MUTATIONAL ANALYSIS OF A PREFUSION MODEL OF THE HERPESVIRUS FUSION PROTEIN

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ABSTRACT

Herpesviruses affect a significant portion of the human population and cause a variety of diseases. The first step of virus infection is entry into a host cell; herpesviruses enter cells by fusing a viral membrane to the host cell membrane. This membrane fusion is mediated by the refolding of the herpesvirus fusion protein (glycoprotein gB) from a prefusion form into a postfusion form. The structure of postfusion structure of gB has been solved; however, the prefusion structure has been modeled only. This research proposes to create new mutants of gB to both validate the proposed prefusion gB model and identify key amino acids involved in the gB transition from its prefusion to its postfusion conformation.

1. INTRODUCTION

Nine herpesviruses infect humans causing diseases that include cold sores, genital herpes, chicken pox, mononucleosis, and certain lymphomas(Roizman, Knipe, and Whitley 2013). All viruses are parasites that take over the host cell machinery in order to replicate their genomes. Herpesviruses are particularly interesting due to their ability to cause lifelong infection. For infection to occur with enveloped viruses such as herpesviruses, the viral membrane and the host cell membrane must fuse together to allow the viral genome to be transported into the host cell.

In herpesviruses, fusion is mediated by virally-encoded glycoproteins that are present on the surface of the virus particle. For herpes simplex virus, four glycoproteins are required for host cell binding and entry: glycoprotein D (gD), gH, gL, and gB (Fig. 1)(Connolly et al. 2011).

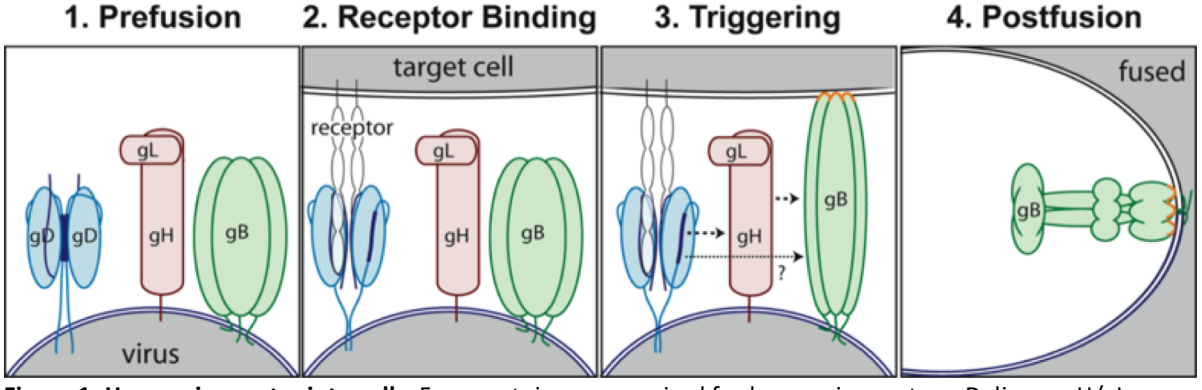


Figure 1. Herpesvirus entry into cells. Four proteins are required for herpesvirus entry: gD dimer, gH/gL heterodimer, and gB trimer (first panel). The receptor binding protein gD binds to host cell receptors (panel 2) and triggers the other proteins (panel 3). The mechanics of triggering are not completely understood; however, gB is the fusion protein that inserts into the target cell and folds back on itself bringing the viral membrane and target cell membrane together (forth panel).

Without these essential viral membrane proteins, viral infection cannot occur, making viral entry glycoproteins interesting subjects of study(Awasthi et al. 2008; Cairns et al. 2007). gD is the receptor-binding protein and it binds to specific receptors present on the host cell plasma membrane, including nectin-1, herpesvirus entry mediator (HVEM), and a modified form of heparan sulfate (Spear 2004). This receptor binding is proposed to transmit a signal to gH and gL, which form a complex known as gH/gL(Atanasiu et al. 2010). This signal is then transmitted to the fusion protein gB, the protein that physically brings the virus and the host membranes together. gB is required for entry of every member of the herpesvirus family. Upon triggering, hydrophobic loops on gB insert into a host cell membrane and the viral protein refolds to bring the host membrane and the viral membrane together(Hannah et al. 2007). This membrane merger creates a pore in the membrane that allows the viral capsid containing the genome to enter the cell.

