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Examining the refolding of the herpesvirus fusion protein, glycoprotein B

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Table of Contents

Abstract

Fusion of the viral and host cell membranes during herpesvirus entry into cells is mediated by glycoprotein B (gB), a class III viral fusion protein that is essential and conserved in all herpesviruses. gB is believed to mediate fusion by inserting into a target cellular membrane and refolding from a prefusion to a postfusion conformation to bring the viral and cellular membranes together. Electron cryotomography revealed two distinct gB conformations and the postfusion gB crystal structure has been solved, however the crystal structure of the prefusion conformation and the details of how gB refolds to execute fusion remain uncertain. A previous mutagenesis study demonstrated that a region in herpes simplex virus (HSV) gB, termed the coil-arm region, contributes to fusion. This coil-arm region resembles the six-helix bundle that is predicted to provide energy to drive fusion in class I fusion proteins. A panel of small molecule compounds that were designed to inhibit interactions of the coil-arm region in HSV gB were screened. One compound significantly decreased HSV1 and HSV2 entry into cells with an IC_{50} in the low μM range. The compound was not cytotoxic and inhibited herpesvirus entry specifically. The compound inhibited entry at a step after virus binding and was able to inhibit fusion in a cell-cell fusion assay. Design of a potent fusion inhibitor could lead to drug candidates that target this conserved fusion protein and clarify how the protein functions, potentially providing a new tool to study the gB conformational change and provide a broader understanding of how similar class III viral fusion proteins refold.

I. Review of Literature

Viral Replication

Viruses are obligate intracellular parasites that are dependent on host cells to replicate. They infect all living organisms, from single-celled bacteria to complex multicellular eukaryotes. Viruses consist of a genome of single- or double-stranded DNA or RNA surrounded by a protective capsid made of protein. This capsid may be surrounded by an additional layer called an envelope that is derived from the host cell membranes. The surface of a virus particle is studded with surface proteins that execute virus entry. Virus replication cycle contains seven key stages: attachment, entry, replication, gene expression, assembly, transport, and release (Fig. 1). (Shors, 2011)

Herpesviruses are double-stranded DNA viruses. The genome is enclosed inside an icosahedral capsid, which is surrounded by tegument proteins and a lipid envelope (Shors, 2011). Like all other enveloped viruses, to enter a cell, HSV must fuse its envelope with the cell membrane. This fusion event is executed by glycoproteins on the virus envelope. At least twelve distinct glycoproteins are present on the surface of the herpesvirus virion, four of which are necessary and sufficient for viral entry: glycoprotein B (gB), gD, gH, and gL (Turner et al., 1998).

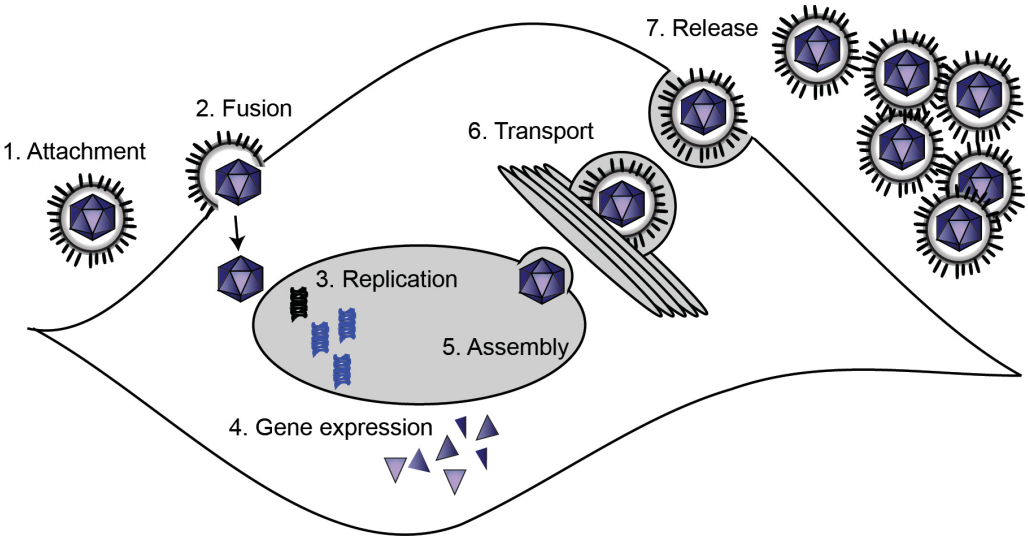

Figure 1: Herpesvirus replication cycle. The seven key steps of replication are outlined. 1. Attachment is mediated by several viral glycoproteins, including gC and gB binding to cell surface proteoglycans and gD binding to receptors including nectin-1, HVEM, and a modified form of heparan sulfate. 2. Entry is achieved by gB mediating fusion of the viral and cellular membranes. Fusion occurs with the cell membrane in some cells types and with an endosomal membrane for other cell types. 3. The viral DNA is replicated in the nucleus. 4. Viral genes are expressed, including structural genes. 5. The genome is packaged into capsid within the nucleus. 6. The capsids bud out of the nucleus and are brought to the cell surface by vesicular transport. 7. Progeny virions are released from the cell.

Human Herpesviruses

Herpesviruses represent a large family of viruses that infect a variety of hosts and cell types (Pellet and Roizman, 2007). Nine distinct herpesviruses establish latent infections in human. HSV1 and HSV2 are prototypical herpesviruses, infecting the epithelial cells and neurons, typically at the mouth and genitals, respectively (Connolly et al., 2011). HSV1 and HSV2 share a broad range of features. They are members of the subfamily *Alphaherpesvirinae* and have genomes of 152 kilobase pairs (Shors, 2011). Both viruses use the same entry receptors and both establish latency in neurons (Shors, 2011).

Most middle-aged adults are seropositive for several human herpesviruses (Shors, 2011). HSV1 prevalence is approximately 70%, while HSV2 prevalence is between 10 and 20% (WHO,

2016). Varicella Zoster Virus (VZV), another member of *Alphaherpesvirinae,* causes chickenpox and shingles and has a seroprevalence in the United States of 85-95%. A live attenuated VZV vaccine has been used since the mid-1990s. Epstein Barr virus EBV, the prototypical member of *Gammaherpesvirinae*, is the leading cause of mononucleosis (80% of cases) and can lead to Burkitt's Lymphoma in immunosuppressed patients. Cytomegalovirus (CMV), the prototypical member of *Betaherpesvirinae*, accounts for the remaining 20% of cases of mononucleosis and has a prevalence of 40-70%. HHV6a, HHV6b, and HHV7 infect most people by the age of two and typically lead to fever and rashes, but may also lead to seizures. Finally, Kaposi sarcoma-associated herpesvirus (KSHV) is not known to have any symptoms during primary infection; however, it can lead to skin cancer, particularly in immune-suppressed AIDS patients. Appearance of this rare form of cancer was an important component in first identifying HIV/AIDs in the 1980s. The high seroprevalence, as well as their important clinical manifestations, make the herpesvirus family a medically relevant group of viruses to study. (Shors, 2011)

Herpesvirus Entry Glycoproteins

Unlike many other enveloped viruses that require only one protein for entry, herpesviruses require the concerted effort of multiple surface glycoproteins to mediate entry into cells (Turner et al., 1998). These glycoproteins facilitate entry into the cell by mediating membrane fusion, causing the viral envelope to become continuous with the membrane of the target cell (Fig. 2). The crystal structures of all four HSV glycoproteins that are required for fusion (gD, gH-gL, and gB) have been solved (Krummenacher et al., 2005) (Heldwein et al., 2006) (Chowdary and Heldwein, 2010).

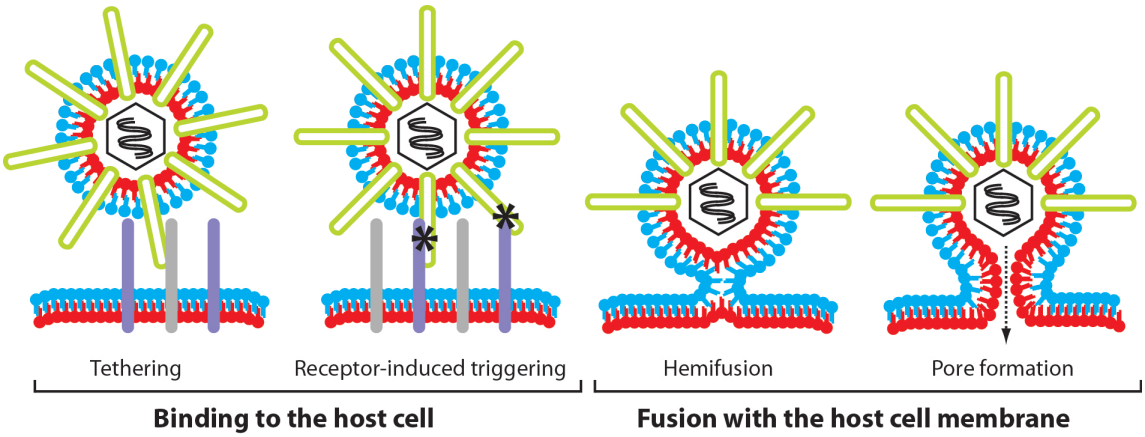

Figure 2: Herpesvirus entry into cells. The initial interaction between viral glycoproteins and cell surface glycosaminoglycans tethers the virus to the cell (panel 1). This promotes entry, but is not required. Following tethering, gD binds specific cell surface receptors (purple) (panel 2). This receptor-binding triggers conformational changes in the viral glycoproteins (green). The fusion protein, gB, refolds to mediate the fusion of the viral and cellular membranes. The outer leaflets of the membranes merge (panel 3) and then a pore forms in the membrane (panel 4) that allows the viral capsid, containing the viral genome, to enter the cell cytoplasm (Connolly et al., 2011).

gD functions as the receptor-binding protein by binding to one of several receptors, including nectin-1, HVEM, or a modified form of heparin sulfate (Fig. 3) (Pertel et al., 2001a). The binding of gD to receptor is thought to activate the gH-gL heterodimer. The role of the gH-gL heterodimer is not well understood, but it is believed to regulate the fusion ability of gB (Atanasiu et al., 2010). Upon triggering, gB undergoes a large conformational change that allows fusion to occur (Connolly et al., 2011). Although the receptor-binding protein varies among members for the *Herpesviridae* family, gB is conserved as the fusion protein for all herpesviruses (Heldwein and Krummenacher, 2008).

