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Cross Talk among the Glycoproteins Involved in Herpes Simplex Virus Entry and Fusion: the Interaction between gB and gH/gL Does Not Necessarily Require gD▽

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The gD, gB, and gH/gL glycoprotein quartet constitutes the basic apparatus for herpes simplex virus (HSV) entry into the cell and fusion. gD serves as a receptor binding glycoprotein and trigger of fusion. The conserved gB and gH/gL execute fusion. Central to understanding HSV entry/fusion has become the dissection of how the four glycoproteins engage in cross talk. While the independent interactions of gD with gB and gD with gH/gL have been documented, less is known of the interaction of gB with gH/gL. So far, this interaction has been detected only in the presence of gD by means of a split green fluorescent protein complementation assay. Here, we show that gB interacts with gH/gL in the absence of gD. The gB-gH/gL complex was best detected with a form of gB in which the endocytosis and phosphorylation motif have been deleted; this form of gB persists in the membranes of the exocytic pathway and is not endocytosed. The gB-gH/gL interaction was detected both in whole transfected cells by means of a split yellow fluorescent protein complementation assay and, biochemically, by a pull-down assay. Results with a panel of chimeric forms of gB, in which portions of the glycoprotein bracketed by consecutive cysteines were replaced with the corresponding portions from human herpesvirus 8 gB, favor the view that gB carries multiple sites for interaction with gH/gL, and one of these sites is located in the pleckstrin-like domain 1 carrying the bipartite fusion loop.

Entry of herpes simplex virus (HSV) into the cell requires a multipartite apparatus made of a quartet of viral glycoproteins, gD, gB, and the heterodimer gH/gL, and a multistep process that culminates in the fusion of the virion envelope with cell membranes (5, 6, 10, 25, 36, 41). gD serves as the receptor-binding glycoprotein, able to interact with alternative receptors, nectin1, herpesvirus entry mediator (HVEM) and, in some cells, modified heparan sulfate (9, 13, 30, 39). It can also be engineered to accept heterologous ligands able to interact with selected receptors present on tumor cells and thus represents a tool to redirect HSV tropism (21, 28, 29, 42). The heterodimer gH/gL and gB execute fusion and constitute the conserved fusion apparatus across the *Herpesviridae* family. gB structure in the postfusion conformation shows a trimer with a central coiled coil (19). gH shows elements typical of type 1 fusion glycoproteins, in particular, helices able to interact with membranes, and two heptad repeats potentially able to form a coiled coil (12, 15–18). The discovery that a soluble form of gD enables entry of gD-null virions revealed that gD serves the additional function of triggering fusion and led to the view that the major roles of gD are to sense that virus has reached a receptor-positive cell and to signal to gB and gH/gL that fusion is to be executed (8). Biochemical and structural analyses showed that the C-terminal region of the gD ectodomain, containing the profusion domain required for fusion but not for receptor binding, can undergo major conformational changes (11, 24). Specifically, it binds the gD core and masks or hinders the receptor binding sites, conferring upon the molecule a closed, auto-inhibited conformation (24). Alternatively, it may unfold, conferring upon gD an open conformation. It was proposed that the C terminus of gD unfolds from gD core at receptor binding and recruits gH/gL and gB to a quaternary complex. A key feature of the model was that complexes among the glycoprotein quartet were not preformed, but, rather, they would assemble at the onset of or at fusion execution.

Central to understanding HSV entry/fusion has become the dissection of the interactions that occur among the members of the glycoprotein quartet and their significance to the process. A first evidence of a gD-gH/gL interaction was provided in coimmunoprecipitation studies (35). Interactions between gD and gH/gL and between gD and gB were subsequently detected by split green fluorescence protein (GFP) complementation assays, implying that gD can recruit gB and gH/gL independently of one another, a result that argues against a stepwise recruitment of the glycoproteins to gD. In agreement with the proposed model, the interaction between gH/gL and gB was detected in the presence of transfected or soluble gD (1, 2). However, further studies highlighted levels of complexity not foreseen in the initial model. Thus, pull-down analyses showed that the interaction sites in gD with gB and with gH/gL lie in part outside the C-terminal portion of the gD ectodomain, that resting virions contain small amounts of gD in complex with gB and with gH/gL prior to encountering cells, and that de novo gD-gB complexes were not detected at virus entry into the cell (14).

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A major objective of current studies was to analyze the interaction of gB with gH/gL. We documented the interaction by two independent assays, i.e., by a complementation assay of split yellow fluorescent protein Venus (herein indicated as YFP) (31) in whole cells and, biochemically, by a pull-down assay. The latter was applied recently in our laboratory and is based on the ability of One-Strep-tagged proteins (e.g., gH) to specifically absorb to Strep-Tactin resin and thus retain any protein in complex (14). To preliminarily search for gB regions critical for the interaction with gH/gL, we engineered chimeric forms of HSV-1 and human herpesvirus 8 (HHV-8) gB in which the cysteines were preserved. While none of the chimeras was completely defective in the interaction, the interactions in the chimeras carrying substitutions in the pleckstrin-like domain 1—the domain that carries the bipartite fusion loops—were hampered. Altogether, the results underscore the ability of gB to interact with gH/gL in the absence of gD and favor the view that sites in gB for interaction with gH/gL involve multiple contacts, one of which is located in the domain that carries the fusion loops.

