP in excess over BirA (40). Cloning these herpesvirus proteins into plasmids with different promoters may help optimize the positive and negative controls for the ProB assay.

Another method related to ProB is called ID-PRIME. This assay takes advantage of the enzyme *E. coli* lipoic acid ligase, which catalyzes the covalent ligation of a coumarin fluorophore
onto a peptide recognition sequence, LAP1 (62). Only when the proteins labeled with either the ligase or LAP1 interact is the LAP1 labeled with coumarin, a blue fluorophore. Coumarin labeling in the absence of the interaction is low. The enzyme was created in such a way that when the interaction is present the labeling is maximally fast, but when the interaction is not present the labeling is undetectably slow (62). The issue with this method is that some endogenous proteins are lipoylated, arising to the same problem as endogenous biotinylation with ProB (62). Other protein-protein interactions will not work for low affinity, transient interactions that occur with the entry glycoproteins.
V. Appendix

Cloning BirA and APm onto the cytoplasmic tails of HSV1 gB, HSV1 gH, and PIV5 F. The cloning strategy was designed using four-primer PCR. In this method, one primer pair is used to amplify the 5’ fragment of a construct and a second primer pair is used to amplify the 3’ fragment of a construct. The 3’ primer of the first PCR and the 5’ primer of the second PCR are designed to have sequence overlap. Thus, the product of the first PCR will base-pair with the product of the second PCR. The 5’ primer of the first PCR and the 3’ primer of the second PCR can then be used to amplify the full-length construct. Incorporation of restriction sites into the 5’ and 3’ primer used in the second round of PCR allows the fragment to be ligated into an expression vector. A benefit to the four-primer PCR approach is that the two halves of the construct can be joined precisely, without any additional residues being added, such as those encoded by restriction site sequences.

The primers were designed so that the BirA and APm fragments would be amplified without any glycoprotein sequence included, so that these PCR products could be use to add BirA or APm to any of the glycoproteins. The 3’ primer for amplifying BirA included a FLAG epitope and the 3’ primer for amplifying APm included an HA epitope.

Primers also were designed to clone each glycoprotein in combination with BirA and APm. The 5’ primer used to amplify the glycoproteins was the same, regardless of whether the BirA or APm was to be added. The 3’ primers included sequence from the glycoprotein and either BirA or APm. This 3’ primer is the primer that includes the sequence overlap required for this four-primer PCR method. Plasmids pPEP100 (pCAGGs-gH), pSG5-EBVgB, and pCAGGs-W3AF (PIV5 F protein), and pECFP-N1-BirA-neurexin were used as templates for amplifications. The sequence of APm is short enough to be entirely encoded in the primer.

To amplify the 5’ fragment of the HSV1 gH-BirA construct, a 5’ primer (rSC22) and a 3’ primer (rSC25b) amplified gH with a 3’ end complimentary to BirA. To amplify the 5’fragment
gH-APm, the same 5’ primer was used in combination with a 3’ primer (PCR28b) to amplify gH with a 3’ end complementary to APm.

To amplify HSV1 gB-BirA, the 5’ primer rSC89 was used in addition to the 3’ primer rSC25b. To amplify HSV1 gB-APm, the 5’ primer rSC18 was used with the 3’ primer rSC28b.

To amplify the 5’ fragments of the PIV5 F constructs, a 5’ primer (rSC90) was used in combination with a 3’ primer including F sequence and either BirA (rSC25c) or APm (rSC28c) sequence.

To amplify the 3’ BirA fragment, the 5’ primer (rSC63) was used in combination with a 3’ primer with the restriction enzyme site for BglII (rSC25b) or EcoRI (rSC25c). To amplify the 3’ APm fragment, the 5’ primer (rSC64) was used in combination with a 3’ primer with the restriction enzyme site for BglII (rSC28b) or EcoRI (rSC28c).

The full-length constructs were amplified using the products of these PCRs and the appropriate primer combinations, from the primers listed above. The primers used in this cloning had varied length, by necessity, and were used in more than one PCR, thus optimization of the PCR conditions was required. The viral glycoproteins have are GC-rich and thus optimization was extensive, including temperature gradient PCR to determine optimal annealing temperatures and the use of different polymerases.