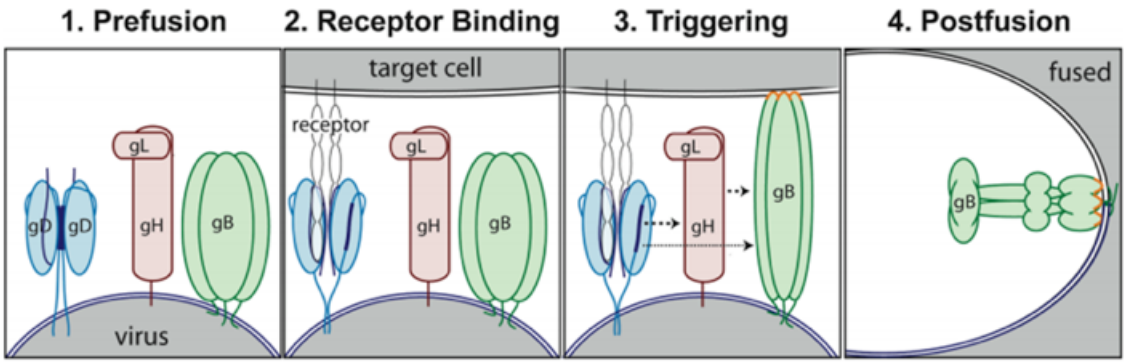

Figure 3: Interactions of HSV entry glycoproteins. 1. The four HSV1 and 2 glycoproteins required for viral entry are gD, gH-gL, and gB. They are present on the surface of the virion. 2. Attachment to a host cell is mediated by gD (blue) binding to receptor (white) on the cell surface. 3. Receptor binding elicits interactions among the viral glycoproteins, including the gH-gL heterodimer (red). These interactions trigger the fusion protein gB (green) to insert fusion loops (orange) into cell membrane, forming an intermediate conformation of gB. 4. gB refolds into a postfusion conformation to drive fusion of the viral and cellular membranes. gD and gH-gL are removed from the final panel for clarity. (Connolly et al., 2011).

The crystal structure of the gB ectodomain in the postfusion form has been solved for several herpesviruses, including HSV1 (Heldwein et al., 2006) (Fig. 4), EBV (Backovic et al., 2009), and HCMV (Burke and Heldwein, 2015; Chandramouli et al., 2015). gB is a 904 residue protein that is well conserved across the herpesvirus family. When the transmembrane domain of gB was removed to facilitate crystallization, the protein folded to a postfusion conformation, suggesting that the transmembrane domain stabilizes the prefusion conformation of the protein (Heldwein et al., 2006).

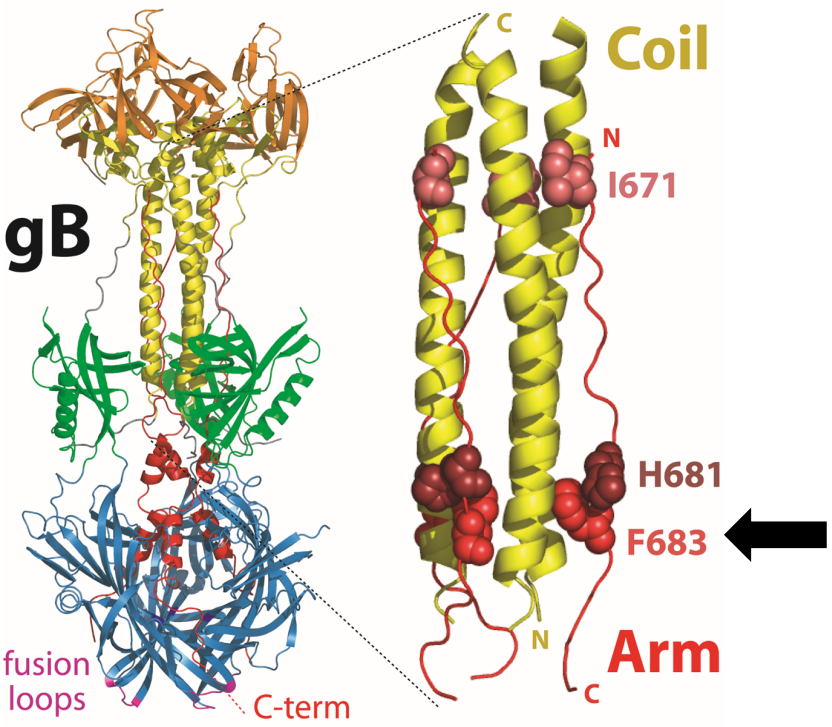

Figure 4. Structure of postfusion gB. The crystal structure of the HSV1 fusion protein gB is shown (Heldwein et al., 2006). Domain I (blue), domain II (green), domain III (yellow), domain IV (orange), and domain V (red) are shown. The C-terminus, truncated prior to the transmembrane domain, is at the end of domain V, at the base of the molecule. The fusion loops (magenta) are present in domain I and are also at the base of the molecule in this conformation of gB. A close up of the central extended coiled-coil core (yellow) and the C-terminal arm of gB (red) is shown. Three residues in the arm that were shown previously by mutagenesis to contribute to fusion are labeled. For this study, compounds were designed to inhibit interactions between critical arm residues H681 and F683 and the coil (arrow).

The prefusion form of gB has not been crystalized for any herpesvirus. Attempts to stabilize a prefusion form of the protein for crystallization have been unsuccessful (Silverman et al., 2010). A model of the prefusion gB conformation has been proposed, based on the structurally similar class III fusion protein vesicular stomatitis virus (VSV) G (Fig 5) (Gallagher et al., 2014). The crystal structures of both the prefusion and postfusion forms of VSV G have been solved (Roche et al., 2006) (Roche et al., 2007). Unlike HSV gB, the VSV G conformational change is reversible and triggered by low pH, which facilitated crystallization of both forms (Albertini et al., 2012).

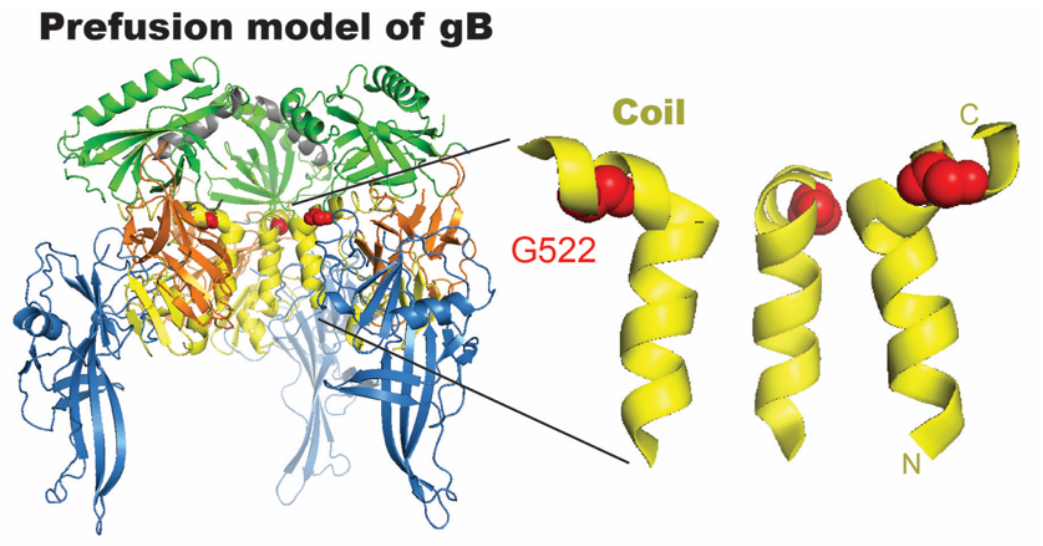

Figure 5: Model of prefusion conformation of gB The prefusion conformation of gB has not been crystalized, however HSV1 gB and the fusion protein G of rhabdovirus have similar postfusion structures (Roche et al., 2007) and this allows modeling of the prefusion form of gB using the prefusion form of G (Eisenberg et al., 2012). The five gB domains are colored as in the previous figure on this hypothetical model of the prefusion gB trimer. A close up of the region of domain III (yellow), which exists as an extended coiled-coil in the postfusion structure, is shown. The coil is broken near residue G522 (red). Domain V, which contains the arm in the postfusion structure, is not present in the model.

Another structural model of gB, potentially in the prefusion conformation, was created using electron cryotomography (cryoET) of gB expressed on cell-derived vesicles (Zeev-Ben-Mordehai et al., 2016). gB present on vesicles displayed two distinct conformations. Tomographic reconstruction showed that one conformation matched the known postfusion crystal structure and the second conformation was a novel shorter form of gB (10 nm compared to the 16 nm postfusion structure) (Zeev-Ben-Mordehai et al., 2016). Several domains from the postfusion structure of gB were fit into the short form reconstruction, but not domains III (coil) and V (arm), which are expected to undergo conformational changes during fusion and are the domains of interest in this project. The extended coil present in domain III would not be able to fit into this short conformation of gB, supporting the prediction in the previous model that this

coil is broken in prefusion gB. A compact conformation of gB on membranes was visualized independently by another group using cryoET (Fontana et al., 2017) and this work suggests that the fusion loops point towards the membrane.

Viral Fusion Proteins

Fusion proteins like gB function by existing in a metastable state until they are triggered by either receptor binding or acidic pH. Upon activation, enveloped virus fusion proteins undergo large conformational changes that bring together the viral lipid bilayer with the host cell membrane (Smith and Helenius, 2004). This fusion event releases the viral capsid, which contains the viral genome, into the host cell (Smith and Helenius, 2004).

Viral fusion proteins are divided into three classes and herpesvirus gB is a class III fusogen (White et al., 2008). Other class III viral fusion proteins include those of rhabdoviruses, such as VSV and chadipura virus, and baculoviruses, an arthropod infecting virus family (Baquero et al., 2015). Class III is the most recently discovered class and it shares similarities with class I, the most well characterized class of fusion proteins (Heldwein et al., 2006). Class III fusion proteins are homotrimers that contain hydrophobic fusion loops (FL) at one end of the molecule (Fig. 4) (Baquero et al., 2015). These FLs directly interact with the host cell membrane. Class III fusion proteins also feature a central α-helical coiled-coil that is primarily responsible for the trimerization of the protein (Fig. 4) (Heldwein et al., 2006).

Class III fusion proteins share several notable features with class I, allowing us to apply the concepts of how class I fusion proteins refold to the lesser understood class III fusion proteins. Class I fusion proteins are homotrimers that form a characteristic bundle of six antiparallel helices when folded in the postfusion conformation (White et al., 2008). This six-

helix bundle is a remarkably stable structure and its formation is proposed to provide the energy for fusion to occur (Russell et al., 2001). When a class I fusion protein is triggered, by receptor binding or acidic pH, three heptad repeat regions (heptad repeat A, HRA) located adjacent to hydrophobic fusion peptides form an extended trimer and propel the fusion peptides into the host cell membrane (Fig. 6) (Lamb and Jardetzky, 2007). Three distal heptad repeat regions (heptad repeat B, HRB) located adjacent to the hydrophobic transmembrane domains then rearrange to bind to the HRA central trimer, forming a six-helix bundle comprised of HRA and HRB. Since HRA is adjacent to the fusion peptide embedded in the host cell membrane and HRB is adjacent to the transmembrane domain embedded in the viral membrane, the antiparallel binding of HRB to HRA brings the viral and host cell membranes together to facilitate fusion (Fig. 6) (Russell et al., 2001).

