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Using proximity biotinylation to detect herpesvirus entry glycoprotein interactions: limitations for integral membrane glycoproteins

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Abstract

Herpesvirus entry into cells requires coordinated interactions among several viral transmembrane glycoproteins. Viral glycoproteins bind to receptors and interact with other glycoproteins to trigger virus-cell membrane fusion. Details of these glycoprotein interactions are not well understood because they are likely transient and/or low affinity. Proximity biotinylation is a promising protein-protein interaction assay that can capture transient interactions in live cells. One protein is linked to a biotin ligase and a second protein is linked to a short specific acceptor peptide (AP). If the two proteins interact, the ligase will biotinylate the AP, without requiring a sustained interaction. To examine herpesvirus glycoprotein interactions, the ligase and AP were linked to herpes simplex virus 1 (HSV1) gD and Epstein Barr virus (EBV) gB. Interactions between monomers of these oligomeric proteins (homotypic interactions) served as positive controls to demonstrate assay sensitivity. Heterotypic combinations served as negative controls to determine assay specificity, since HSV1 gD and EBV gB do not interact functionally. Positive controls showed strong biotinylation, indicating that viral glycoprotein proximity can be detected. Unexpectedly, the negative controls also showed biotinylation. These results demonstrate the special circumstances that must be considered when examining interactions among glycosylated proteins that are constrained within a membrane.

Keywords

proximity biotinylation; herpesvirus; protein-protein interactions; virus entry

1. Introduction

Herpesviruses cause lifelong infections in a wide variety of hosts and the average person carries multiple herpesviruses (Longnecker, Kieff, and Cohen, 2013; Roizman, Knipe, and

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Whitley, 2013). Human herpesvirus infections can result in diseases ranging from the relatively benign (cold sores, chickenpox, or mononucleosis) to life-threatening conditions, including encephalitis and lymphomas. Most enveloped viruses use a single transmembrane glycoprotein to bind to and fuse with a host cell, however herpesviruses encode receptor-binding and fusion functions on separate proteins (Connolly et al., 2011; Eisenberg et al., 2012; Krummenacher et al., 2013). Thus, communication among multiple herpesvirus entry glycoproteins is required for entry into a cell. This work uses proteins from the model herpesviruses herpes simplex virus type 1 (HSV1) and Epstein Barr virus (EBV) to explore glycoprotein interactions.

Three entry glycoproteins are conserved in all herpesviruses: gH, gL, and gB (Connolly et al., 2011; Eisenberg et al., 2012; Krummenacher et al., 2013). gL is tethered to the virus by gH, which is anchored in the viral membrane (Fig. 1). The gH/gL heterodimer binds to cellular receptors and triggers fusion mediated by gB. gB is a class III fusion protein that inserts into the host cell membrane and undergoes a conformational change to fuse the viral and cell membranes. For EBV entry into epithelial cells, gH/gL serves as the receptor-binding complex and gB is the fusion protein, thus gH/gL and gB are sufficient for EBV fusion with epithelial cells (Hutt-Fletcher and Chesnokova, 2010). For HSV1 entry and EBV entry in to B cells, an additional receptor-binding protein is required (Borza and Hutt-Fletcher, 2002). HSV1 gD binds to one of several cellular receptors and communicates with gH/gL and/or gB to trigger fusion (Krummenacher et al., 2005). Thus, four HSV1 entry glycoproteins (gD, gH/gL, and gB) are necessary and sufficient for HSV1 fusion (Pertel et al., 2001; Turner et al., 1998). EBV lacks a gD homolog, but EBV gp42 serves a similar receptor-binding function during B cell fusion (Mullen et al., 2002). EBV gp42 binds to receptor and communicates with gH/gL and gB to trigger EBV fusion with B cells (Sathiyamoorthy et al., 2014).

The crystal structures of all of these proteins (HSV1 gD, gH/gL, gB and EBV gp42, gH/gL, gB) have been solved (Connolly et al., 2011; Eisenberg et al., 2012), and many aspects of herpesvirus entry into cells are well understood. The sequence of protein-protein interactions and the precise interaction sites among the entry glycoproteins are either not fully defined or disputed. This is likely because the glycoprotein interactions are low affinity or transient. Determining the sites of interactions between the glycoproteins may provide targets for antiviral intervention, including structure-based small molecule inhibitors. Additionally, a better understanding of the interaction sites may present candidates for subunit vaccines. Multiple conformational changes in several glycoproteins likely occur during herpesvirus entry and mapping the protein interactions will help us characterize these transformations. Due to the conservation of the entry glycoproteins, information learned about one virus may impact the other clinically relevant and economically significant members of the herpesvirus family.

Herpesvirus entry glycoprotein interactions have been probed using multiple approaches. Functional interactions between gD and gH/gL have been reported (Atanasiu et al., 2010; Fan, Longnecker, and Connolly, 2014), but demonstrating a physical interaction has been challenging. Glycoproteins can be cross-linked on the virus surface and during virus entry (Handler, Cohen, and Eisenberg, 1996a; Handler, Eisenberg, and Cohen, 1996b). Pull-down

assays demonstrating entry glycoprotein associations have been reported (Gianni, Amasio, and Campadelli-Fiume, 2009), however the native glycoproteins exist in membrane-anchored states on the virion envelope and the detergents required for a pull-down assay may perturb the normal interactions. In live cells, bimolecular fluorescence complementation (BiFC) has been used to show physical interaction among the glycoproteins. In BiFC, two glycoproteins are tagged with complementary halves of a yellow fluorescent protein (YFP). When the two tagged proteins interact, the intact YFP is restored and fluorescence is detected. Using BiFC, gD has been reported to interact physically with both gH/gL and gB (Atanasiu et al., 2007; Avitabile, Forghieri, and Campadelli-Fiume, 2007). A receptor-dependent interaction between gH/gL and gB has also been reported (Atanasiu et al., 2007) but is disputed (Avitabile, Forghieri, and Campadelli-Fiume, 2009). The inherent affinity of the two halves of YFP can complicate BiFC and contribute to false positive signals (Connolly et al., 2009). The two halves of YFP may be unable to dissociate after formation, and thus may alter function during transient interactions (Kerppola, 2006).