The postfusion structure of gB is has been crystallized using a purified soluble form of the protein(Heldwein et al. 2006).The postfusion structure of gB, the human herpesviruses Epstein Barr virus (EBV), and cytomegalovirus (CMV) were solved subsequently(Backovic, Longnecker, and Jardetzky 2009; Chandramouli et al. 2015; Burke and Heldwein 2015). The prefusion conformation of gB has not been crystallized because the secreted gB protein folds to its postfusion form (Silverman et al. 2010). Although gB lacks sequence homology to rhabdovirus fusion protein G, the postfusion structures of the two fusion proteins are remarkably similar (Roche et al. 2007). Since both the prefusion and postfusion conformations of the rhabdovirus G protein have been crystallized, the similarity between gB and G allowed modeling of the prefusion form of gB(Gallagher et al. 2014).

A comparison of the existing postfusion gB structure and the proposed prefusion gB structure highlights amino acids that appear to be important in the folding from prefusion to postfusion (Fig. 2).

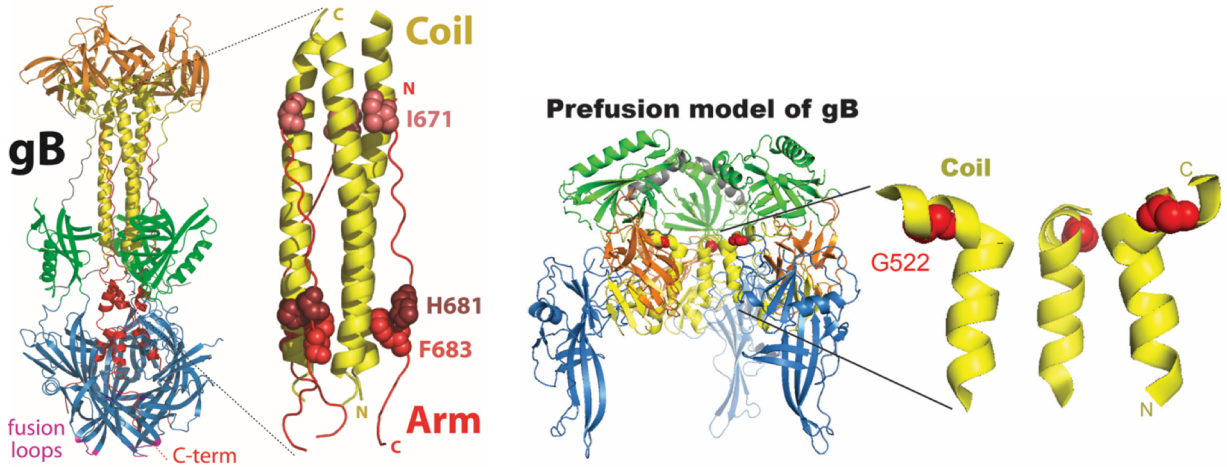


Figure 2. Structure of gB. (A) Five domains on the crystal structure of the postfusion gB trimer (Heldwein et al. 2006) are colored, and a close up of the coiled core (yellow) and the C-terminal arm of gB (red) is shown. Formation of the coil-arm complex is thought to promote refolding into the postfusion conformation. Residues on the arm previously shown by mutagenesis to contribute to fusion are labeled. (B) The prefusion conformation of gB has not been solved, however HSV 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(Gallagher et al. 2014). To test this prefusion model in the current study, gB mutations were created based on this prefusion model. Glycines were added near G522 (red) to increase the flexibility of the coil (yellow) and potentially stabilize a prefusion gB conformation.