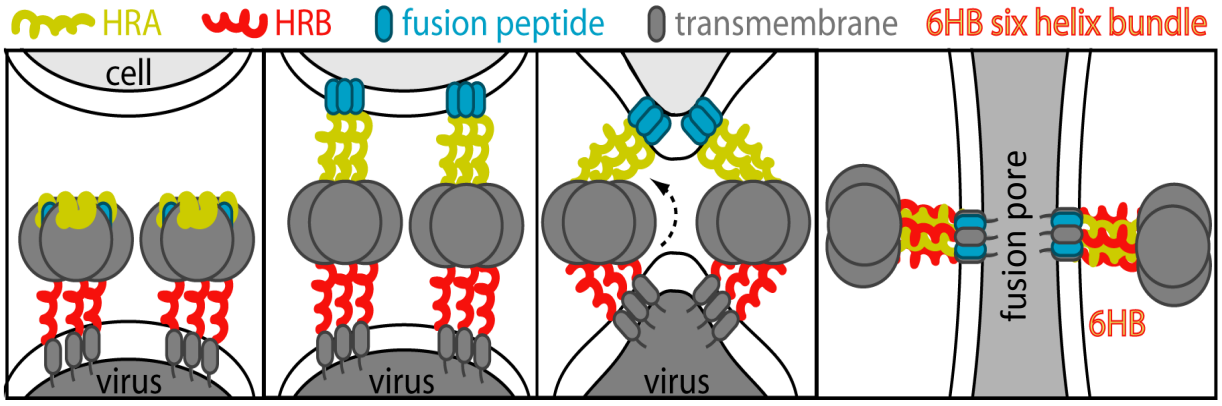

Figure 6. Model of class I fusion protein refolding. The class I fusion protein exists in a prefusion form on the virion (first panel). Heptad repeat A (HRA, yellow) is not helical in this confirmation. The fusion protein is triggered by low pH and/or receptor-binding to insert hydrophobic fusion peptides (blue) into the target cell membrane (second panel). Insertion creates an intermediate conformation of the protein that is anchored in both the viral and host cell membranes. An extended trimeric coiled-coil of HRA is now present (yellow). The protein refolds, packing heptad repeat B (HRB, red) against HRA (third panel). HRB and HRA form a stable six-helix bundle (6HB) and virus/cell fusion is complete (fourth panel). Formation of the

6HB is proposed to provide the energy required for creation of a fusion pore, merging the viral and cellular membranes and allowing the viral genome to enter the cell.

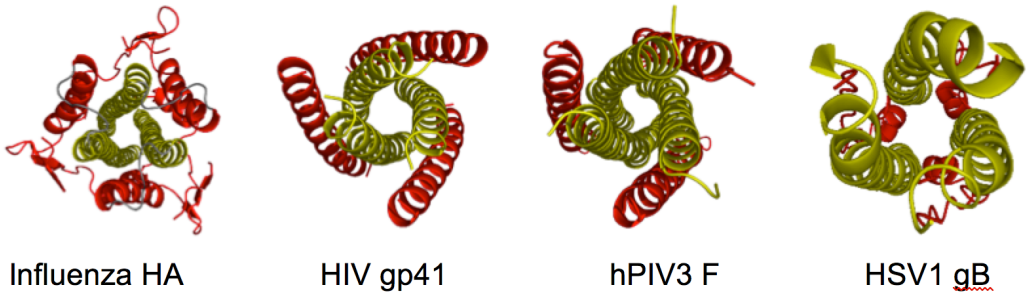

Figure 7. Class I fusion protein six-helix bundles compared to the coil-arm region of HSV gB. The herpesvirus fusion protein gB is a class III fusion protein rather than a class I fusion protein. gB contains a coil-arm region that resembles a six-helix bundle (6HB). The coil-arm complex of HSV1 gB (far right), is shown, viewed from above. The 6HB from three class I fusion proteins are shown also, including influenza hemagluttinin (Bullough et al., 1994), human immunodeficiency virus gp41 (Chan et al., 1997), and human parainfluenza virus 3 F protein (Yin et al., 2005).The gB coil or HRA are shown in yellow. The gB arm or HRB are shown in red.

Although class III fusion proteins do not form a six-helix bundle, antiparallel interactions between a central coil and an extended arm of the postfusion form of gB resemble the interactions of the class I six-helix bundle (Fig. 7). gB domain III features a 44-residue α-helix in each gB protomer that forms a trimeric central coiled-coil, resembling the HRA of class I fusion proteins (Fig. 4). gB domain V consists of an long arm that extends from top to bottom of the gB ectodomain and packs against the coiled-coil in an antiparallel orientation (Fig. 4)(Connolly and Longnecker, 2012). Although domain V is not helical, each domain V arm packs against the domain III coils of two other gB protomers to form a "coil-arm" complex in a manner similar to how HRB packs against the HRA trimer to form a six-helix bundle (Fig. 7). This thesis project focuses on two domains III and V of gB, examining their role in the refolding of gB during fusion.

Class III fusion proteins demonstrate distinct differences from class I fusion proteins. Unlike class I fusion proteins, gB uses hydrophobic fusion loops rather than fusion peptides to insert into the host cell membrane (Cooper and Heldwein, 2015). Fusion peptides have a free N-terminus, whereas fusion loops are internal sequences. More significantly, coil-arm complex is not immediately adjacent to the gB fusion loops and transmembrane domains, unlike the six-helix bundle of class I fusogens (Fig. 4). The positioning of the coil-arm complex relative to the membrane-anchored portions of the protein may impact its contribution to fusion promotion.

Glycoprotein B Coil-Arm Function and Thesis Objectives

In a previous study, the role of the coil-arm complex in fusion was investigated by site-directed mutagenesis (Connolly and Longnecker, 2012). Residues in the domain V arm that faced the coil and had multiple side-chain contacts with coil residues in the postfusion gB crystal structure were mutated to alanine and the effect of the mutations on fusion was determined. gB residues H681 and F683, two residues that pack against the base of the coil, were shown to be important for gB-mediated cell-cell fusion and virus entry into cells.

This thesis project builds upon this mutagenesis study, using a different method to investigate the importance of the coil-arm complex to fusion. Small molecule compounds were designed to inhibit the H681 and F683 interaction with the coil. If the coil-arm complex of gB drives fusion similarly to six-helix bundle of class I fusion proteins, disrupting the formation of the coil-arm complex should inhibit fusion. Inhibiting gB function with small molecule compounds may inhibit virus entry and provide new tools to study prefusion gB and the gB conformational change.

II: Materials and Methods

Cells, viruses, and plasmids

African green monkey kidney cells (Vero) were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 µg/mL penicillin streptomycin (PS). Vero cells were used because HSV can enter and produce high titer virus in these cells and they are used commonly in HSV entry studies. Chinese hamster ovary (CHO-K1), which are not susceptible to HSV because they lack entry receptors, and CHO-nectin-1 cells (CHO-K1 cells that stably express the HSV receptor nectin-1) were cultured in Ham's F12 media supplemented with 10% FBS and 10 µg/mL PS. Virus strains that encode a β-galactosidase (βgal) reporter gene include HSV1 strain KOS tk12(Warner et al., 1998), HSV2/Gal (strain 333)(Taylor et al., 2007), and vaccinia strain vSIJC-20 (Whitbeck et al., 2009). tk12 carries βgal under control of the HSV1 ICP4 promoter, HSV2/Gal carries βgal under control of the hCMV immediate-early promoter, and vSIJC-20 carries βgal under control of the p7.5 early/late vaccina viral promoter. HSV2 strain 333 ZAG 1.1.1, which encodes green fluorescent protein (GFP) reporter gene cellular *Egr1* promotor, was used also (HSV2-GFP). All viruses were prepared by infecting three confluent T150 flasks of Vero cells for 3 days at 37°C. Cells were released and sonicated 3 times for 30 seconds each to release virus from cells. Cells were pelleted for 10 minutes at 3000 RPM. Virus stocks were stored at -80°C in 1:1 ratio of DMEM/sterile milk. Plasmids used included pSG5-HSV1 gB, pCAGGS-gD (pPEP99), pCAGGS-gH (pPEP100), pCAGGS-gL (pPEP101), pCAGGS-T7 polymerase, and a plasmid encoding luciferase under the control of the T7 promoter (Pertel et al., 2001b).

Development of Compounds

Compounds were designed in collaboration with the Buolamwini lab at the Rosalind Franklin University College of Pharmacy. Using the Schrodinger modeling suite protein preparation wizard, postfusion gB was uploaded and a model of the coil-arm region was constructed. Using the LOPAC 1,280-compound library (Sigma-Aldrich Corporation) compounds were ranked based on how tightly they would bind to the gB coil. From the top 100 compounds, 26 were picked and subsequently screened in virus entry assays. An additional 51 compounds were tested due to structural similarities to the most promising compound candidate, compound 5. Images of compounds are not shown in this thesis for proprietary reasons.

Qualitative GFP Virus Entry Assay

Compounds were screened for entry inhibition using HSV2-GFP. Compounds diluted to 10 μM or DMSO (vehicle control) were incubated with 3000 plaque forming units (pfu), which represents the number of infectious virus particles, HSV2-GFP in 50 μL media and incubated at 4° for 1 hour. The virus/compound mixture was added to Vero cells that had been plated in 96 well dish 24 hours prior. Cells were incubated at 37°C for 5 hours and imaged with a fluorescence microscope. As a control for off target effects, a duplicate plate was prepared with compound added one hour into 37°C incubation. After one hour, a majority of virus should have entered cells and an entry inhibitor should have little effect on GFP signal.

Quantitative β-Galactosidase Virus Entry Assay

Vero cells were seeded in 96-well plates at 6×10^4 cells/well and grown to confluency overnight at 37°C. HSV1 KOS tk12 ($2x10^5$ pfu/well) or HSV2/Gal 333 ($2x10^4$ pfu/well) were

added to cells in triplicate wells. The pfu/well used was optimized by titrating each strain to determine the sensitivity of β-galactosidase expression. One set of cells (virus entry screen), 17 μM compounds diluted in DMEM were added simultaneously. For another set of cells (off-target screen), DMEM alone was added. The cells were incubated on ice for one hour to allow receptor binding to occur without virus entry. The cells were transferred to 37°C for two hours to allow virus entry. Cells were washed and treated with citrate buffer (40 mM sodium citrate, pH 3, 10 mM KCl, 135 mM NaCl) for three minutes to inactivate extracellular virus. For the virus entry screen samples, DMEM was added. For the off-target screen samples, 17 μM compounds diluted in DMEM was added to examine whether the compounds showed direct effects on gene expression. Cells were incubated at 37°C for three hours to allow expression of β-galactosidase reporter gene. Cells were lysed with 50 μL 1% NP40 in PBS (Gibco, pH 7.4 10x; final concentration 9 g/L NaCl [155 mM], 795 mg/L Na_2HPO_4-$_7H_2O$ [2.97 mM], 144 mg/L KH_2PO_4 [1.06 mM]). Substrate Chlorophenol red-β-D-galactopyranoside (CPRG) (50 μL of 3.3 mg/mL stock) was added to lysed cells and cells were placed at 37°C for 90 minutes to allow enzymatic reaction. Absorbance was read at 560 nm as a surrogate measure of virus entry.