This work explores using a promising technique, proximity biotinylation (ProB), to characterize the herpesvirus entry glycoprotein interactions. Proximity biotinylation uses an enzyme and substrate pair to detect candidate protein interactions (Fernandez-Suarez, Chen, and Ting, 2008; Thyagarajan and Ting, 2010). One candidate protein is tagged with the *Escherichia coli* biotin ligase enzyme (BirA), and a second candidate protein is tagged with an acceptor peptide substrate (AP), consisting of 15 amino acids (GLNDIFEAQKIEWHE). Exogenous biotin is supplied and, if BirA and AP come into close proximity, BirA will ligate biotin to the AP covalently (Fig. 1A). The interaction can be observed by western blot using avidin to visualize the biotinylated proteins. Mutants of the AP target peptide with differing affinities for BirA have been created, providing options for optimization of signal over background (Fernandez-Suarez et al., 2008). The low affinity mutant peptide (APm) used in this work differs in three C-terminal residues GLNDIFEAQKIEGEF.

ProB offers multiple advantages over other methods of detecting protein-protein interaction. Labeling occurs in live cells and does not require membrane disruption. The interaction between biotin and avidin is high affinity and sensitive. A low false positive rate across a range of protein expression levels has been reported (Fernandez-Suarez et al., 2008). Endogenous mammalian biotin ligase does not recognize the AP target peptide. Since the AP is only 15 amino acids, it is less likely to alter the glycoprotein function. BirA and AP are able to dissociate after an interaction so candidate proteins are free to move in the membrane as they normally do during virus entry. A major advantage to ProB is that the biotin is linked to the AP tag covalently, thus the biotin tagging serves as a permanent marker that an interaction occurred, even if the proteins do not remain associated. The interaction between proteins can be clearly identified and quantified by western blot detection of biotinylated proteins. ProB does not require the use of a reporter gene to demonstrate interaction, as is required in a membrane two hybrid assay (Petschnigg et al., 2014). Cells could be transfected with different combinations of viral glycoproteins in the presence and absence of receptor to determine the sequence of physical interaction among

glycoproteins. Adjusting the timing of the pulse of exogenous biotin could allow modification of the timing of the capture of the interactions.

2. Materials and Methods

2.1. Cells, antibodies, and plasmids

Chinese hamster ovary (CHO-K1) cells lack HSV-1 and EBV receptors and were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO₂. CHO-nectin-1 cells (Geraghty et al., 1998) stably express nectin-1, an entry receptor for HSV1. They are grown in the same media as CHO-K1 cells with 250 µg/mL G418 added during alternate passages. Trypsin-EDTA was used to detach these adherent cells.

The antibodies used include anti-HSV1 gD polyclonal antibody (PAb) R7 (Isola et al., 1989), anti-HSV1 gD 340–356 PAb (Eisenberg et al., 1985), anti-HSV1 gD monoclonal antibodies (MAbs) III-114 and III-174 ascites (Para et al., 1985), anti-EBV gB MAb CL55 (Wu, Borza, and Hutt-Fletcher, 2005), anti-EBV gB PAb (Garcia, Chen, and Longnecker, 2013), anti-hemagglutinin (HA) tag MAb 12CA5 hybridoma supernatant (Niman et al., 1983), anti-FLAG M2 MAb (Sigma F9291), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG PAb (GAM-HRP, Pierce PI32230), HRP-conjugated goat anti-rabbit IgG PAb HRP (GAR-HRP, Pierce PI32260), IRDye 800-conjugated goat anti-rabbit PAb (Licor, GAR-800), and IRDye 800-conjugated goat anti-mouse PAb (Licor, GAM-800). Protein A/G PLUS agarose (Santa Cruz SC2003) were used for immunoprecipitation and HRP-conjugated streptavidin (Pierce PI21140) was used to detect biotin.

Wild-type HSV1 glycoproteins were encoded in the pCAGGS vector (pPEP98 [HSV1 gB], pPEP99 [HSV1 gD], pPEP100 [HSV1 gH], and pPEP101 [HSV1 gL]) (Niwa, Yamamura, and Miyazaki, 1991; Pertel et al., 2001). Wild-type EBV gB, EBV gB767, and EBV gB816 were encoded in the pSG5 vector (Garcia et al., 2013). HSV1 gD-BirA, HSV1 gD-AP, HSV1 gD-APm, EBV gB-BirA, EBV gB-AP, and EBV gB-APm and were cloned into the pSG5 vector (Agilent). Using four-primer PCR, the AP and APm constructs were tagged on the C-termini of the glycoprotein cytoplasmic domains with an HA tag (YPYDVPDYASL), followed by a di-glycine linker and the AP or APm. The BirA constructs were tagged on the C-termini of the glycoprotein cytoplasmic domains with the BirA ligase, followed by a di-glycine linker and a FLAG tag (DYKDDDDK). Primer sequences used in cloning included cgggaattcaccatggcgccggctgccgcag,ggcacggtgttatcctcatgtaaaacaaggcggtgtgcg, and tcgggcacgtcgtagggttagtaaaacaaggcggtgtgcg for HSV1 gD constructs; cgggaattcaccatgactcggcgtaggggtgct,ggcacggtgttatcctcataaactcagtcctgcctccc,tcgggcacgtcgtagggttagtaaaactcagtcctgcctccc for EBV gB constructs; atgaaggataacaccgtgcc and gcggatcctactgtcgtcgtcgtcctttagtcacctccttttctgcactacgcaggg for BirA constructs; and taccctacgacgtgccccgactacgccagcctgggagggcgccctgaacgata,gcggatcctactcgtgccactcgtatcttgggcctcgaagatatcgttcaggccgcctc,gcggatcctagaactgcctcgtatcttgggcctcgaagatatcgttcaggccgcctc for AP and APm constructs. Template plasmid pECFP-N1-BirA-neurexin was obtained from Addgene (Thyagarajan and Ting, 2010). All cloned constructs were sequenced. Plasmid DNA was purified using the Zippy plasmid miniprep kit (Zymo Research).