An extended alpha-helical coiled-coil region comprises the core of the gB trimer in its postfusion form (Heldwein et al. 2006). In the prefusion model, this coil is broken distinctly in half. Mutation of the amino acids at the site of the break in the coil may allow for examination of the effects of these mutations on gB fusion function. The introduction of glycine residues at this site may increase the flexibility of the region and thereby stabilize the prefusion form of gB. Similar methods such as the introduction of cysteine mutations in HSV gD, have been shown to stabilize gD and allow for the separation of binding and fusion(Lazear et al. 2008).

A previous mutation that truncates the cytoplasmic tail of gB causes enhanced levels of fusion (hyperfusion) (Baghian et al. 1993). How hyperfusion is caused by the truncation is unknown, but it is proposed to stabilize prefusion gB through interactions between the cytoplasmic tail and the viral (Chowdary et al. 2010). Cytoplasmic regions of other fusion proteins from other viruses containing cytoplasmic tail regions also have been shown to impede fusion, including paramyxoviruses and retroviruses (Waning et al. 2004; Wyss et al. 2005).

If the glycine mutations designed for this study stabilize the prefusion form of gB, the gB mutants should demonstrate reduced fusion capacity. The addition of the hyperfusogenic mutations may restore some level of fusion because the hyperfusogenic mutations in the cytoplasmic tail and they hypofusogenic mutations in the ectodomain may compensate for one another (Connolly and Longnecker 2012). For this reason, the hyperfusogenic gB mutation 876t will be used as a comparison throughout this study.

2. MATERIALS AND METHODS

*2.1 Cell*s

Chinese hamster ovary (CHO-K1) cells, chosen because they lack HSV-1 receptors, were maintained at 37℃ in 5% CO_2 in Ham's F-12 growth medium with 10% fetal bovine serum (FBS) with penicillin-streptomycin (PS). CHO-nectin-1 cells (Geraghty et al. 1998), cells stably expressing the HSV entry receptor nectin-1, were maintained at 37℃ in Ham's F12/10%FBS/1xPS. CHO-nectin-1 cells were given 250 μg/mL of antibiotic G418 once every 10 passages to maintain expression of the nectin-1 receptor. Trypsin-EDTA was used to detach cells during passage procedures.

2.2 Syncytia Assay

Syncytia assays were performed to demonstrate that wildtype viral glycoproteins (gB, gH, gL, gD) were functioning properly. CHO-nectin1 cells were diluted to 6×10^5 cells/mL, and 1 mL was seeded to each of 6 wells on a 6-well plate. After overnight incubation at 37℃, CHO-nectin1 cells were transfected with 900 ng of DNA/well in 500 μL using 3.5 μL/well lipofectamine. After 3 hours at 37℃, wells were aspirated and 1 mL/well Ham's F12/10%FBS/1xPS was added for an overnight incubation at 37℃. Cells were fixed using 1 mL/well methanol for 30 minutes. Syncytia were visualized by staining with Giemsa stain. Using an inverted tissue culture microscope, cells were imaged at 400X magnification using Micron software. Results are depicted in Figure 3 below.

2.3 gB Mutant Design

Based on the prefusion model of gB, the break in the central gB coil occurs around residue G522. Residues C-terminal to G522 were substituted with glycine to increase flexibility in the coil and potentially promote a prefusion gB conformation. Insertion mutants were also designed. Construct gB-522+1G has one glycine inserted between residues 522 and 523. Construct gB-522+2G has two glycines inserted between residues 522 and 523. A tryptophan residue at this site was also mutated to alanine to determine if this bulky residue contributes to the stability of the prefusion form of gB. Specific mutations are depicted in Figure 4 below.

2.4 Primer Design

Mutagenic primers (Table 1) were designed using *http://www.genomics.agilent.com/primerDesignProgram.jsp*. The primers were designed to introduce mutations into the gB coding sequence that are predicted to increase flexibility in the coil region. Synthesized primers were ordered from AGCT. All primer stocks were diluted to 100 μM and stored at -20°C.