To determine the efficacy of selected compounds, the assay was performed as above with serial dilutions of compound added simultaneously with virus. Virus entry was also screened against the unrelated vaccinia virus vSIJC-20 ($2x10^5$ pfu/well) encoding a βgal reporter gene.

Entry Inhibition After Virus Binding

To determine which step of virus entry (receptor binding or fusion) is inhibited by compound, the virus entry assay was modified to adjust the timing of the addition of the compound. Cell densities were the same as the virus entry assay described above. Cell

populations underwent two treatments. For the first treatment, virus binding to cells was permitted prior to adding compound. In the second treatment, compound was added to cells at the same time as virus. For the first treatment, 50 μL/well virus was added to cells and the cells were incubated on ice for one hour in the absence of compound, to allow the virus to bind to the cells. Fusion does not occur on ice. Serial dilutions of compound were then added to the wells and the cells were incubated on ice for an additional 30 minutes to allow for compound binding, followed by an incubation at 37°C for 5 hours to allow for expression of the βgal reporter gene. For the second treatment, serial dilutions of compound were added to the cells at the same time that the virus was added. The cells were incubated on ice for 1.5 hours and then the cells were shifted to 37°C for 5 hours. β-galactosidase expression was quantified as above.

Luciferase Assay (Quantitative Cell-cell Fusion assay)

CHO-K1 cells (called effectors) were seeded at 6 x 10^4 cells/well in 96 well plates and CHO-nectin-1 cells (called targets) were seeded in 6 well plates at 10^6 cells/well. Cells were grown overnight at 37°C. Effector cells were transfected with 110 ng DNA/well in OptiMEM, including plasmids encoding HSV 1 gB, gD, gH, gL and T7 polymerase at a 3:2:2:2:2 ratio, using Lipofectamine 2000 (0.35 μL/well in 50 μL total volume). Target cells were transfected with 2.2 μg/well DNA with plasmid encoding luciferase under the control of the T7 promoter. The cells were incubated at 37°C for three hours and effector cells were rinsed with PBS supplemented with MgCl (0.2 g/L MgCl2-6H2O (0.98 mM)) and $CaCl_2$ (0.2 g/L CaCl2 (1.8 mM)) (PBS+). Serial dilutions of compound (50 μL/well) were added to the effector cells and the cells were incubated for 30 minutes at 37°C to allow for compound binding. Target cells were rinsed with versene (0.2 g/L EDTA in PBS) once and then released into 200 μL/well

versene. Target cells were resuspended in 1.5 mL/well OptiMEM. Target cells (50 µL/well) cells were overlaid onto the effector cells (in the presence of compound). Cells were incubated for 12-16 hours at 37°C. Cells were lysed with 50 µL/well passive lysis buffer (Promega) for 10 minutes at room temperature. 50 µL/well luciferase substrate (Promega) was added and luminescence was immediately recorded (Promega GloMax luminometer).

Cytotoxicity Assay

The toxicity of selected compounds to CHO-K1 and Vero cells was assessed using Promega Cyto-Tox-Glo^{TM} Cytotoxicity Assay. This assay measures protease activity to quantify the number of dead cells in a sample. Cells with compromised membrane integrity release proteases. This kit provides a peptide substrate that results in luminescence when cleaved by these proteases. The substrate cannot penetrate intact cells. Cells were seeded at 6 x10^4 cells/well overnight at 37°C in a 96 well plate. Cells were rinsed with 200 µL/well OptiMEM. Serial dilutions of compounds diluted in OptiMEM were added to cells (75 µL/well). Cells were incubated for 16 hours, the maximum experimental time used in the cell-cell fusion assay. Following the manufacturer's protocol, cell death was recorded by adding 50 µL/well Cytotoxicity Assay Reagent. Plates were briefly shaken on an orbital shaker and incubated at room temperature for 15 minutes. Luminescence was measured to determine the total amount of dead cells (Promega GloMax luminometer). Then 50 µL/well lysis reagent was added to lyse all of the living cells. The plates were briefly shaken on an orbital shaker and incubated at room temperature for 15 minutes. Luminescence was measured to determine the total amount of luminescence after all cells had been lysed. To determine the viable cell luminescence, the dead

cell luminescence (read before cell lysis) was subtracted from the total luminescence (read after cell lysis), according to the manufacturer's protocol.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7. Figure 9, figure 11, and figure 13 results were analyzed using one-way ANOVA with Tukey's post hoc test to determine if samples with compound present were significantly different from control with 0 μM compound. For figure 10, IC_{50} was determined using regression analysis.

III: Results

Screen for Virus Entry Inhibitors

An initial panel of 26 small molecules designed to inhibit the interaction of HSV gB arm residues H681 and F683 with the central coil of gB were screened for HSV entry inhibition. Virus entry was assayed qualitatively using a HSV2 carrying a GFP reporter gene (Fig. 8) or quantitatively using HSV1 carrying a β-galactosidase (βgal) reporter gene (Fig. 9). Cells were seeded in wells overnight and then infected with virus in the presence or absence of compounds at 17 μM. Cells were incubated at 4°C for one hour to allow virus binding and then shifted to 37°C for five hours to allow for virus entry and reporter gene expression. To control for off-target effects, such as global effects on gene expression, compounds were added either simultaneously with virus or one hour after virus entry had occurred. Compounds that reduce reporter gene expression when added simultaneously with virus, but not when added one hour after virus entry, were considered viable candidates for entry inhibitors.

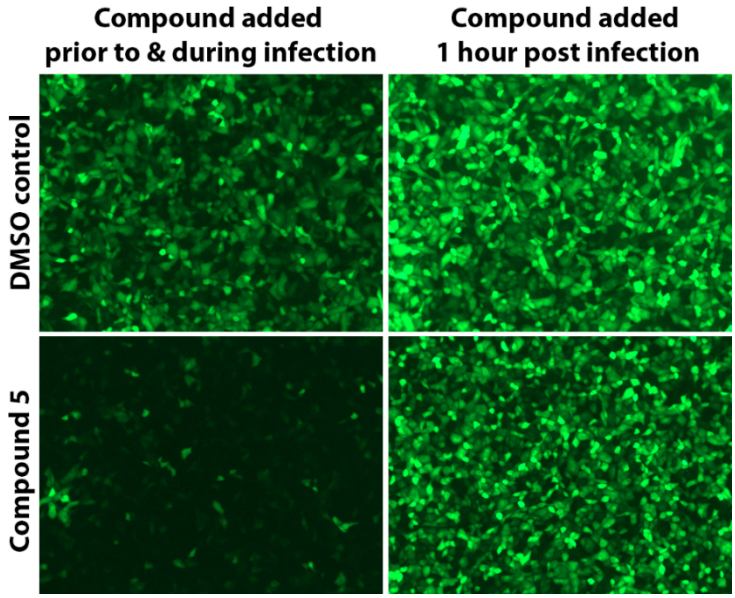

Figure 8. Qualitative screen for virus entry inhibition. HSV2 ZAG1.1.1, a strain that encodes a GFP reporter gene, was incubated with a panel of 26 compounds at 17 μM or DMSO vehicle for one hour at 4°C. The mixtures were added to Vero cells at 37°C for five hours and cells were imaged for GFP as an indication of virus entry (left). As a control, virus was allowed to enter cells for an hour at 37°C prior to adding a compound or DMSO (right). Results for compound 5 are shown.

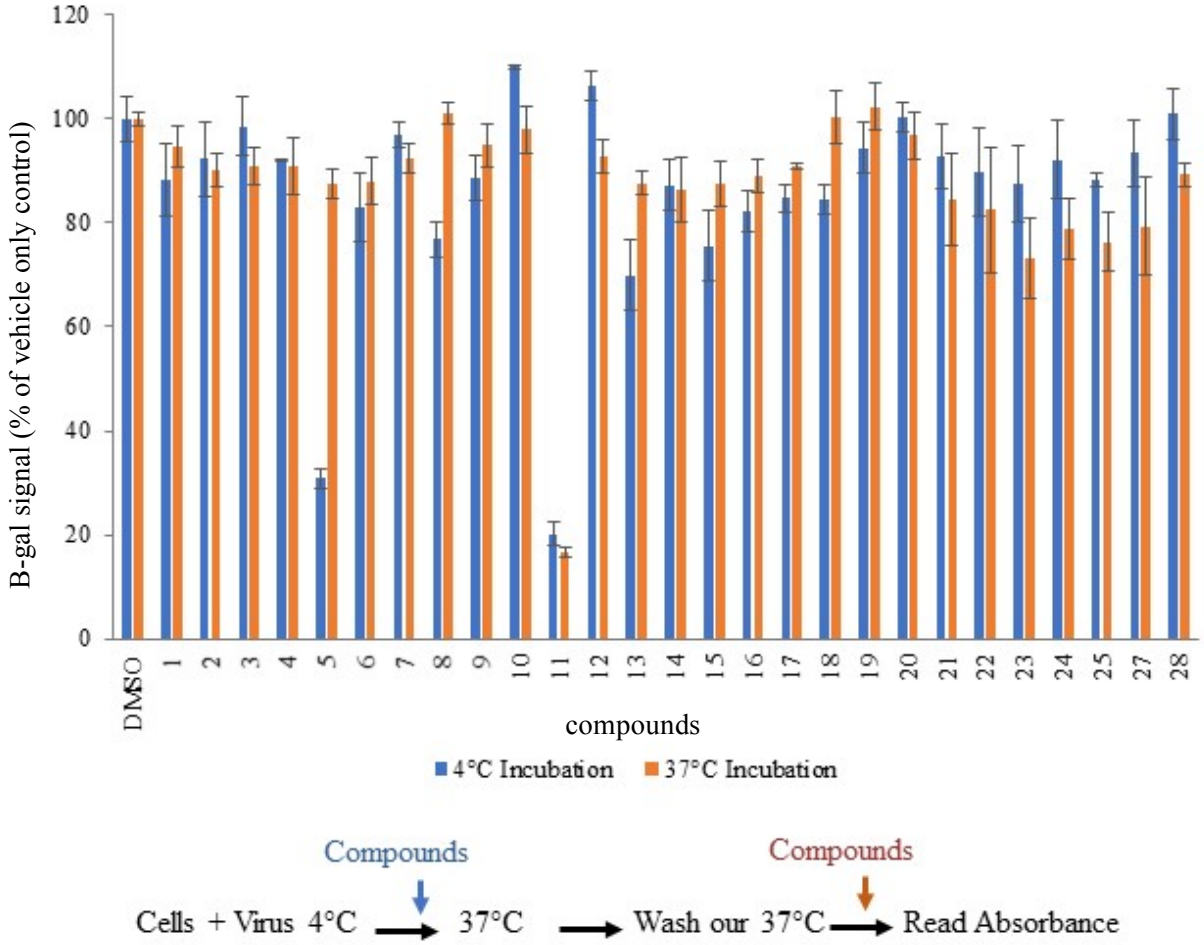

Figure 9. Quantitative screen for virus entry inhibition. A panel of 26 compounds was tested for inhibition of HSV1 KOS tk12, a strain that encodes β-galactosidase (βgal) as a reporter gene, entry into cells. HSV1 (2 x 10^5 pfu/well) plus 17 μM compound (blue bars) or media (orange bars) were added to Vero cells in 96 well plates on ice for one hour. Cells were transferred to 37°C for two hours and then treated with a citrate buffer to inactivate extracellular virus. After rinsing, media (blue bars) or 17 μM compound (orange bars) was added to the cells. Cells were incubated at 37°C for three hours and then lysed with NP40. Entry levels were determined by adding the βgal substrate CPRG and reading absorbance at 560 nm. Data are expressed as a percentage of the DMSO/vehicle control for each treatment group. Error bars show standard deviation of three replicate wells from a representative experiment. The experimental design is drawn to demonstrate when compound was added for the two treatment groups.