2.2. Immunoprecipitation (IP) of wild-type HSV1 gD and EBV gB

CHO-K1 cells were seeded overnight at 1.3×10^6 cells/well in 6 well plates and transfected with vector and/or plasmids encoding wild-type HSV1 gD or EBV gB, using 2.2 μ g DNA (1.1 μ g per construct) and 7 μ L Lipofectamine 2000 (Invitrogen) per well in Opti-MEM (Gibco). After four hours, the transfection mixtures were replaced with Ham's F-12 media supplemented with 10% FBS. After an overnight incubation, cells were rinsed with PBS+ (phosphate buffered saline [PBS] containing 0.5 mM Mg^{2+} , 0.9 mM Ca^{2+}) and lysed in 450 μ L/well ice-cold mild lysis buffer (1% Triton X-100, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 20 mM Tris, pH 8). Protease inhibitors were added to the buffer immediately prior to use (1 μ g/mL leupeptin, 10 mM NaF, 1 mM $NaVO_3$, and 1 mM PMSF). Cell lysates were collected and insoluble debris was pelleted. Cell lysate supernatants were aliquoted into duplicate tubes holding 170 μ L lysate. For the HSV1 gD IP samples, 5 μ L each of III-114 and III-174 MAb ascites fluid was added to each tube. For the EBV gB IP samples, 5 μ L of CL55 MAb was added to each tube. Samples were rotated at 4°C for two hours and 15 μ L of a 50% slurry of protein A/G agarose was added to each sample for one hour at 4°C. Agarose was pelleted and washed three times with 1 mL cold mild lysis buffer. Each pellet was resuspended in 100 μ L of 2X SDS loading buffer (4% SDS, 350 mM β -mercaptoethanol). Samples were boiled for 5 minutes and pelleted. Duplicate polyacrylamide gels (Bio-Rad) were loaded with 40 μ L sample per well. Samples were separated by SDS-PAGE and transferred to 0.2 μ m nitrocellulose membranes (Bio-Rad). Blots were blocked in PBS with 1% bovine serum albumin (BSA). Blots were probed with either anti-HSV1 gD 340–356 PAb (1:2,000 dilution) or anti-EBV gB PAb (1:1,000 dilution) in PBS with 0.3% Tween (PBS-T) and 1% BSA. Blots were washed three times in PBS-T, probed with fluorescently-labeled GAR-800 (1:10,000 dilution) for an hour, washed three times in PBS-T, and imaged using an OdysseyFc imager (Licor).

2.3. Evaluation of total cell protein expression

CHO-K1 cells were seeded overnight at 3×10^5 cells/well in 24 well plates and transfected using 440 ng DNA and 1.4 μ L lipofectamine 2000 per well in Opti-MEM. After 3–5 hours, the transfection mixtures were replaced with Ham's F-12 media supplemented with 10% FBS. After an overnight incubation, cells were chilled and lysed using cold RIPA buffer (radioimmunoprecipitation assay buffer: 1% NaDOC, 1% TX100, 0.1% SDS, 10 mM Tris-Cl pH 7.4) including a protease inhibitor cocktail (Amresco M250). Cell lysates were collected and insoluble debris was pelleted. SDS loading buffer containing β -mercaptoethanol was added to the lysate supernatants and samples were boiled for 5 minutes. Samples were loaded on polyacrylamide gels and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and blots were blocked with 5% dry milk in PBS for 30 minutes at room temperature (RT) or overnight at 4°C. Blots were incubated for 2 hours at RT with anti-HSV1 gD 340–356 PAb (1:2,000 dilution) or anti-EBV gB PAb (1:1,000 dilution) in PBS-T with 5% milk. Blots were washed three times with PBS-T. GAR-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 chemiluminescent substrate (ECL, Pierce) and imaged using an HD2 imager (ProteinSimple).

2.4. Cell-based enzyme-linked immunosorbent assay (CELISA) for surface expression

CHO-K1 cells were seeded overnight at 6×10^4 cells/well in a 96-well plate. Triplicate wells were transfected with 110 ng/well of DNA using 0.35 μ L Lipofectamine 2000 per well (50 μ L/well total volume). After an overnight incubation at 37°C, the wells were blocked for 30 minutes at RT with PBS+BSA (PBS+ with 3% [wt/vol] BSA). Cells were incubated with anti-gD R7 PAb diluted 1:2,500 or anti-gB CL55 MAb diluted 1:4,000 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 GAR-HRP at 1:3,500 dilution or GAM-HRP at 1:2,000 dilution for 1 hour at RT. Expression was determined by adding Ultra TMB peroxidase substrate (Thermo Scientific) and measuring the absorbance at 670 nm on a Biotek Cytation 3 Cell Imaging Multi-Mode Reader.

2.5. Proximity biotinylation

CHO-K1 cells seeded overnight at 3×10^5 cells/well in 24 well plates were transfected with vector and/or plasmids encoding gD or gB constructs using Lipofectamine 2000 in Opti-MEM. Each well received 150–220 ng/construct (300–400 ng/well total DNA), as described in the figure legends. After 3–5 hours, the transfection mixtures were replaced with Ham's F-12 media supplemented with 10% FBS. After an overnight incubation, media was replaced by a solution of 20 μ M biotin freshly dissolved in PBS+. This biotin pulse at 37°C lasted 5–15 minutes, as indicated in the figure legends. After this pulse, cells were rinsed with PBS+ and immediately lysed in cold RIPA buffer containing a protease inhibitor cocktail.