Table 1: Mutagenic Primers. Mutagenic primers were designed to mutate gB coding sequence. Two primers were used per mutation induced.

rSC120 5'HSVgB-W528A	gcgttgccatcgcg**gc**gtgcgagctacaga
rSC121 RC3'HSVgB-W528A	tctgtagctcgcac**gc**cgcgatggcaacgc
rSC122 5'HSVgB-G522+1G	acgatatgttgggc**gga**cgcgttgccatcgc
rSC123 RC3'HSVgB-G522+1G	gcgatggcaacgcg**tcc**gcccaacatatcgt
rSC124 5'HSVgB-G522+2G	acgatatgttgggc**ggaggt**cgcgttgccatcgc
rSC125 RC3'HSVgB-G522+2G	gcgatggcaacgcg**acctcc**gcccaacatatcgt
rSC126 5'HSVgB-523G	cgatatgttgggc**g**gcgttgccatcgc
rSC127 RC3'HSVgB-523G	gcgatggcaacgc**c**gcccaacatatcg
rSC128 5'HSVgB-523G2	cgatatgttgggc**g**gcg**g**tgccatcgcgtgg
rSC129 RC3'HSVgB-523G2	ccacgcgatggca**c**cgc**c**gcccaacatatcg
rSC130 5'HSVgB-523G3	cgatatgttgggc**g**gcg**g**tg**g**catcgcgtggtgc
rSC131 RC3'HSVgB-523G3	gcaccacgcgatg**c**ca**c**cgc**c**gcccaacatatcg
rSC132 5'HSVgB-523G4	tcaacgatatgttgggc**g**gcg**g**tg**g**c**gg**cgcgtggtgcgagctac
rSC133 RC3'HSVgB-523G4	gtagctcgcaccacgcg**cc**g**c**ca**c**cgc**c**gcccaacatatcgttga
rSC134 5'HSVgB-523G5	gtcaacgatatgttgggc**g**gcg**g**tg**g**c**gg**cg**g**gtggtgcgagctacagaa
rSC135 RC3'HSVgB-523G5	ttctgtagctcgcaccac**c**cg**cc**g**c**ca**c**cgc**c**gcccaacatatcgttgac

2.5 Quikchange PCR Mutagenesis

Mutagenesis of plasmid-encoded gB with was performed according to the Quikchange 2015manual (2015). The template plasmid used is named pSG5-HSV1 gB and encodes an ampicillin resistance gene. Quikchange mutagenesis is a procedure for site directed mutagenesis *in vitro*. Polymerase Chain Reaction (PCR) thermal cycling denatures the DNA template by breaking the hydrogen bonds at 95°C, anneals mutagenic primers at 60°C, and extends the primers to replicate the DNA at 68°C. This process is repeated multiple times to create a several copies of mutant DNA. The produce of the PCR is a full-length plasmid containing the desired mutations.

Reactions used conditions were:

5 μL of 10x reaction buffer
1 μL (10 μM) of plasmid encoding HSV-1 gB (pSG5-gB)
1 μL (125 μM) of primer #1
1 μL (125 μM) of primer #2
5 μL of dNTP mix [250 μM)
37 μL of water (ddH_2O)
Then add 1 μL of *PfuTurbo* DNA polymerase (2.5 U/μL)

The PCR cycle was 1× 95°C 60 sec // 18 × 95°C 50 sec, 60°C 50 sec, 68°C 7.5 min // 1× 68°C 7 min // 1× 4°C continuous.

To remove template DNA (parental, non-mutant DNA that has been methylated in a bacterial cell), reactions were treated with DpnI, (1 μL/tube), an enzyme that digests methylated DNA, for 1-2 hours at 37℃. The newly synthesized DNA that was created by PCR and carries the mutations will lack methylation. The resulting samples (4 μL) were transformed into competent *Escherichia coli* cells, chemically altered cells that easily take-up DNA. Using the Zymo Mix and Go kit, 4 μL PCR was added to 50 μL cells in pre-chilled microcentrifuge tubes and left on ice for 30 minutes. Samples were plated on agar plates containing ampicillin (100 μg/mL) using rattler beads, and incubated overnight at 37℃.