Compound 5 was the most interesting candidate, showing notable inhibition of reporter gene expression using both the GFP- and βgal-expressing viruses. Compounds 5 was the only compound that inhibited over 50% virus entry and was significantly different from vehicle only control when added at 4°C incubation, with a p value < 0.01 (determined by ANOVA and

Tukey's post hoc analysis), but not significantly different when added at 37°C time point. The assay had a Z'-factor of 0.84 in HSV1 and 0.83 in HSV2. Compound 11 also inhibited reporter gene expression using both viruses, but this inhibition was observed also when compound 11 was added to cells after virus entry, suggesting that compound 11 impacts overall gene expression. An additional 75 compounds were screened using the quantitative βgal-virus entry assay, including 51 compounds selected for structural similarity to compound 5 (data not shown). All 77 compounds were screened in the same assay with HSV2 and the results were similar to HSV1 (data not shown). One compound that was related to compound 5, called SINS, inhibited virus entry well, however additional experiments showed that this compound was less potent than compound 5 (data not shown), so the further experiments were focused on compound 5.

Compound 5 Potency

To determine the potency of compound 5 inhibition of virus entry, serial dilutions of compound 5 were added to cells simultaneously with βgal-expressing HSV1. Cells were incubated on ice for an hour for virus binding and then shifted to 37°C for virus entry. Compound 5 inhibited HSV1 entry in a dose-dependent manner (Fig. 10). To examine the specificity of compound 5 inhibition, the compound was simultaneously tested against HSV2 and vaccinia (a virus from an unrelated family) that encode βgal. Vaccinia is also an enveloped virus that is able to infect Vero cells and shows comparable βgal activity in the same time frames as HSV. Compound 5 inhibited both HSV1 and HSV2 entry, however it did not inhibit the unrelated vaccinia virus (Fig. 10). The IC_{50} (inhibitory concentration that reduces entry by half) for compound 5 was 3.1 μM for HSV1 and 2.5 μM for HSV2. HSV1 and HSV2 are closely

related viruses that encode functionally interchangeable gB genes, thus inhibition of both HSV1 and HSV2 was expected. The lack of inhibition of vaccinia virus by compound 5 suggests that compound 5 inhibits herpesvirus entry specifically, rather than inhibiting a cellular process that impacts membrane fusion/virus entry in a non-specific manner. None of the additional compounds screened had an IC_{50} lower than that of compound 5 (data not shown).

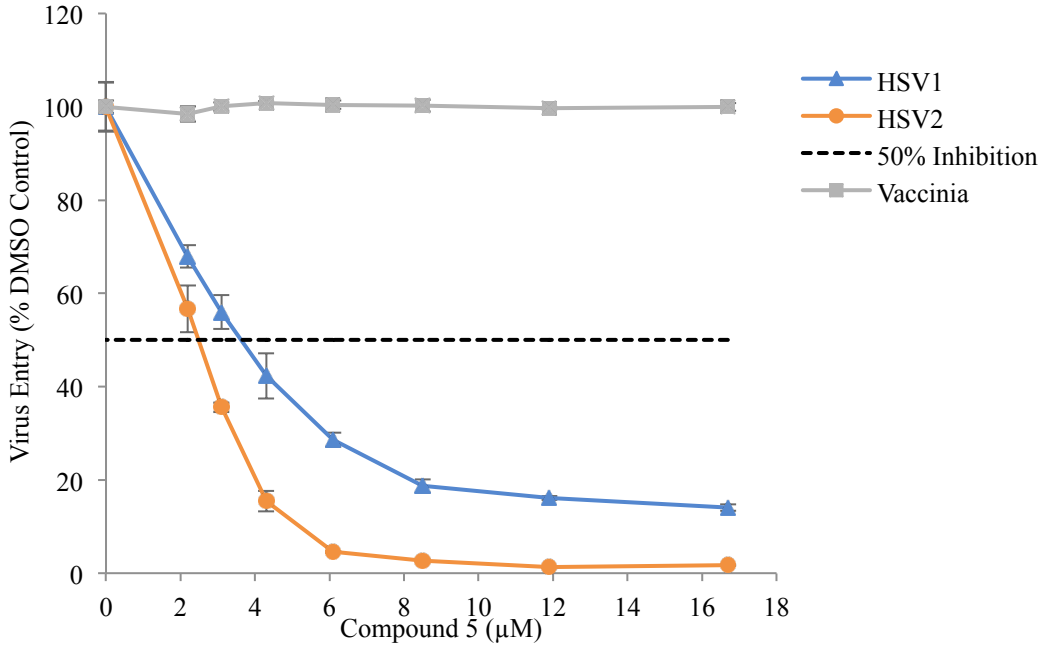

Figure 10. Dose-dependent inhibition of virus entry. Serial dilutions of compound 5 plus β-galactosidase reporter viruses HSV1 KOS tk12 ($2x10^5$ pfu/well), HSV2/Gal (2×10^4 pfu/well), or vaccinia virus vSIJC-20 ($2x10^5$ pfu/well) were added to Vero cells on ice for one hour. Cells were transferred to 37°C for two hours and treated with citrate buffer to inactivate extracellular virus. Cells were incubated at 37°C for three hours and lysed with NP40. Entry was determined by adding CPRG and reading absorbance at 560 nm. 100% virus entry was set to the vehicle only control (DMSO) for each virus. The IC_{50} (highlighted by a dashed line) of HSV1 is 3.12 µM and of HSV 2 is 2.45 µM. Experiments were performed three times and error bars represent standard deviation of triplicate samples of a single representative experiment.

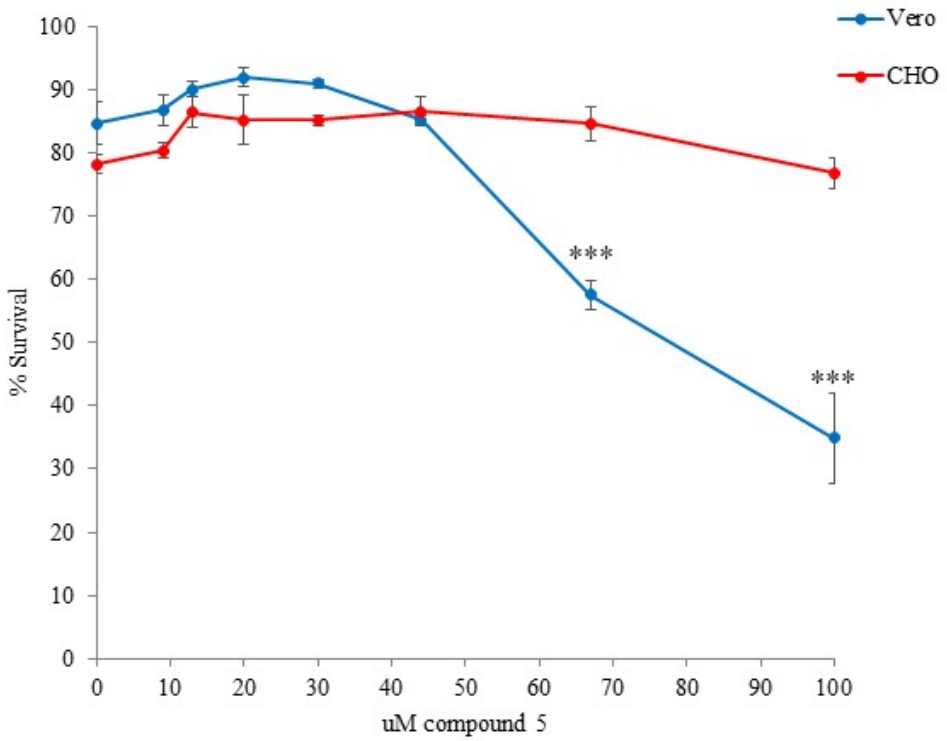

Figure 11. Cytotoxicity of compound 5. The cytotoxicity of compound 5 was assessed in Vero and CHO-K1 cells. Serial dilutions of compound 5 were added to cells in a 96-well plate and the cells were incubated at 37°C for 16 hours. The viability of the cells was determined by using Promega Cyto-Tox-Glo^{TM} Cytotoxicity Assay. This assay measures the release of dead cell protease using a luminogenic cell-impermeant peptide substrate. To determine the viable cell percentage, the luminescence was read after 16 hour incubation to determine the dead cell luminescence. Cells were lysed and luminescence was read again to determine the total cell luminescence. Dead cell luminescence was subtracted from the total luminescence (luminescence detected after cell lysis) and expressed as a percentage of the vehicle only (DMSO) control. Experiments were performed three times. The experiment was performed in triplicate. Data represent the mean of three replicate wells of a representative experiment with standard deviation shown. Statistical significance was determined as different from 0 µM compound by ANOVA and Tukey's post hoc test (*** denotes P <0.001).

Cytotoxicity of Compound 5

Cell toxicity was tested using the Promega Cyto-Tox-Glo^{TM} Cytotoxicity Assay. Compound 5 was determined not to be cytotoxic up to 44 µM on Vero cells, the cell line used in the virus entry assay, and up to 100 µM in CHO cells, the cell line used later for the cell-cell

fusion assay (Fig. 11). Compound 5 was toxic to Vero cells at 66 μM, but this concentration is over a log greater than the IC_{50} for compound 5 (Fig. 11).

Inhibition After Virus Binding

Virus entry occurs in two major steps: glycoprotein binding to host cell receptors and fusion of the viral and cellular membranes. In HSV1 and HSV2, gD acts to bind to the cell and gB acts as the fusion protein. To determine which stage of virus entry compound 5 inhibits, the timing of the addition of compound to the virus entry assay was varied. As seen previously, when virus and compound were added to the cell simultaneously, virus entry was inhibited (Fig. 12). When virus was added to cells on ice and allowed to bind for 1 hour prior to adding compound 5 and shifting the cells to 37°C, virus entry was still inhibited. This suggests that compound 5 is able to inhibit entry at a post-binding step of virus entry. Although, this result does not demonstrate that compound 5 targets gB, this result is consistent with compound 5 inhibiting the membrane fusion step of entry, not gD-receptor binding.