Cells lysates were collected and insoluble debris was pelleted. Lysate supernatants were separated by SDS-PAGE using duplicate gels. Proteins were transferred to nitrocellulose and membranes were blocked with PBS with 1% BSA for 30 minutes at RT or overnight at 4°C. BSA was used as the blocking agent because milk can inhibit the biotin-avidin interaction. To detect biotinylated proteins, one membrane was incubated with streptavidin-HRP (diluted 1:7,000–10,000) in PBS-T with 1% BSA for 1 hour. To detect total protein expression, the duplicate membrane was incubated for 2 hours at RT with either anti-EBV gB PAb (diluted 1:1,000) or anti-HA MAb 12CA5 (diluted 1:2,000) in PBS-T with 1% BSA, as described in the figure legends. This membrane was washed three times in PBS-T and incubated with either GAR-HRP (1:10,000 dilution) or GAM-HRP (1:7,000 dilution) in PBS-T with 1% BSA for 1 hour at RT or overnight at 4°C. Both blots were washed three times with PBS-T and visualized using ECL as described in section 2.3. The duplicate blot then was stripped using two 7 minute washes in a mild stripping buffer (200 mM glycine, 3.5 mM SDS, 1% Tween-20, pH 2.2), followed by two rinses in PBS and two rinses in Tris-buffered saline with 0.1% Tween-20. Signal loss was confirmed by imaging the stripped blot using ECL. This stripped blot was re-probed for 2 hours at RT using either anti-gD 340–356 PAb (1:2,000 dilution) or anti-FLAG MAb (1:7,000 dilution) in PBS-T with 1% BSA, as described in the figure legend. The blot was washed in PBS-T, incubated with GAM-HRP or GAR-HRP as above, washed in PBS-T, and visualized using ECL.

2.6. Depletion of biotin from medium

Ham's F12 medium supplemented with 10% FBS and 1% PS was depleted of free biotin by adding of 50 µg/mL of soluble streptavidin (New England Biolabs 721). To determine if depletion affected endogenous biotinylation, CHO-K1 cells were transfected as described in section 2.5, but the transfection mix was removed after 3–5 hours and biotin-depleted media was added for the overnight incubation.

2.7. Proximity biotinylation with avidin pull-down

CHO-K1 or CHO-nectin-1 cells were seeded overnight in 6 well plates and transfected with 2.2 µg DNA/well total (1.1 µg DNA/construct) using 7 µL Lipofectamine 2000. Empty vector was used as needed to bring each transfection to 2.2 µg DNA total. After 5 hours, the transfection mixture was replaced with Ham's F12 medium supplemented with 10% FBS. After an overnight incubation, the media was replaced with 20 µM biotin in PBS+ for 5 minutes at 37°C. One sample received a mock pulse of PBS+ without biotin. Cells were rinsed with PBS+ and lysed in 400 µL/well of cold RIPA buffer containing protease inhibitors. For each lysate, 270 µL was reserved for the pull-down (see below). The remaining cell lysates were analyzed by SDS-PAGE by loading duplicate polyacrylamide gels with 40 µL lysate per lane and western blotting, as described in section 2.5. Anti-HA MAb 12CA5 (1:2,000 dilution) or anti-FLAG (1:7,000 dilution) MAbs were used as primary antibodies and fluorescently-labeled GAM-800 (1:10,000 dilution) was used as the secondary antibody. Blots were visualized using an OdysseyFc (Licor).

To pull-down the biotinylated proteins, 20 µL of a 50% slurry of neutravidin agarose beads (Thermo Scientific) were added to the 270 µL of cell lysate and samples were incubated for 2 hours at 4°C on a rotator. Beads were pelleted and washed three times with 1 mL cold RIPA buffer. Each pellet was resuspended in 45 µL of 2X SDS loading buffer (4% SDS, 350 mM β-mercaptoethanol). Samples were boiled for 5 minutes and pelleted. Samples (40 µL/well) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blot was blocked in Odyssey blocking solution (Licor) and probed with anti-HA MAb 12CA5 (1:2,000 dilution) to detect APm constructs. The proteins were visualized using GAM-800, as described above.

3. Results

3.1. Positive and negative ProB controls

HSV and EBV entry glycoproteins form multimers and thus detection of proximity between the monomers of a glycoprotein can be used a positive control for the ProB assay (Fig. 1C). Although HSV and EBV use a homologous set of glycoproteins to enter cells, the glycoproteins of EBV cannot substitute functionally for those of HSV and vice versa. Since glycoproteins from the two viruses do not interact functionally, they are not expected to interact physically, and thus they provide a negative control for the ProB assay. The crystal structures of HSV1 and EBV gB demonstrate a common fold for these homologous proteins; however, extensive sequence variation between the homologs should preclude the interaction of gB with heterotypic herpesvirus entry glycoproteins (Backovic, Longnecker, and Jardetzky, 2009; Heldwein et al., 2006). HSV1 gB and EBV gB share 29% identity and

43% amino acid similarity (Backovic et al., 2009). EBV does not encode a homolog for glycoprotein gD. EBV entry into B cells is triggered by gp42, which does not share sequence homolog with HSV1 gD (Mullen et al., 2002).

To confirm that HSV1 gD and EBV gB do not physically interact, co-immunoprecipitation of the two wild-type proteins was attempted (Fig. 2). CHO-K1 cells were transfected overnight with HSV1 gD and/or EBV gB alone or in combination. Cells were lysed in a mild detergent buffer and lysates were divided in half. HSV1 gD or EBV gB was precipitated from the duplicate samples using MAbs. Precipitated proteins were loaded onto gels for western blot analysis. Duplicate blots were probed with PABs specific for either HSV1 gD or EBV gB. HSV1 gD failed to co-precipitate with EBV gB (Fig. 2A lane 8) and EBV gB failed to co-precipitate with HSV1 gD (Fig. 2B, lane 4). The heavy chains of the MAbs specific for HSV1 gD migrate just below the HSV1 gD band (Fig. 2A).