The amplified full-length constructs were digested and ligated into the vector pSG5. This vector has only three restriction sites in the multiple cloning site, so directional cloning was not possible. The ligations were transformed into competent cells and the proper orientation of the fragment was confirmed either by diagnostic restriction digest of DNA isolated from a transformant by miniprep or by PCR amplification directly from an isolated colony.
Table 1. Primer name and corresponding sequences for HSV1 gB, gH, and PIV5 F protein.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene/Plasmid</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>rSC18</td>
<td>5’ EcoRI-HSV1 gB</td>
<td>CGGAATTCACCATGCACCAGGGCGCCCCCTC</td>
</tr>
<tr>
<td>rSC22</td>
<td>5’ BglIII-HSV1 gB</td>
<td>CGAGATCTACCATGGGGAAATGTTTATGGTT</td>
</tr>
<tr>
<td>rSC25b</td>
<td>RC3’ BirA-FLAG-BglII</td>
<td>GCAGATCTCTACTTTGTCGTGTCGTCTCTGTAGTCACCTCCTTT TTGCACACTACGGG</td>
</tr>
<tr>
<td>rSC25c</td>
<td>RC3’ BirA-FLAG-EcoRI</td>
<td>GCAGATCTCTACTTTGTCGTGTCGTCTCTGTAGTCACCTCCTTT TTGCACACTACGGG</td>
</tr>
<tr>
<td>rSC28b</td>
<td>RC3’ APmut-FLAG-BglII</td>
<td>GCAGATCTCTAGAATCCTGCTCAGTGTCAGTTTCTGGGCTCGAAG ATATCGTTCAGGCGCTCT</td>
</tr>
<tr>
<td>rSC28c</td>
<td>RC3’ APmut-HA-EcoRI</td>
<td>GCAGATCTCTAGAATCCTGCTCAGTGTCAGTTTCTGGGCTCGAAG ATATCGTTCAGGCGCTCT</td>
</tr>
<tr>
<td>rSC57</td>
<td>RC3’ HSV gB-BirA</td>
<td>GGCACGGTGTTATCTCTCATCAGGTCTCCTCGTGCGGT</td>
</tr>
<tr>
<td>rSC58</td>
<td>RC3’ HSV gB-AP</td>
<td>TCGGGCAGTCTAGGGGTACAGGTCGTCTCCTCGTGCGGT</td>
</tr>
<tr>
<td>rSC61</td>
<td>RC3’ HSV gH-BirA</td>
<td>GGCACGGTGTTATCATCTCTCATCAGGTCTCCTCGTGCGGT</td>
</tr>
<tr>
<td>rSC62</td>
<td>RC3’ HSV gH-AP</td>
<td>TCGGGCAGTCTAGGGGTATCTCTCATCAGGTCTCCTCGTGCGGT</td>
</tr>
<tr>
<td>rSC63</td>
<td>5’ BirA</td>
<td>ATGAAGGATAACACCGTGCC</td>
</tr>
<tr>
<td>rSC64</td>
<td>5’ HA-AP</td>
<td>TACCCCTACGACGTGGCCGACTAGCCAGGGCTGGAGCGCGCTGAACGATA</td>
</tr>
<tr>
<td>rSC89</td>
<td>5’ EcoRI-KOS gB</td>
<td>GCGGAATTCACCATGCACCAGGGTGCACTCCTCGTG</td>
</tr>
<tr>
<td>rSC90</td>
<td>5’ EcoRI-PIV5 F</td>
<td>GCGGAATTCACCATGGGTACTATAATTCAATTTC</td>
</tr>
<tr>
<td>rSC91</td>
<td>RC3’ PIV5 F-BirA</td>
<td>GGCACGGTGTTATCTCTCATCCTTATGATAAAACAAAATTTA</td>
</tr>
<tr>
<td>rSC92</td>
<td>RC3’ PIV5 F-AP</td>
<td>TCGGGCAGTCTAGGGGTATTTATGATAAAACAAAATTTA</td>
</tr>
</tbody>
</table>
## Table 2. Optimal PCR conditions to amplify 5’ and 3’ fragments