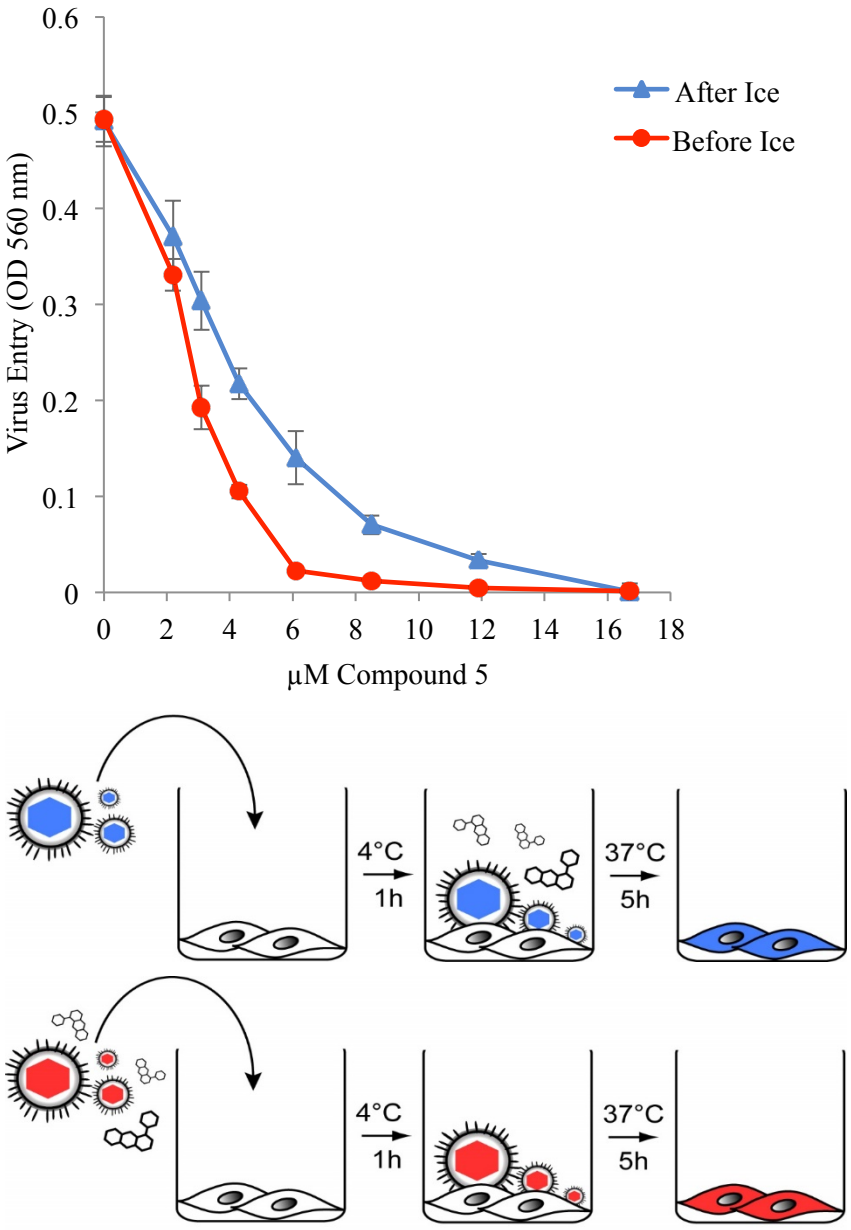

Figure 12. Inhibition of virus entry before and after binding. HSV2/Gal (2 x 10^4 pfu/well) was added to Vero cells on ice for one hour with either compound 5 (red line) or DMEM (blue line). Cells were rinsed with DMEM to remove compound and unbound virus. Compound 5 (blue line) or DMEM (red line) was added to the cells on ice for 30 minutes. Cells were transferred to 37°C for five hours and lysed with NP40. βgal expression was measured to indicate the level of virus entry. Error bars represent standard deviation of triplicate samples of a single representative experiment. The experiments were performed three times. The experimental design is drawn to show when the compound was added for each condition.

Inhibition of cell-cell fusion

To directly examine the effect of compound 5 on fusion, a cell-cell fusion assay was performed. In this assay, one population of cells (called effector cells) is transiently transfected with all four glycoprotein that are required for HSV1 fusion (gD, gH, gL, and gB), along with T7 polymerase. A second population of cells (called target cells) that stably express the HSV receptor nectin-1 are transiently transfected with a plasmid encoding luciferase under the control of the T7 promoter. The two cell populations are co-cultured overnight at 37°C and, if cell-cell fusion occurs, the T7 polymerase from the effector cells drives expression of the luciferase reporter gene from the target cells. Luciferase expression coincides with the level of cell-cell fusion and is detected using luminescence. This assay allows isolation of the entry/fusion step of the virus replication cycle, eliminating dependence on the downstream events that must happen after virus entry to allow for reporter gene expression, such as transport of the capsid to the nucleus and viral gene expression.

The cell-cell fusion assay was performed and dilutions of compound 5 were added at the time of co-culture. Compound 5 was shown to significantly reduce cell-cell fusion at a concentration of 19.5 μM and greater ($p<0.05$) (Fig. 13). Inhibition of fusion in the cell-cell fusion assay required higher a concentration of compound 5 than was required to inhibit virus entry (IC_{50}, of 3.1 μM), however this difference may be due to differences in the amount of gB present in the assays. The number of gB trimers available on a cell surface to mediate cell-cell fusion may exceed the number of gB trimers available on the virion surface to mediate virus-cell fusion. These results suggest that compound 5 is inhibiting the fusion step of HSV virus entry.

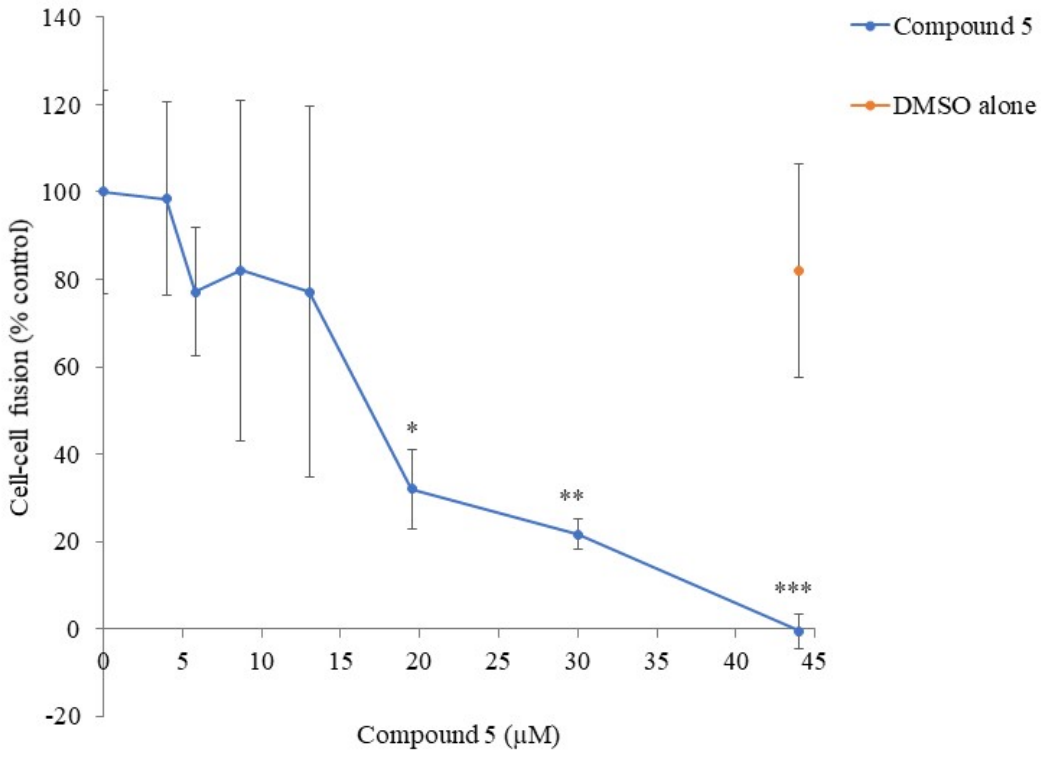

Figure 13. Inhibition of cell-cell fusion. CHO-K1 cells (effector cells) were transfected with plasmids encoding entry glycoproteins gB, gD, gH, gL and T7 polymerase. CHO-nectin-1 cells (target cells) were transfected with a plasmid encoding luciferase under the control of the T7 promotor. Three hours post-transfection, target cells were overlaid onto effectors cells and serial dilutions of compound 5 were added. After an overnight incubation, cells were lysed and luciferase expression was assayed to indicate the level of cell-cell fusion that occurred. Fusion detected in the absence of compound was set as 100%. Experiments were performed three times. Error bars represent standard deviation of triplicate samples of a single representative experiment. Statistical significance was determined as different from 0 μM compound by ANOVA with Tukey's post hoc test (* $P<0.05$, ** $P<0.01$, *** $P<0.001$)

IV: Discussion

Targeting Virus Entry for Inhibition

The HSV envelope glycoproteins facilitate virus entry into the cell, making them ideal targets for antiviral intervention. Entry inhibitors could serve as prophylactic or therapeutic drugs to reduce herpesvirus disease incidence and severity, similar to the peptide-based entry inhibitor Fuzeon that targets the HIV fusion protein and is currently used to treat HIV (Eckert and Kim, 2001). The current treatments for herpesvirus infections are nucleoside analogs, such as acyclovir, that prevent viral DNA replication (Gnann et al., 1983). Small molecule inhibitors of HSV entry do not yet exist. A panel of compounds was designed to inhibit function of the herpesvirus fusion protein gB by interfering with coil-arm interactions. Previous work suggested that the formation of the coil-arm complex may provide a driving force for membrane fusion, analogous to the function of the six-helix in class I viral fusion proteins (Connolly and Longnecker, 2012). These compounds were designed to disrupt the contacts between the gB arm residues H681 and F683 and the central coil-coil present in the postfusion structure of gB.

Compound 5 Inhibition and Specificity

Of 77 compounds tested, compound 5 showed the most promise as an entry inhibitor. Compound 5 inhibited virus entry of both HSV1 and HSV2, when tested using a GFP reporter HSV2 (Fig. 8) and βgal reporter HSV1 and HSV2 (Fig 10). Due to conservation of gB across the herpesvirus family, a compound that inhibited either HSV1 was expected to inhibit HSV2 as well. Compound 5 inhibited HSV entry with an IC_{50} in the low micromolar range (Fig. 10), but did not inhibit βgal or GFP expression when added to cells after virus entry (Fig. 9). Compound 5 did not inhibit the entry of vaccinia virus, an unrelated DNA virus that infects the same cell

line as HSV in the same time frame using the same βgal reporter gene, suggesting that compound 5 inhibition is specific to herpesviruses (Fig. 10). Compound 5 also was able to inhibit HSV-mediated fusion using a cell-cell fusion assay, in which the only the glycoproteins required for entry (gB, gD, and gH-gL) were expressed (Fig. 13). This suggests that the fusion step of virus entry is the target of compound 5 inhibition, rather than a later stage in the virus replication cycle. Future experiments will use the cell-cell fusion assay to test the effect of compound 5 on cells expressing unrelated viral fusion proteins to demonstrate whether inhibition of cell-cell fusion is specific to HSV. Compound 5 was not cytotoxic at the concentrations used in these assays (Fig. 11).