To evaluate ProB as a viable method to detect interactions between the HSV1 glycoproteins necessary for entry, ProB constructs were cloned for HSV1 gD and EBV gB (Fig. 1B). The cytoplasmic tails of these proteins were linked to either biotin ligase (BirA) or its target acceptor peptide (AP or APm). APm has three amino acid substitution at the C-terminus of the AP (WHE to GEF) that was shown to reduce its affinity for BirA and reduce background signals (Fernandez-Suarez et al., 2008). The BirA-linked constructs have a C-terminal FLAG epitope tag and AP-linked constructs have a hemagglutinin (HA) epitope tag between the glycoprotein cytoplasmic domain and the AP. The AP was placed at the C-terminus to enhance its exposure to BirA.

3.2. Expression of HSV1 gD and EBV gB 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 using an anti-gD PAb anti-peptide 340–356. The wildtype HSV1 gD, gD-AP, and gD-APm migrated to 50 kDa, while gD-BirA migrated to 75 kDa (Fig. 3A), as expected. HSV1 gD-BirA was expressed at lower levels than wildtype gD.

Herpesviruses acquire the viral envelope and glycoproteins from the Golgi or trans-Golgi network (Johnson and Baines, 2011), but cell surface expression of the glycoproteins can be used to assess proper folding of the proteins. Cell surface expression of these gD proteins in CHO-K1 cells was determined by cell-based ELISA (CELISA). All the HSV1 gD constructs were present on the cell surface, suggesting that they were processed properly (Fig. 3B). 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. A comparison of these assays suggests that although overall gD-BirA expression is reduced, the protein is surface expressed preferentially.

The total cell expression of the EBV gB constructs also was detected by western blotting of transfected CHO-K1 cell lysates and CELISA of transfected CHO-K1 cells. Wild-type EBV gB is known to be expressed at the cell surface poorly, so two additional gB mutants (gB767 and gB816) that were shown previously to express at high levels on the cell surface were included as controls for the detection of gB expression (Garcia et al., 2013). 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. 3C). 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 (Garcia et al., 2013), wild-type gB expression on the cell surface was barely detectable (Fig. 3D). The ProB constructs also were detected on the cell surface poorly. As expected, the gB truncation mutants were expressed at higher levels on the cell surface, indicating that the assay was capable of detecting gB. The increase in surface expression for the gB tail mutants without a concurrent increase in total cell expression is consistent with previous reports (Garcia et al., 2013).

3.3. Initial ProB assay 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 μ M biotin for 15 minutes and lysed in 150 μ L RIPA buffer. The biotin is membrane-permeable and thus labeling can occur within the cell. Duplicate gels were loaded with 40 μ L lysate/lane and the proteins were separated by SDS-PAGE. Protein biotinylation was detected using streptavidin-HRP and protein expression was detected by western blot with antibodies specific for HSV1 gD or EBV gB. Coexpression of the homotypic pair HSV1 gD-BirA and HSV1 gD-AP resulted in strong biotinylation of the gD-AP target (Fig. 4A, lane 3, ~50 kDa). Coexpression of the homotypic pair EBV gB-BirA and EBV gB-AP also resulted in strong biotinylation of the gB-AP target (Fig. 4A, lane 8, ~140 kDa). Thus, proximity was detected in the homotypic positive controls

Unexpectedly, coexpression of the heterotypic pairings also resulted in biotinylation of the AP targets. Coexpression of HSV1 gD-BirA with EBV gB-AP resulted in biotinylation of gB-AP (Fig. 4A, lane 4, ~140 kDa); however, the biotinylation was weaker than that observed for homotypic gB-BirA and gB-AP expression (Fig. 4A, lane 8). The other heterotypic combination (EBV gB-BirA and HSV1 gD-AP) also showed a weak biotinylation of the gD-AP (Fig. 4A, lane 9, ~50 kDa). The band migrating just above 50 kDa in lanes 4 and 8 is probably a degradation product of the biotinylated EBV gB-AP, since it is present exclusively in samples containing gB-AP. Endogenous biotinylated cellular proteins appear at ~140 kDa and ~75 kDa in every lane, including those lacking any BirA construct.

Using the duplicate blot, gD-BirA, gD-AP, and wildtype gD expression was detected using a PAb specific for an HSV1 gD peptide. Both ProB gD constructs were expressed, but HSV1 gD-AP was expressed at a greater level than HSV1 gD-BirA (Fig. 4C), consistent with the previous expression results. Expression of EBV gB-BirA, AP, and wildtype was detected with a polyclonal antibody specific for EBV gB. Expression of both ProB gB constructs also is detected; however, EBV gB-BirA expression in the last two lanes is weak (Fig. 4B).

Results were similar whether the AP or APm constructs were used (Fig. 4D–F). The APm peptide differs from AP peptide in its three C-terminal residues and is reported to have a

lower affinity for BirA (Fernandez-Suarez et al., 2008). Unfortunately, the lower affinity of APm for BirA did not impact the heterotypic biotinylation observed.

For the ProB assay to serve as a useful tool to examine glycoprotein interactions, specific interactions (i.e. interactions driven by the candidate proteins and not an inherent affinity of BirA for AP) must be detected at greater levels than nonspecific interactions. In this assay, the homotypic combinations must result in greater biotinylation than the heterotypic combinations (Fig. 1C). In attempt to diminish detection of the heterotypic biotinylation, the biotin exposure time was lessened and amount of DNA transfected was reduced. A five minute exposure to 20 μ M biotin was determined to be sufficient for labeling (data not shown). Unfortunately, reducing the amount of DNA transfected to below 150 ng/well per construct in a 24-well plate resulted in protein expression levels that were too low to detect reliably (data not shown).

In this initial ProB trial, biotinylation was enhanced when gD-AP or gD-APm was coexpressed with homotypic gD-BirA rather than heterotypic gB-BirA. Similarly, biotinylation was enhanced when gB-AP or gB-APm was coexpressed with homotypic gB-BirA rather than heterotypic gD-BirA. Thus, the assay detected the homotypic interactions more readily than the heterotypic interactions, however a fair comparison of the homotypic versus heterotypic biotinylation requires a comparison of the expression levels both BirA constructs.