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR1d (5’ BirA, BglIII) pg 2-3</td>
<td>Vent</td>
<td>pECFP-N1-BirA-neurexin</td>
<td>rSC63, rSC25b</td>
<td>98C, 60s</td>
<td>98C, 30s</td>
<td>66.5C, 60s</td>
<td>72C, 2:40</td>
<td>72C, 5 min</td>
</tr>
<tr>
<td>PCR5 (5’ gH, BirA) pg 1</td>
<td>Vent</td>
<td>pEP100 (pCAGGS-gH)</td>
<td>rSC22, rSC61</td>
<td>98C, 60s</td>
<td>98C, 30s</td>
<td>52C, 60s</td>
<td>72C, 2:40</td>
<td>72C, 5 min</td>
</tr>
<tr>
<td>PCR6 (5’ gH, APm) pg 1</td>
<td>Vent</td>
<td>pEP100 (pCAGGS-gH)</td>
<td>rSC22, rSC62</td>
<td>98C, 60s</td>
<td>98C, 30s</td>
<td>52C, 60s</td>
<td>72C, 2:40</td>
<td>72C, 5 min</td>
</tr>
<tr>
<td>PCR3 (5’ AP, BglIII) pg 1</td>
<td>Vent</td>
<td>N/A</td>
<td>rSC64, rSC28b</td>
<td>98C, 60s</td>
<td>98C, 30s</td>
<td>52C, 60s</td>
<td>72C, 2:40</td>
<td>72C, 5 min</td>
</tr>
<tr>
<td>PCR9 (5’ PIV5, BirA) pg 1</td>
<td>Vent</td>
<td>pCAGGS-W3AF</td>
<td>rSC90, rSC91</td>
<td>98C, 60s</td>
<td>98C, 30s</td>
<td>52C, 60s</td>
<td>72C, 2:40</td>
<td>72C, 5 min</td>
</tr>
<tr>
<td>PCR2e (5’ BirA, BglIII) pg 3</td>
<td>Vent</td>
<td>pECFP-N1-BirA-neurexin</td>
<td>rSC63, rSC25b</td>
<td>98C, 60s</td>
<td>98C, 30s</td>
<td>47C, 60s</td>
<td>72C, 60s</td>
<td>72C, 5 min</td>
</tr>
<tr>
<td>PCR10 (5’ PIV5, APm) pg 1</td>
<td>Vent</td>
<td>pCAGGS-W3AF</td>
<td>rSC90, rSC92</td>
<td>98C, 60s</td>
<td>98C, 30s</td>
<td>52C, 60s</td>
<td>72C, 2:40</td>
<td>72C, 5 min</td>
</tr>
<tr>
<td>PCR4 (5’ AP, EcoRI) pg 1</td>
<td>Vent</td>
<td>N/A</td>
<td>rSC64, rSC28c</td>
<td>98C, 60s</td>
<td>98C, 30s</td>
<td>52C, 60s</td>
<td>72C, 2:40</td>
<td>72C, 5 min</td>
</tr>
<tr>
<td>PCR7 (5’ gB, BirA enhancer pg 23</td>
<td>Q5, GC enhancer</td>
<td>pCAGGS-HSV1-gB</td>
<td>rSC89, rSC57</td>
<td>98C, 30s</td>
<td>98C, 10s</td>
<td>71C, 30s</td>
<td>72C, 1:45</td>
<td>72C, 2 min</td>
</tr>
<tr>
<td>PCR8n (5’ gB, AP enhancer pg 26</td>
<td>Q5, GC enhancer</td>
<td>pCAGGS-HSV-gB</td>
<td>rSC18, rSC58</td>
<td>98C, 30s</td>
<td>98C, 10s</td>
<td>70C, 30s</td>
<td>72C, 2:15</td>
<td>72C, 2 min</td>
</tr>
</tbody>
</table>
Table 3. Optimal PCR conditions to amplify fragments of glycoprotein with tag