Virus entry occurs in two stages: attachment of the virus to cell surface by viral glycoproteins binding to cellular receptors and fusion of the viral envelope with the cell membrane. HSV attachment is primarily executed by gD and virus fusion is mediated by the refolding of gB. To determine which stage of HSV entry compound 5 acts upon, compound 5 was added to the virus entry assay either before or after virus-cell binding. Compound 5 inhibited entry when added after virus binding to cells was permitted on ice for one hour (Fig. 12), suggesting that it inhibits entry at a step after gD attachment. Interestingly, entry was inhibited when compound 5 was added during the virus-cell binding step on ice and the cells were rinsed prior to incubation at 37°C. This suggests that compound 5 can bind to HSV on ice, prior to the fusion event. Compound 5 inhibited entry somewhat better when added during virus-cell binding, rather than after binding, suggesting that the compound may impact on virus-cell binding to some degree.

Future Directions

Relatives of compound 5 were developed and tested, but, thus far, all of these compounds had a higher IC_{50} than compound 5. To develop a more potent HSV inhibitor, more derivatives will be developed. An ideal drug candidate would have a high affinity for gB, a low IC_{50}, and no cytotoxicity at the relevant concentrations.

To demonstrate that compound 5 is binding to gB, a biotinylated form of compound 5 could be added to the entry assay. Precipitation of the compound using streptavidin-conjugated beads followed by a western blot probing for HSV gB would determine whether the compound is binding directly to gB. Adding the biotinylated compound before and after the binding step of entry could determine when the compound binds gB. A caveat to these studies is that biotinylation of compound 5 may impact its ability to inhibit virus entry, but this could be examined by determining the IC_{50} of biotinylated gB using the virus entry assay.

An alternative approach to identify the viral target of compound 5 is to map the location of mutations that provide resistance to compound 5. If compound 5 binds to gB at the coil-arm interface, mutations at this site may prevent compound 5 binding. By growing the virus in the presence of compound 5, viral escape mutants would be selected and their genomes would be sequenced. The impact of these mutations on inhibition by compound 5 could be defined using the virus entry assay and co-precipitation with biotinylated gB. In attempt to identify viral escape mutants, HSV1 or HSV2 (100 pfu/well) and compound 5 were added to Vero cell monolayers in six-well plates. After two hours at 37°C, extracellular virus was inactivated with a low pH citrate buffer, cells were overlaid with methylcellulose to restrict virus spread to local plaques. After three days at 37°C, plaques were visualized by staining the cells with Giemsa stain. Unexpectedly, compound 5 did not reduce the number of plaques formed (data not shown), so no

escape mutants could be selected. Despite attempts to optimize this protocol, inhibition of plaque formation failed. In both the virus entry assay and the plaque formation assay, entry is permitted for two hours in the presence of compound 5 prior to virus inactivation using a citrate buffer. This step is followed by a five hour incubation in the entry assay and a three day incubation in the plaque formation assay. One potential explanation for the discrepancy between the two assays is that compound 5 somehow protects the extracellular virus from inactivation and the virus is able to infect the cells when given a three day window.

Development of a compound that blocks interactions between amino acid residues in the gB coil-arm complex may provide a viable drug candidate for the prevention or treatment of HSV infection. If the coil-arm complex is vulnerable site in HSV, development of compounds targeting this site could be expanded to include other herpesviruses, since gB is conserved across the virus family. The development of a compound that inhibits gB also would provide a useful tool to study gB. The structure of the prefusion form of gB has not been crystalized because removal of the gB transmembrane domain caused the protein to fold to its postfusion conformation. Inhibiting folding to the postfusion form could allow the capture of a prefusion or an intermediate state of gB and facilitate the crystallization of an alternate gB conformation. Trapping an alternative conformation of gB also may stabilize its interaction with the other entry glycoproteins, such as gH-gL, allowing mapping of the interaction sites between these proteins and elucidating the interactions that occur among the glycoproteins.

V. Appendix: Mutagenesis of the gB Coil

Abstract

Herpesviruses are ubiquitous and most adults are seropositive for several herpesviruses. Common diseases caused by herpes virus include oral and genital herpes, chicken pox, and mononucleosis. The first step of virus infection is entry into a host cell. Enveloped viruses, including herpesviruses, enter cells by fusing a viral membrane with the host cell membrane and releasing the virus contents into the cytoplasm. Herpes simplex virus (HSV) entry is complex and involves four distinct proteins, unlike many viruses that require only one entry protein. The HSV fusion protein, gB, mediates fusion by refolding of the from a prefusion form into a postfusion form, merging the virus envelope with the host cell membrane. The structure of postfusion structure of gB has been solved; however, the prefusion structure has yet to be crystalized. Two models of prefusion gB have been proposed. A panel of gB mutants was created introducing substitutions in the central coiled-coil region to attempt to stabilize the prefusion conformation of gB. The mutants were designed to examine a proposed prefusion gB model and identify key amino acids involved in the gB transition from its prefusion to its postfusion conformation.

Introduction

Virus-cell fusion is facilitated by glycoproteins on the surface of the virus envelope. In HSV1 and HSV2, four glycoproteins are necessary and sufficient for virus entry: gD, gH, gL, and gB. gB functions to bring the viral envelope together with the cell membrane, releasing the DNA containing capsid into the host cell. To undergo this fusion event, gB is triggered to undergo a large conformational change, refolding from a prefusion to a postfusion state. The postfusion crystal structure of gB has been solved for several herpesviruses (Fig. 4) (Backovic et al., 2009; Burke and Heldwein, 2015; Chandramouli et al., 2015; Heldwein et al., 2006). The prefusion structure has yet to be crystalized, however models of the prefusion conformation have been made (Gallagher et al., 2014; Zeev-Ben-Mordehai et al., 2016).

One model was constructed based on the structurally similar viral fusion glycoprotein, VSV G (Fig. 5) (Gallagher et al., 2014). VSV G has been crystalized in both prefusion and postfusion. VSV G is triggered by pH and the conformational change is reversible, facilitating crystallization of the prefusion form. The postfusion structure of gB features an extended 44 residue α-helix that forms a central coiled-coil structure in the trimer. In this prefusion model, this helix is broken. This helix contributes to trimerization of postfusion gB and may extend upon triggering in a similar to fashion to VSV G. To test the validity of this prefusion gB model, mutants were designed to stabilize prefusion gB. Introducing proline mutants in the coil of gB may make the conformational change from prefusion to postfusion more difficult by stabilizing a broken helix in prefusion gB.

Materials and Methods

Cells and plasmid constructs

Chinese hamster ovary cells (CHO-K1) that lack HSV receptors and the derived cell line CHO-nectin-1 cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS) supplemented with penicillin and streptomycin. Plasmids used included pSG5-HSV1 gB (wild-type [WT] or a truncated version 876t), pCAGGS-gD (pPEP99), pCAGGS-gH (pPEP100), pCAGGS-gL (pPEP101), pCAGGS-T7 polymerase, and a plasmid encoding luciferase under the control of the T7 promoter (Pertel et al., 2001b). gB mutants were constructed by QuikChange site-directed mutagenesis (Agilent Genomics), according to manufacturer protocol. DNA was transformed into *E.coli* strain XL Blue. *E. coli* was made competent using Mix & Go kit (Zymo Research) and transformations were performed according to manufacturer protocol onto LB agar/ampicillin plates. Individual clones were grown overnight at 37°C in LB broth/ampicillin (100 μg/mL). Minipreps were prepared with Zyppy Plasmid Miniprep Kit (Zymo Research) according to manufacturer protocol.

Luciferase Assay (Quantitative cell-cell fusion assay)

Effector cells (CHO-K1 cells) were seeded in white 96-well plates at 6 x 10^4 cells per well. Target cells (CHO-nectin-1 cells) were seeded in 6-well plates, overnight at 37°C. The effector cells were transfected in triplicate with 30 ng of gB (WT or 876t) or empty vector and 20 ng each of plasmids encoding HSV1 gD, gH, gL, and T7 polymerase (110ng/well of total DNA) in Opti-MEM (50μL/well) using Lipofectamine 2000 (0.35μL/well). The target cells were transfected in duplicate with a plasmid encoding luciferase (2.2 μg/ well) in Opti-MEM (1 mL/well), using 7 μL/ well Lipofectamine 2000. Three hours post-transfection, media was

removed from the effector cells and cells were rinsed twice with PBS supplemented with MgCl and CaCl2 (PBS+). Target cells were rinsed twice with 2 mL of versene and cells were released in 200 μL/well versene for 5 minutes. Target cells were resuspended in Opti-MEM (1.5 mL/well) and overlaid onto the effector cells in the 96 well dish (50 μL/well). Cells were co-cultured for 12-16 hours at 37°C. The wells were rinsed with PBS+, and 50 μL/well lysis buffer was added (Promega). After a 10 minute incubation at room temperature, 50 μL/well of luciferase substrate was added and the luminescence was recorded using a luminometer (Promega GloMax).

Cell-Based Enzyme Linked Immunosorbent Assay (CELISA)

CELISA was used to assess the levels of gB expressed on the surface of the fusion assay. A 96 well plate of effector cells (CHO-K1) was transfected with 110 ng of plasmid encoding gB using lipofectamine 2000 (0.35 μL/well) in 100 μL OptiMEM. Plates were incubated at 37°C for three hours and rinsed with 200 μL/well PBS+. The media was replaced with DMEM/10%FBS/1xPS (100 μL/well) and incubated overnight at 37°C. Cells were fixed with 3% paraformaldehyde in PBS for one hour. Cells were rinsed three times with 200 μL/well PBS. Cells were incubated with primary polyclonal antibody anit-HSV1 ab9533 (Promega) diluted 1:2000 in 5% milk in PBS for 1-2 hours. This antibody recognizes several HSV1 glycoproteins, including gB. Cells were rinsed as above and incubated with secondary antibody goat anti-rabbit polyclonal horseradish peroxidase diluted 1:1000 in 5% milk in PBS. Cells were rinsed as above and 50 μL/well BioFX TMB One Component HRP Microwell Substrate was added. Plates were incubated at room temperature for 30 minutes and absorbance was measured at 620 nm.