MATERIALS AND METHODS

Cells. COS-7 and 293T cells were grown in Dulbecco's modified Eagle minimal essential medium containing 5% fetal bovine serum. Cells were transfected 16 to 20 h after seeding when they reached about 80% confluence.

Antibodies. Monoclonal antibodies (MAbs) 30 and H170 to gD, H1817 and H233 to gB, 53S and H12 to gH, and H633 to gC were described previously (4, 20, 22, 33, 34, 38). MAb to the V5 epitope was from Invitrogen (Milano). Polyclonal antibody to gH/gL was a gift from H. Browne (University of Cambridge, United Kingdom).

Construction of YFP chimeric glycoproteins. The chimeras made of HSV glycoproteins plus YFP N- or C-terminal fragments were derived from the previously described chimeras containing the enhanced GFP (EGFP) fragments (2) by replacing the EGFP fragments with the corresponding YFP fragments. Inasmuch as YFP (31) carries a valine insertion at position +2, the ATG codon of YFP is usually referred to as −1, relative to the GFP start codon. The YFP N and C fragments spanned amino acids (aa) −1 to 157 and 158 to 238, respectively. They were PCR amplified from plasmid Venus/pCS2, kindly provided by Atsushi Miyawaki (Hirosawa, Wako-city, Japan). The amplimers were cloned in the glycoproteins' endodomain, 1 to 10 aa upstream of the stop codon, following insertion of appropriate restriction sites by site-directed mutagenesis (2). $gB_{\Delta 867}$ (herein designated gB_{Δ}) carries a stop codon at aa 867, which functionally removes the endocytosis motifs and phosphorylation site (3). The chimeras were designated gB_C, gH_N, gC_C, $gB_{\Delta C}$, where the N and C subscripts identify the YFP fragments. For gH_N the YFP N amplimer was amplified by means of oligonucleotides 5′-TTTGCATGCTTATGGTGAGCAAGGGCGAGGAGCTGGT-3′ (forward) and 5′-GGGAGATCTCTACTGCTTGTCGGCGGTGATATAGAC GTTG-3′ (reverse) and ligated into SphI/BglII-predigested gH plasmid. For $gB_{\Delta C}$, the YFP C amplimer was amplified by means of oligonucleotides 5′-CC CAGATCTAAGAACGGCATCAAGGCCAACTTCA-3′ (forward) and 5′-GG GAAGCTTTTACTTGTACAGCTCGTCCATGCCGAG A-3′ (reverse) and ligated into BglII/HindIII-predigested gB_{Δ} plasmid. For full-length gB_C, the EcoRV restriction site was inserted in the plasmid encoding full-length gB in pcDNA3.1 (pcDNA-gB) by site-directed mutagenesis in place of the stop codon by means of oligonucleotide 5′-GAGGACGACCTGGATATCGGGGTTTGTT G-3′. The YFP C amplimer was generated with oligonucleotides 5′-GCCGAC GATATCAAGAACGGCATCAAGGCCAAC-3′ (forward) and 5′-GGGAAG CTTTTACTTGTACAGCTCGTCCATGCCGAGA-3′ (reverse) and then ligated into EcoRV-HindIII-digested pcDNA-gB. For gC_C the YFP C amplimer was amplified by means of oligonucleotides 5′-CCCGCTAGCTAAGAACGGC ATCAAGGCCAACTT CA-3′ (forward) and 5′-GGGAGATCTTACTTGTAC AGCTCGTCCATGCCGAGA-3′ (reverse) and ligated into NheI/BglII-digested gC plasmid.

Construction of $gB_{H1/H8}$ chimeras. The backbone of the HSV-1/HHV-8 gB ($gB_{H1/H8}$) chimeras was a form of gB_{Δ} (3) in which the lysine-rich tract spanning aa 68 to 77 (ATG; aa 1) was replaced with the epitope recognized by MAb 5E1 ($gB_{\Delta 867\text{-}5E1}$). BamHI and EcoRI restriction sites were engineered by site-directed mutagenesis at aa 68 and 77 of pcDNA-gB_{Δ}, respectively, by means of the oligonucleotides 5′-CCAACGGGGGACACGGATCCGAAGAAGAACAA A-3′ and 5′GAACAAAAAACCGAATTCCCCACCGCCGCCGCGCCC-3′. The BamHI/EcoRI intervening fragment was replaced with the 5E1 epitope (40), generated by extension of two partially overlapping synthetic oligonucleotides: 5′-CTATTCGGATCCGAGTCGACCAGGAAGCACTACACCCTCTGGGA ACTCTGCAAGATATGGG-3′ and 5-AGTAATGAATTCGGAGTTATACTT CTAGGTGTGTTATTCCCATATCTTGCAGAGTTCCCAG-3′. The DTKPK KNKKPKNPP sequence between aa 66 and 79 in wild type gB (wt-gB) became DTDPSRPGSTTPSGNSARYGNNTPRSITPNSPP in $gB_{\Delta 867\text{-}5E1}$, including the modifications introduced by the restriction sites.