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR11b (gH-BirA) pg 33</td>
<td>Vent</td>
<td>5, 1d</td>
<td>rSC22, rSC25b</td>
<td>98C 1m</td>
<td>98C, 30s</td>
<td>54C, 1m</td>
<td>72C, 4m</td>
<td>72C, 5m</td>
</tr>
<tr>
<td>PCR12d (gH-APm) pg 34</td>
<td>Vent</td>
<td>6, 3</td>
<td>rSC22, rSC28b</td>
<td>98C 1m</td>
<td>98C, 30s</td>
<td>57C, 1m</td>
<td>72C, 4m</td>
<td>72C, 5m</td>
</tr>
<tr>
<td>PCR13e (F-BirA) pg 41</td>
<td>Q5, no enhancer</td>
<td>9, 2e</td>
<td>rSC90, rSC25c</td>
<td>98C 1m</td>
<td>98C, 30s</td>
<td>70C, 3m</td>
<td>72, 1:40m</td>
<td>72C, 2m</td>
</tr>
<tr>
<td>PCR14 (F-APm) pg 4</td>
<td>Vent</td>
<td>10, 4</td>
<td>rSC90, rSC28c</td>
<td>98C 1m</td>
<td>98C, 30s</td>
<td>52C, 1m</td>
<td>72C, 4m</td>
<td>72, 5m</td>
</tr>
<tr>
<td>PCR15k (gB-BirA) pg 26</td>
<td>Q5, with enhancer</td>
<td>7e, 1d</td>
<td>rSC89, rSC25b</td>
<td>98C, 30s</td>
<td>98C, 10s</td>
<td>70C, 30s</td>
<td>72C, 2:15</td>
<td>72C, 2m</td>
</tr>
<tr>
<td>PCR16j (gB-APm) pg 27</td>
<td>Q5, no enhancer</td>
<td>8n, 3</td>
<td>rSC18, rSC28b</td>
<td>98C, 30s</td>
<td>98C, 10s</td>
<td>71C, 30s</td>
<td>72C, 2:15</td>
<td>72C, 5m</td>
</tr>
</tbody>
</table>
Table 4. Confirmation of ligation of the PCR fragments into pSG5 vector.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Lab Notebook Page</th>
<th>Restriction Enzyme(s)</th>
<th>Expected product</th>
<th>Date</th>
<th>Agarose Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR11b (gH-BirA)</td>
<td>40</td>
<td>XbaI</td>
<td>1292</td>
<td>2/13/14</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>PCR12d (gH-APm)</td>
<td>45</td>
<td></td>
<td>2.5 kb</td>
<td>3/26/14</td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>PCR13e (F-BirA)</td>
<td>47</td>
<td></td>
<td>2.6 kb</td>
<td>3/26/14</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>PCR14 (F-APm)</td>
<td>31</td>
<td>BglII</td>
<td>4933, 463, 361</td>
<td>1/7/14</td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>PCR15k (gB-BirA)</td>
<td>39</td>
<td>NotI, BglII</td>
<td>3585</td>
<td>1/28/14</td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
</tbody>
</table>

** This is the lane with the correct insert.
<table>
<thead>
<tr>
<th>PCR16j (gB-APm)</th>
<th>39</th>
<th>NotI, BglII</th>
<th>2678</th>
<th>1/31/14</th>
<th>kB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[Image: gel electrophoresis with bands at 3.0 and 2.0 kB]
VI. References

1. **Atanasiu D, S. W., Cohen GH, Eisenberg RJ.** 2010. Cascade of events governing cell-cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB. J Virol **84:**12292-12299.

2. **Atanasiu D, W. J., Cairns TM, Reilly B, Cohen GH, Eisenberg RJ.** 2007. Bimolecular complementation reveals that glycoproteins gB and gH/gL of herpes simplex virus interact with each other during cell fusion. Proc Natl Acad Sci **104:**18718-18723.


5. **Avitabile E, F. C., Campadelli-Fiume G.** 2009. Cross Talk among the Glycoproteins Involved in Herpes Simplex Virus Entry and Fusion: The Interaction between gB and gH/gL Does Not Necessarily Require gD. J Virol **83:**10752-10760.


12. Chesnokova LS, N. S., Hutt-Fletcher LM. 2009. Fusion of epithelial cells by Epstein-Barr virus proteins is triggered by binding of viral glycoproteins gHgL to integrins avB6 or avB8. Proc Natl Acad Sci 106:20464-20469.


45. **Maresova, L., T. J. Pasieka, and C. Grose.** 2001. 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 75:9483-9492.


51. **Nemerow GR, M. C., Schwend VK, Tollefson V, Cooper NR.** 1987. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. J virol 61:1416-1420.