3.4. ProB assay optimization: detection using epitope tags

Epitope tags allow for direct comparison of heterotypic protein levels. Since both of the BirA enzyme constructs have FLAG tags and both of the APm substrate constructs have HA tags, the ProB assay was performed as previously using the HA or FLAG epitopes to assess protein expression levels. Since the AP and APm constructs gave similar results, assay optimization was conducted using APm constructs only. Reduced amounts of DNA (150 ng/construct) were used to enhance potentially the detection of specific interactions only. Biotinylation was apparent in the homotypic combinations for both gD (Fig. 5A, lane 2, ~50 kDa) and gB (Fig. 5A, lane 6, ~140 kDa). The heterotypic combinations revealed weaker but detectable biotinylation signals (Fig. 5A, lane 3 and 7). Although all of the APm and BirA constructs were expressed, the gB constructs were expressed at greater levels than their gD counterparts (Fig. 5B and 5C). The uneven expression of gD-BirA and gB-BirA is relevant to the interpretation of specificity of the assay.

In addition, reducing the amounts of DNA transfected decreased overall gD and gB expression levels. With these lower levels of expression, the endogenous biotinylated species that migrate to ~140 kDa and ~75 kDa in all lanes became more problematic. The ~140 kDa endogenous protein co-migrates with gB-APm and obscures its detection.

3.5. ProB assay optimization: endogenous biotin depletion

In attempt to decrease the impact of the endogenous biotinylated species, cells were depleted of free biotin by the addition of streptavidin (Fernandez-Suarez et al., 2008). The ProB assay was performed as previously, except streptavidin was added to the media after transfection and maintained for the duration of the overnight incubation prior to the biotin pulse. As with

the previous ProB trials, the homotypic combinations yielded biotinylation signals (Fig. 6, lanes 2 and 6). The heterotypic combination yielded weaker biotinylation signals than the homotypic interactions (Fig. 6, lanes 3 and 7). Unfortunately, depletion of the free biotin failed to decrease the signal from the endogenous protein that co-migrates with gB-APm.

3.6. ProB assay optimization: avidin pull-down

To reduce detection of the endogenous biotinylated species, biotinylated proteins were precipitated from the samples using avidin beads and the biotinylated APm proteins were detected by western blot using an antibody specific for the APm constructs. As previously, CHO-K1 cells were transfected with combinations of APm and BirA constructs overnight, pulsed with 20 μ M biotin for 5 minutes, and then lysed. Avidin beads were added to aliquots of the cell lysates to precipitate any biotinylated proteins. Precipitated proteins then were separated by SDS-PAGE, blotted, and probed with an anti-HA MAb. In parallel, total expression of the APm and BirA constructs was detected by loading cell lysates directly onto gels and western blotting using the epitope tag antibodies.

The addition of the avidin precipitation step eliminated the endogenous band that previously obscured the gB-APm band (Fig. 7A). As seen previously, both homotypic (lanes 3 and 6) and heterotypic (lanes 4 and 7) interactions were apparent. gB-APm was biotinylated at equivalent levels by both gB-BirA and gD-BirA (lanes 6 and 7), suggesting that this assay does not discern specific oligomeric interactions among gB monomers from nonspecific heterotypic interactions. gD-APm was biotinylated at approximately five-fold greater levels when co-expressed with its homotypic partner gD-BirA (lane 3) versus its heterotypic partner gB-BirA (lane 4). This may reflect that the assay detects gD oligomeric interactions more readily than nonspecific HSV1 gD-EBV gB interactions. Although this indicates a degree of specificity in the homotypic combination over the heterotypic combination, the overall strong biotinylation of the heterotypic partners provide evidence that the specificity of the assay is insufficient. All of the constructs were expressed as expected (Fig. 7B and C).

3.7. ProB assay in the presence of receptor

Crosslinking studies suggest that HSV1 gD is a dimer on the viral envelope (Handler et al., 1996b). Although the ectodomain of gD crystallizes as a monomer when expressed as a soluble protein (Carfi et al., 2001), a soluble gD dimer can be stabilized by adding a disulfide bond to the membrane-proximal end of ectodomain (Krummenacher et al., 2005). Crystal structures suggest that the membrane-proximal region that forms the gD dimerization face overlaps with the receptor binding site on gD (Carfi et al., 2001; Di Giovine et al., 2011; Krummenacher et al., 2005). To determine if the presence of an HSV entry receptor would influence the glycoprotein interactions, the ProB assay with avidin pull-down was repeated using cells that express the HSV entry receptor nectin-1, CHO-K1-nectin-1 (Fig. 8). As seen previously, both homotypic and heterotypic interactions were detected in the presence of nectin-1. The ratio of homotypic versus heterotypic biotinylation by gD-BirA was not changed notably by the presence of nectin-1.

4. Discussion

These experiments demonstrate that the ProB assay can detect the proximity of homotypic monomers within HSV1 gD or EBV gB oligomers. Unfortunately, biotinylation among heterotypic combinations, intended to serve as negative controls, was detected also. Using a lower affinity AP substrate (APm) and reducing the duration of the biotinylation pulse or the amount of DNA transfected did not eliminate the heterotypic biotinylation. Although very few endogenous proteins were biotinylated at levels sufficient to appear on a western blot, two species of endogenous biotinylated proteins did obscure the evaluation of gD and gB biotinylation. Overnight depletion of free biotin from the medium did not remove the endogenous biotinylated proteins, but precipitation of biotinylated proteins prior to western blotting for the APm constructs allowed unobstructed detection of the gD-APm and gB-APm biotinylated species.

Differences in gD versus gB expression impact the interpretation of specificity for this assay. Using epitope tags allowed direct comparison of expression of heterotypic BirA and APm constructs. To demonstrate the detection of specific interactions, the homotypic combinations tested ideally would have shown an indisputable increase in biotinylation compared to the heterotypic combinations. Although the gD homotypic interaction showed enhanced biotinylation (Fig. 7), biotinylation of the heterotypic combination remained strong. These results fail to demonstrate specificity of the proximity biotinylation assay in this context.