Results

Mutations in the gB coil were designed to decrease the stability of the postfusion conformation of gB and potentially increase stability of the prefusion conformation of gB. In the postfusion gB conformation, the coil exists as a 44-residue extended central coiled-coil. In one model of prefusion gB (Gallagher et al., 2014), the coil is broken near residue G522 (Fig. 14). In another model of prefusion gB. gB is too short to accommodate an extended coiled-coil (Zeev-Ben-Mordehai et al., 2016). To examine the function of the coiled-coil, residues G522 to W528 were individually substituted with proline (Fig. 14). The amino acid proline chosen due to its low propensity to form α-helices (Pace and Scholtz, 1998). If the extended coil is critical to gB function, these mutations should inhibit fusion by inhibiting gB refolding from a prefusion to a postfusion form. If the extended coil is broken at this site in the prefusion form of gB, these proline mutations may favor a prefusion conformation, thereby reducing fusion. If the mutations hinder fusion by favoring a prefusion conformation (rather than disrupting the global conformation of gB), then their fusion function may be restored by the addition of a known hyperfusogenic mutation (876t) that truncates the cytoplasmic tail of gB (Baghian et al., 1993). Using site-directed mutagenesis, the mutations were introduced into WT gB and a hyperfusogenic form of gB (876t). 876t carries a mutation that truncates the cytoplasmic tail of gB. Mutations in the gB cytoplasmic tail enhance fusion, potentially by altering interactions with other entry glycoproteins (Cooper and Heldwein, 2015).

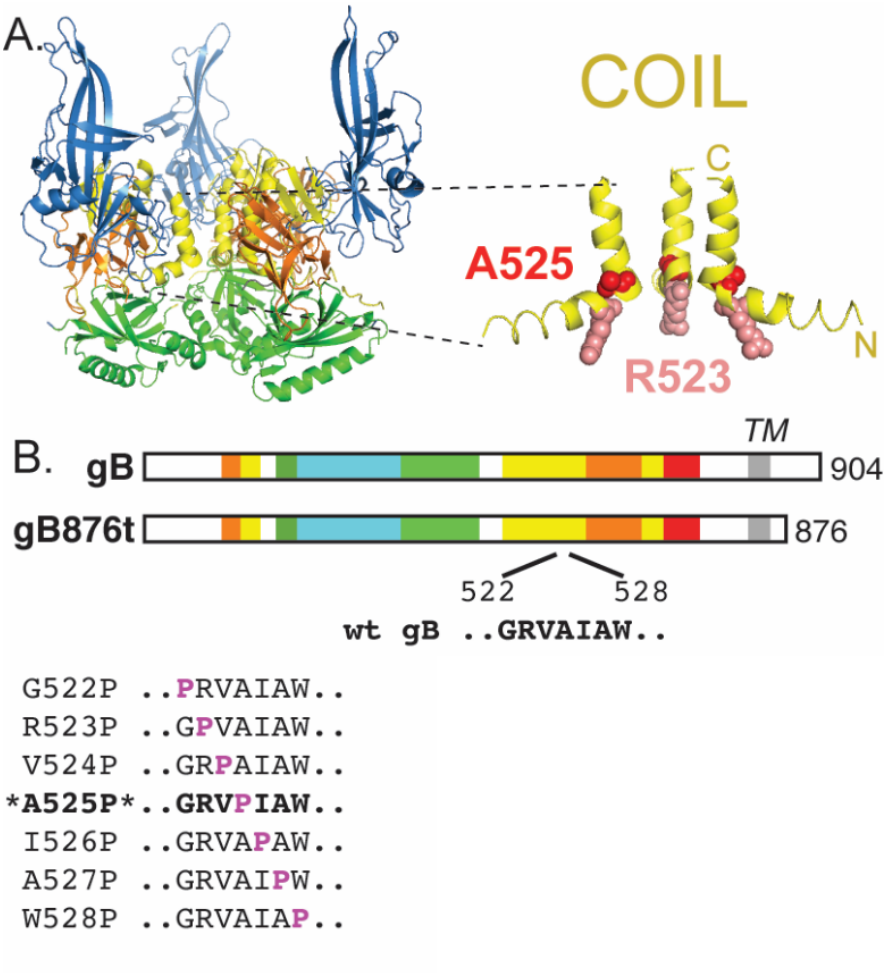

Figure 14. Site-directed mutagenesis of the gB coil. (A) Prefusion model of gB (Gallagher et al., 2014). Domains colored as above figure. The extended coiled-coil in postfusion gB is broken in this prefusion model. Residues A525 (red) and R523 (pink) in the coil are highlighted. (B) Proline mutations (purple) were introduced near the break in the coil (at G522) in both wt gB and hyperfusogenic gB876t. TM indicates transmembrane domain.

The gB constructs were confirmed by sequencing. Mutants were examined for function using a quantitative cell-cell fusion assay (Fig. 15) and for expression using a cell-based ELISA (Fig. 16). The addition of any of the mutations to WT gB reduced cell-cell fusion severely, to below the level of detection (Fig 16). Unfortunately, surface expression of the proteins was decreased severely as well, to nearly undetectable levels, complicating the interpretation of the results. The diminished fusion observed may be due to decreased expression rather than to an

effect on the conformation of gB. When the hyperfusogenic mutation 876t was added to the proline mutations, fusion was restored for gB-A525P. Expression of gB-A525P was restored partially also. This suggests that gB-A525P retains some function despite its low level of expression.

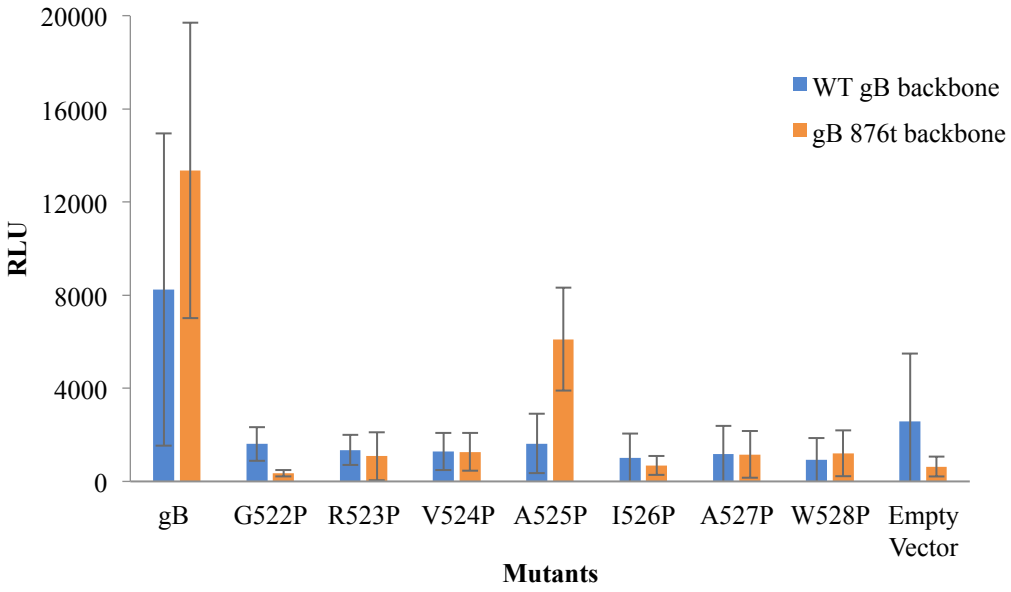

Figure 15. Cell-cell Fusion. Effector cells (CHO-K1) were transfected with plasmids encoding T7 polymerase, gD, gH, gL, and a gB mutant. The gB mutations were in the background of either WT gB (blue bars) or hyperfusogenic gB 876t (orange bars). Target cells (CHO nectin-1) were transfected with a plasmid encoding luciferase under the T7 promoter. Cells were co-cultured for 12-16 h and the luciferase activity was recorded as a measure of cell-cell fusion. Error bars represent standard deviation of triplicate samples in one representative experiment.

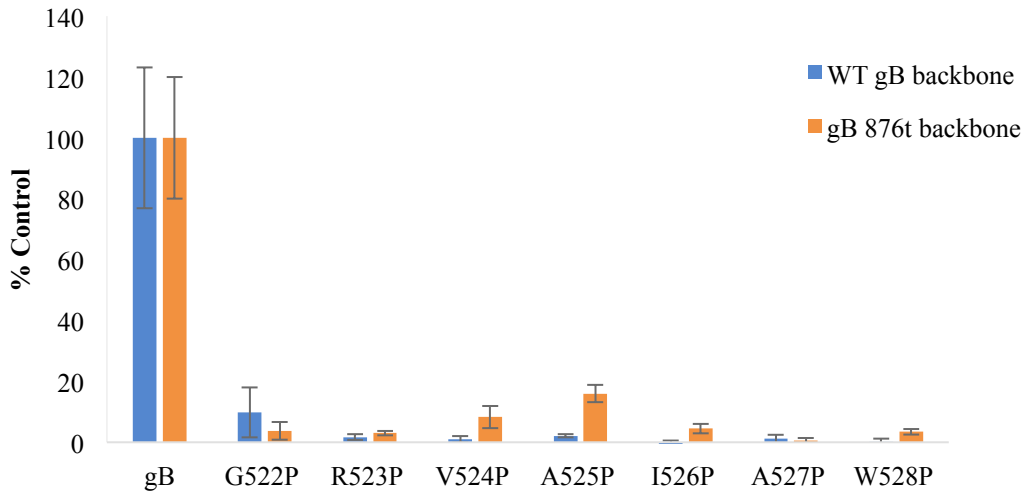

Figure 16. Cell surface expression of mutants. Surface expression of gB mutants in both WT gB or the hyperfusogenic gB (876T) background was determined by CELISA using anti-HSV polyclonal antibody ab9533. Cell were transfected with gB, cultured for 12 hours, and fixed with PFA. Wild-type and gB 876t expression levels were set to 100%. Error bars represent standard deviation of triplicate samples of one representative experiment.

Discussion

Mutations in the coil severely impacted both cell-cell fusion and surface expression of gB, in both a WT and hyperfusogenic (876t) gB background. One mutant, A525P, showed some surface expression in the 876t background (15.9% ±2.8 of WT). In the cell-cell fusion assay, this mutant mediated fusion at appreciable levels (45.7% ±16.5 of WT). These findings suggest that the residue A525 is partially amenable to substitution mutations. The substitution of other amino acids in this region of the coil may impact fusion function without decreasing cell surface expression

This panel of mutations showed over all low levels of surface expression, demonstrating how sensitive gB is to mutations. This sensitivity has been seen in other studies, specifically in the coil region, demonstrating its importance for proper protein formation and trafficking (Vitu et al., 2013).

Testing the function of these mutations at a lower incubation temperature may provide another opportunity to test the effect of these mutations on fusion function. Incubating the cells at 32°C for a longer time, rather than at 37°C for 12 hours, may increase the trafficking of misfolded protein to the surface, this increase has been shown in gB expression (Gallagher et al., 2014). Given that the 876t mutation restored gB-A525P function partially, the function of gB-A525P at 32°C would be the most interesting. If gB-A525P is expressed on the surface at 32°C, its function in a WT background compared to the 876t background could suggest whether this mutant favors a prefusion conformation.

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