2.6 Preparation of DNA from clones

Up to 3 colonies (depending on number of colonies available on the plate) were inoculated individually into 5 mL Luria-Bertani broth (LB) + ampicillin (1 μL/mL). Culture were incubated overnight at 37℃, shaking at 250 rpm. DNA was isolated from the cultures using a Zymo Zyppy Plasmid Miniprep Kit. The miniprep procedure was as follows:

Spin in culture tubes in large centrifuge at 3000 RPM for 3 min.
Pour off LB+amp. Resuspend in 600 μl of water and transfer to a microcentrifuge tube.
Add 100 μl of 7x lysis buffer and mix by inverting the tube 4-6 times.
Add 350 μl of cold Neutralization buffer and mix thoroughly within two minutes.
Centrifuge at 11,000 x g for 3 minutes.
Transfer the supernatant into the Zymo-spin IIN column.
Place column into a collection tube and centrifuge for 15 seconds.
Discard the flow-through and place the column back into the same collection tube.
Add 200 μl of Endo-Wash Buffer to the column. Centrifuge for 30 seconds.
Add 400 μl of Zyppy Wash Buffer to the column. Centrifuge for 1 minute.
Transfer the column into a clean 1.5 ml microcentrifuge tube.
Add 30 μl of Zyppy Elution Buffer directly to the column matrix and let stand for 1 minute at room temp.

A Nanodrop spectrophotometer was used to determine DNA concentration and purity by reading absorbance at 260 and 280 nm. DNA was stored at -20℃.

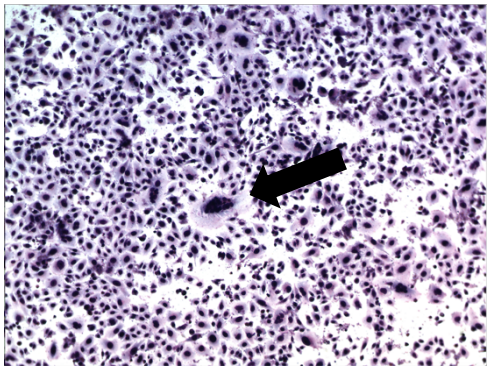
2.8 Luciferase Assay

To quantify fusion, CHO-K1 cells (termed effector cells) were seeded at 8×10^5 cells/well in a 96-well plate. CHO-nectin1 cells (termed target cells) were seeded at 6×10^5 cells/well in a 6-well plate. Effector cells were transfected in 50 μL using 0.35 μL/well lipofectamine 2000 and 110 ng/well DNA including plasmids encoding HSV1 gB, gD, gH, gL, and T7 polymerase at a 3:2:2:2:2 ratio. Target cells were transfected in 1 mL using 7 μL/well lipofectamine and 2.2 μg/well of plasmid encoding the luciferase enzyme under the control of the T7 promoter. After 3 hours at 37℃, versene was used to release the target cells from the plate on which they were cultured, and target cells were overlaid onto effector cells for 12-18 hours at 37℃. If cell-cell fusion occurs, the T7 polymerase will gain access to the plasmid carrying luciferase under the control of the T7 promoter. Expression of the luciferase enzyme can be quantified using luciferase substrate and luminescence. Each well of target cells was used to overlay 30 wells of effector cells. 50 μL/well of passive lysis buffer was added to each well to lyse the cells. After 10 minutes at room temperature, 50 μL/well luciferase substrate was added. Luminescence was detected using a Promega GloMax luminometer with a 0.5 second integration time.

3. RESULTS

3.1 Syncytia Assay

As an initial experiment, the syncytia assay was performed, to become acquainted with general methods and to visually demonstrate cell-cell fusion. When nectin-1 expressing cells were transfected with all four HSV entry glycoproteins, large multi-nucleated cells were observed. This indicated that both the nectin-1 cells and the wildtype herpesvirus entry proteins were able to facilitate fusion (Figure 3). Visualizing cell-cell fusion allows for an understanding of the broader purpose of these experiments, and confirms results in later experiments, such as the luciferase assay.