The proximity biotinylation assay has been used successfully to demonstrate specific interactions among several other protein pairings (Fernandez-Suarez et al., 2008; Kulyassov et al., 2011; Liu et al., 2013; Steel, Murray, and Liu, 2014; Thyagarajan and Ting, 2010). In fact, the assay recently has been adapted to select for novel interaction partners (BioID) (Kim et al., 2014; Lambert et al., 2014; Roux, Kim, and Burke, 2013; Roux et al., 2012). The reason this assay lacks sufficient specificity when detecting herpesvirus entry glycoprotein interactions may be the confounding fact that these entry glycoproteins are membrane-anchored. Restricting proteins to the essentially two-dimensional boundary of the membrane may increase the frequency of random collisions, some of which are sufficient for biotinylation to occur. While the proximity biotinylation assay may discern interactions among cytosolic proteins accurately, the restricted mobility of transmembrane proteins may increase the sensitivity of the assay and hinder its specificity. Nonspecific interactions among membrane-anchored virus entry proteins have been reported similarly using bimolecular complementation (Connolly et al., 2009). In addition, the glycans on these glycoproteins may contribute to nonspecific interactions. Although HSV1 gD and EBV gB do not co-immunoprecipitate (Fig. 2), the glycans may promote sufficient gD-gB contact to permit proximity biotinylation. This work emphasizes the need to control for false positive interactions when using proximity biotinylation to examine interactions among membrane-anchored glycosylated proteins.

5. Conclusions

The proximity biotinylation assay offers several advantages over other protein-protein interaction assays, especially its ability to mark a transient interaction in live cells permanently by attaching biotin covalently. The assay must be designed carefully, with special consideration for negative controls and avoiding false positive results. Proteins that are tethered to a membrane, including herpesvirus entry glycoproteins, present a higher risk of false positive results. This assay is limited in its utility for detecting specific protein-protein interactions of membrane-anchored proteins and is unlikely to be useful for studying herpesvirus entry glycoprotein interactions.

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Abbreviations

HSV	herpes simplex virus
EBV	Epstein Barr virus
ProB	proximity biotinylation
BirA	biotin ligase enzyme
AP	acceptor peptide
APm	acceptor peptide mutated
CHO-K1	Chinese hamster ovary-K1 cells
SDS	sodium dodecyl sulfate
BSA	bovine serum albumin
PBS	phosphate buffered saline
PBS+	PBS with magnesium and calcium
PBS-T	PBS with tween
PBS+BSA	PBS with magnesium, calcium and BSA
RT	room temperature
ECL	enhanced chemiluminescence reagent
CELISA	cell-based enzyme-linked assay
FBS	fetal bovine serum
IP	immunoprecipitation
RIPA	radioimmunoprecipitation assay

HA	hemagglutinin
YFP	yellow fluorescent protein
PAb	poyclonal antibody
MAb	monoclonal antibody
GAR	goat-anti-rabbit
GAM	goat-anti-mouse
HRP	horseradish peroxidase
BiFC	bimolecular fluourescence complementation

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Highlights

- Proximity biotinylation detects proximity between herpesvirus entry glycoproteins.
- Specificity of the proximity biotinylation assay is an important concern.
- Proximity biotinylation is limited for examining membrane-anchored interactions.

Fig. 1.

Proximity biotinylation (ProB) uses an enzyme and substrate pair to examine protein-protein interactions. (A) One candidate protein is tagged with the *Escherichia coli* biotin ligase BirA and another candidate protein is tagged with the BirA substrate, a 15 amino acid acceptor peptide (AP). If an interaction occurs between the two candidate proteins, BirA will covalently ligate biotin (star) to the AP. Labeling occurs in live cells and the proteins are free to dissociate after biotinylation. The four HSV1 glycoproteins (gD, gH/gL, and gB) that are necessary and sufficient for HSV1 fusion are shown. In this example, gD-BirA biotinylates a gH-APm target. Biotinylation of an unrelated construct (such as EBV gB-APm) would not be expected. (B) Schematic representation of HSV1 gD and EBV gB constructs created. BirA, AP, or APm were added to the cytoplasmic tails of the glycoproteins. APm is a mutant form of AP with lower affinity for BirA. The dark blue and green boxes represent the transmembrane domains. BirA-linked constructs include a C-terminal FLAG epitope tag. AP- and APm-linked constructs include a HA epitope tag upstream of the AP or APm. (C) Heterotypic and homotypic ProB construct combinations. Coexpression of heterotypic glycoproteins is not expected to result in biotinylation (negative control on left), but biotinylation (red stars) within homotypic oligomers of gD or gB is expected (positive controls on right).

Fig. 2.

Immunoprecipitation of HSV gD and EBV gB. CHO-K1 cells were transfected with plasmids encoding HSV1 gD or EBV gB and/or empty vector, as noted above the lanes. Cells were lysed in mild buffer and lysates were immunoprecipitated using a mixture of MAbs III-114 and III-174 that are specific for HSV-1 gD (four left lanes) or MAb CL55 that is specific for EBV gB (four right lanes). Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with PAb sera specific for HSV1 gD (top panel) or EBV gB (bottom panel). Molecular weight markers are shown on the left. The asterisk denotes HSV1 gD and the arrowhead denotes EBV gB.

Fig. 3.

Expression of HSV1 gD and EBV gB constructs. (A) Lysates of CHO-K1 cells transfected overnight with the gD constructs or empty vector were resolved by SDS-PAGE. Proteins were blotted and probed with anti-HSV1 gD 340–356 PAb. Molecular weight markers are indicated in kDa. (B) CHO-K1 cells were transfected overnight with the gD constructs or empty vector and intact cells were probed for cell surface expression of gD using PAb R7. Absorbance at 670 nm was measured after the addition of GAR-HRP followed by HRP substrate. Raw data are shown with standard deviations. (C) Lysates of CHO-K1 cells transfected overnight with the gB constructs or empty vector were resolved by SDS-PAGE. Proteins were blotted and probed with anti-EBV gB PAb. (D) CHO-K1 cells were transfected overnight with the gB constructs or empty vector and intact cells were probed for

cell surface expression of gB using MAb CL55. Absorbance at 670 nm was measured after the addition of GAM-HRP followed by HRP substrate. The raw data average of three samples is shown with standard deviations.

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Fig. 4.

Proximity biotinylation trial. (A–C) CHO-K1 cells in 24-well plates were transfected overnight with wild-type HSV1 gD, HSV1 gD-BirA, HSV1 gD-AP, wild-type EBV gB, EBV gB-BirA, EBV gB-AP, and/or empty vector, as listed above each lane. Wells were transfected with 220 ng per construct and empty vector was used as needed to bring the total DNA added to each well to 440 ng. Cells were pulsed with 20 μ M biotin for 15 min and then lysed. Cell lysates were analyzed by SDS-PAGE and duplicate blots were generated. (A) Biotinylated proteins were detected by probing with streptavidin-HRP. The arrowhead denotes biotinylated EBV gB-APm and the asterisk denotes a doublet of biotinylated HSV1 gD-APm. (B) Total EBV gB expression was detected by probing a duplicate blot with anti-gB PAb. (C) The blot from part B was stripped and re-probed with anti-gD 340–356 PAb to detect total HSV1 gD expression. Molecular weight markers are indicated on the left in kDa. The arrowheads denote EBV gB constructs and the asterisks denote HSV1 gD constructs. (D–F) The experiment above was repeated, substituting APm constructs for the AP constructs, as listed above each lane.

Fig. 5.

ProB assay optimization using epitope tags. CHO-K1 cells in 24-well plates were transfected overnight with HSV1 gD-APm, HSV1 gD-BirA, EBV gB-APm, EBV gB-BirA, and/or empty vector. Wells were transfected with 150 ng per construct, as listed above each lane. Empty vector was used as needed to bring the total DNA added to each well to 300 ng. Cells were pulsed with 20 μ M biotin for 5 min and then lysed. Cell lysates were analyzed by SDS-PAGE and duplicate blots were generated. (A) Biotinylated proteins were detected by probing with streptavidin-HRP. (B) gD-APm and gB-APm expression was detected by probing a duplicate blot with anti-HA tag 12CA5 MAb. (C) The blot from part B was stripped and re-probed with anti-FLAG M2 MAb to detect gD-BirA and gB-BirA expression. Molecular weight markers are indicated on the left in kDa. For each blot, the arrowhead denotes EBV gB and the asterisk denotes HSV1 gD.

Fig. 6.

ProB assay with depletion of free biotin from the media. CHO-K1 cells in 24-well plates were transfected with HSV1 gD-APm, HSV1 gD-BirA, EBV gB-APm, EBV gB-BirA, and/or empty vector. Wells were transfected with 150 ng per construct, as listed above each lane. Empty vector was used as needed to bring the total DNA added to each well to 300 ng. After 3 hours, the transfection mixture was replaced with medium supplemented with 10% FCS and 50 μ g/mL soluble streptavidin to bind endogenous biotin. After an overnight incubation in this streptavidin-containing medium, cells were pulsed with 20 μ M biotin for 5 min and then lysed. Cell lysates were analyzed by SDS-PAGE and blotted. Biotinylated proteins were detected by probing with streptavidin-HRP. The arrowhead denotes biotinylated EBV gB-APm and the asterisk denotes biotinylated HSV1 gD-APm. Molecular weight markers are indicated on the left in kDa.

Fig. 7.

ProB assay with avidin pull-down. CHO-K1 cells in 6-well plates were transfected overnight with gD-APm, gD-BirA, gB-APm, gB-BirA, and/or empty vector. Each well received 1.1 μ g DNA per construct, as listed above each lane. Empty vector was used as needed to bring the total DNA added to each well to 2.2 μ g. Cells were pulsed with 20 μ M biotin for 5 min and then lysed. Lane 1 received a mock pulse lacking biotin. Lysates were divided for biotin pull-down (A) or direct loading onto a gel (B and C). (A) Biotinylated proteins were precipitated from the lysates using neutravidin-agarose beads. Precipitated proteins were separated by SDS-PAGE and blotted. APm constructs were detected by probing with 12CA5 MAb. (B and C) Total cell lysates were analyzed by SDS-PAGE and duplicate gels were

blotted. Total gD-APm and gB-APm was detected by probing anti-HA 12CA5 MAb. Total gD-BirA and gB-BirA was detected by probing with anti-FLAG M2 MAb. The arrowheads denote EBV gB constructs and the asterisks denote HSV1 gD constructs. Molecular weight markers are indicated on the left in kDa.

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Fig. 8.

ProB with avidin pull-down in cells expressing the nectin-1 receptor. CHO-nectin-1 cells in 6-well plates were transfected overnight with gD-APm, gD-BirA, gB-APm, gB-BirA, and/or empty vector. Each well received 1.1 μ g DNA per construct, as listed above each lane. Empty vector was used as needed to bring the total DNA added to each well to 2.2 μ g. Cells were pulsed with 20 μ M biotin for 5 min and then lysed. Lane 1 received a mock pulse lacking biotin. Lysates were divided for biotin pull-down (A) or direct loading onto a gel (B and C). (A) Biotinylated proteins were precipitated from the lysates using neutravidin-agarose beads. Precipitated proteins were separated by SDS-PAGE and blotted. APm constructs were detected by probing with 12CA5 MAb. (B and C) Total cell lysates were analyzed by SDS-PAGE and duplicate gels were blotted. Total gD-APm and gB-APm was detected by probing anti-HA 12CA5 MAb. Total gD-BirA and gB-BirA was detected by probing with anti-FLAG M2 MAb. The arrowheads denote EBV gB constructs and the asterisks denote HSV1 gD constructs. Molecular weight markers are indicated on the left in kDa.