1a. 

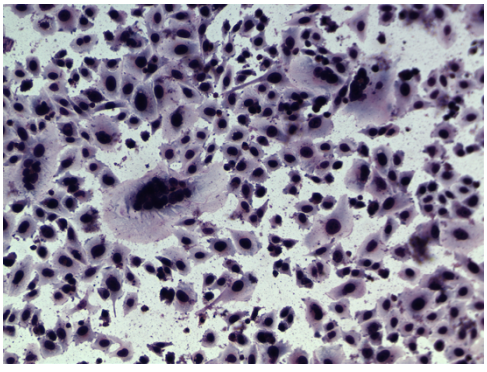
1b. 

Figure 3: Syncytia are large, multinucleated cells. The nuclei of the cells are stained dark purple. 1a. One large syncytia in the center of the frame is indicated by the arrow. 1b. Zoomed in view of large syncytia. Individual nuclei are seen inside one cellular membrane.

3.2 Quikchange Mutations

Mutations in gB may provide insight into the mechanisms of fusion and validate the prefusion model of gB. Mutations can to highlight specific amino acid residues that may be key factors in the refolding of gB during viral entry. In these experiments, substitutions or insertions of glycine residues were made in an attempt to inhibit the transition of the fusion protein from a prefusion to a postfusion form.

In the prefusion model of gB, the central coil is broken at residue G522. We hypothesize that flexibility at this site could stabilize the prefusion conformation. To further increase flexibility at this site, glycine insertions and substitutions were designed (Fig. 4).

Figure 4. Mutations were introduced at residue 522 in attempt to increase flexibility in the coil region, potentially stabilizing a prefusion confirmation of gB. Seven glycine insertion or substitution mutants were created. The W528A substitution mutant was created to determine if this bulky residue near this site in this coil contributes to fusion.

Amplification of DNA by quikchange PCR was confirmed by agarose gel electrophoresis, Transformation of DpnI-digested PCR into competent cells yielded transformants, however colony counts were low. Depending on colony numbers, either two or three isolated clones were selected. The colonies were inoculated into broth and DNA was isolated from the selected clones. Mutations were confirmed via sequencing to ensure that no unintended mutations were present.

3.3 Luciferase Assay

To determine the effects of mutations on gB function, a quantitative cell-cell fusion assay using a luciferase reporter gene was performed (Figure 5). The luciferase assay was successfully preformed twice; one representation is depicted in Figure 5. Samples were tested in triplicate for each experiment.

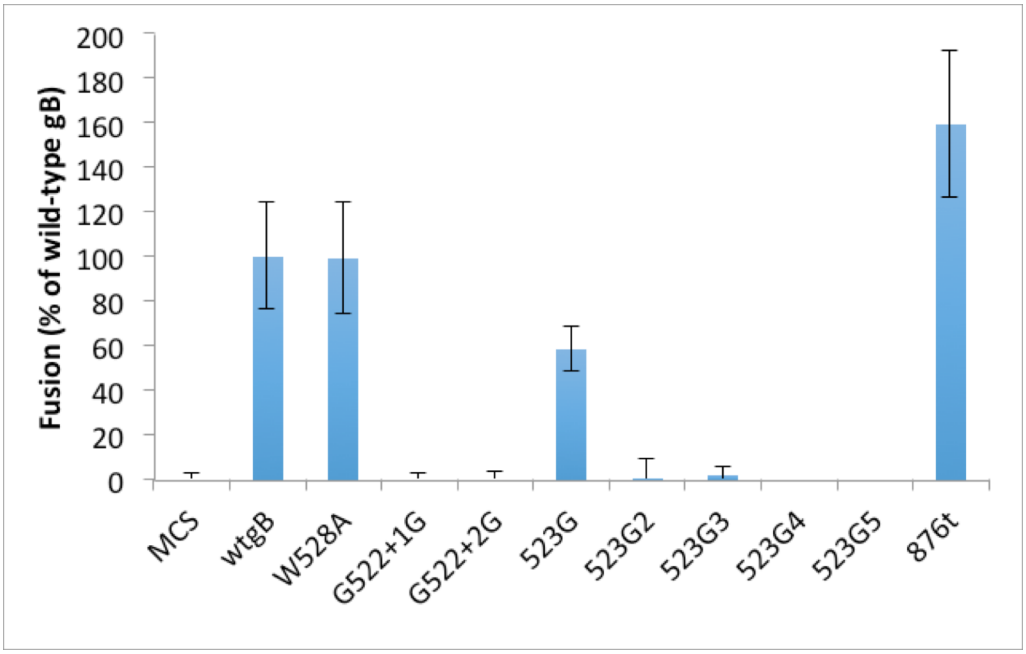


Figure 5. gB mutagens were tested for fusion in a luciferase assay. Controls were empty vector (-) (MCS), wildtype gB(+), and 876t gB(-). Mutant 2b3(W528A) had fusion similar to wildtype, and mutant 5b3(523G) had slightly decreased fusion. The other mutants did not express fusion. The error bars represent standard deviation of the average of triplicate samples in one experiment.

The luciferase assay demonstrated fusion in positive controls, when effector cells were transfected with luciferase, gD, gH, gL, and either wildtype gB (wtgB) and hyperfusogenic gB (876t). There was no fusion in the negative control, when effector cells were transfected with empty vector (MCS). In the depicted trial, cells expressing the gB mutant W528A demonstrated fusion very similar to that mediated by wtgB. The gB mutant 523G demonstrated reduced fusion, approximately 60% of wtgB; however, in another trial, this mutant demonstrated wildtype levels of fusion, so additional trials are necessary. . Mutants G522+1G, G522+2G, 523G2, 523G3,523G4 and 523G5 showed little to know fusion. Further experiments are needed to determine if the mutants loss fusion due to the intended insertion/substitutions stabilizing a prefusion conformation (as hypothesized) or to a lack of gB expression altogether due to misfolding.

4. DISCUSSION

Insertion of mutations into gB as successful; however, this project is ongoing. Whether the loss of fusion observed for some of the mutants is due to the mutations inhibiting the folding of gB to its postfusion form remained to be determined. The mutations may prevent fusion by inhibiting gB expression on the cell surface due to global misfolding of gB. The next set of experiments performed will use antibody-antigen detection in a cell-based enzyme-linkes immunosorgance assay (CELISA) to determine whether expression maintained. If the mutants are expressed at wild-type levels on the cell surface, this suggests that the loss of fusion is not due to misfolding of the protein. The proper folding of the mutants can also be examined using a panel of conformationally-dependent monoclonal antibodies.

Even without expression assays, the findings of the effects of these mutations on fusion are intriguing. The addition of one or two glycine residues at residue 522 prevents fusion, but the substitution of one glycine residue at residue 523 does not prevent fusion. The substition of two glycines inhibits fusion. These results suggest that the sequence of this coil region is important in fusion, but the expression assay (CELISA) must be performed before final conclusions can be drawn.

The hypothesis that glycine mutations stabilize a prefusion form of gB would be supported by the following results: (1) The introduction of glycine residues in the gB coil reduces gD fusion function. This result was observed in the luciferase assay. (2) The glycine mutants retain wild-type levels of expression on the cell surface. This has yet to be tested using CELISA. (3) The glycine mutants react at wild-type levels with a panel of conformationally-dependent monoclonal antibodies. This has yet to be tested using CELISA. (4) The addition of the hyperfusogenic 876t mutation in the cytoplasmic tail of gB restores some degree of fusion for the gB glycine mutants. This has not yet been tested, however the 876t mutation has been added to the plasmids encoding the gB glycine mutants (data not shown). Although it is unknown how 876t imparts a hyperfusogenic phenotype, its ability to rescue the function of the glycine mutants would suggest that the glycine mutants are properly folded and able to achieve a functional prefusion confirmation.

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