In the constructs named $gB_{1\text{-}3(H8)}$, $gB_{3\text{-}4(H8)}$, $gB_{4\text{-}5(H8)}$, $gB_{5\text{-}6(H8)}$, $gB_{6\text{-}7(H8)}$ the HHV-8 gB sequence replaced the endogenous HSV-1 gB sequence between the pair of cysteines 1 and 3, 3 and 4, 4 and 5, 5 and 6, and 6 and 7, respectively. Each construct was generated by a two-step procedure, as follows; first, the appropriate restriction sites were inserted adjacent to the selected pair of cysteines (cysteines were preserved); second, the HHV-8 gB fragment bracketed by the selected pair of cysteines was generated by PCR amplification and ligated into the predigested gB plasmid. To exemplify the procedure, in the case of $gB_{1\text{-}3(H8)}$, the HindIII and XbaI restriction sites were inserted at aa 112 and 217 of HSV-1 gB; the primers for insertion of the restriction sites by site-directed mutagenesis were 5′-GAACACCGATGCAAGCTTTTACGTGTGCCC-3′ and 5′-GTACGTGCGCAACAATCTAGAGACCACCGCGTT-3′, respectively; the oligonucleotides for amplification of the appropriate HHV-8 gB sequence were 5′-GACTTTTAAAGCTTCAGAGTGTGTAGTGC-3′ and 5′-GTCAGTAAAT GTGTTTTCTAGACCGTTG-3′. The other $gB_{H1/H8}$ chimeras were engineered following a similar procedure; the primers used and the relevant coordinates are given in Table 1.

Immunofluorescence assay (IFA). Subconfluent cultures of 293T or COS-7 cells, grown on glass coverslips in 24-well plates, were transfected with 300 to 400 ng of plasmid DNA for each plasmid by means of Arrest-In (Open Biosystem, Celbio, Milan, Italy). At 20 to 48 h after transfection cells were fixed in methanol or 3% paraformaldehyde (neutralized with 1 M glycine) and permeabilized or not with 0.1% Triton X-100. The indicated MAbs were diluted 1:400 in phosphate-buffered saline containing 20% newborn calf serum. Secondary fluorescein isothiocyanate-conjugated or rhodamine-conjugated immunoglobulins (Jackson Laboratories, Soham, Cambridgeshire, United Kingdom) were diluted 1:500. Samples were observed after they were mounted with Fluoromount (Southern Biotech, Hatfield PA) by means of a Nikon Eclipse 90i Microscope equipped with a Nikon digital camera and Nis-Elements Ar (version 2.30) software for image acquisition or with an Axioplan Zeiss microscope equipped with a Kodak DC120 digital camera and Kodak Digital Science 1D LE, version 3.0, software.

Split YFP complementation assay. 293T and COS-7 cells in 24-well dishes were cotransfected by means of Arrest-In (Celbio, Milan, Italy), usually with combinations of five plasmids (125 to 150 ng of each plasmid/well; maximum amount of transfected DNA, 750 ng/well), as detailed previously (2). The plasmids included expressed either the combination gH_N, gB_C, wt-gL, and wt-gC with or without wt-gD or the combination gH_N, gC_C, wt-gL, and gB, with or without wt-gD. When gD was omitted, the transfection mixture contained a plasmid encoding epidermal growth factor receptor 2 with a deletion of signaling sequences (37) to equal the amount of transfected DNA in the other combinations. To evaluate the effect of brefeldin A (BFA), COS-7 cells were cotransfected with 125 ng/well of each plasmid for 4 h in serum-free medium and thereafter exposed for 24 h to BFA (1 μg/ml) (Sigma Aldrich, Milan, Italy) diluted in Dulbecco's modified Eagle's medium containing 2.5% fetal bovine serum. At 20 h (293T) or 40 h (COS-7) after transfection, cells were mounted with Fluoromount, with or without prior fixation, and observed with a Leica TCS-SL confocal microscope, set at 100% excitation at 514 nm with emission between 530 and 570 nm. Images were collected with a 63× Leica oil immersion objective (numerical aperture, 1.62); confocal slices were 1.5 to 2.0 μm thick. For each experimental series, images were collected on the same day, under the same settings, applying 1,024-by 1,024-pixel resolution and an 8-bit intensity scale. As detailed elsewhere (2), the first sample to be observed in a series was the gC_C-containing negative one; in subsequent observations of samples belonging to the same series, the settings were not modified. Observations shown in Fig. 4 were carried out with a Nikon Eclipse 90i Microscope.

Pull-down experiments by means of gH_{st}. The ability of One-Strep-tagged gH (gH_{st}) to pull down other glycoproteins was assayed in cells transfected with a mixture of plasmids encoding gH_{st}, gL carrying the V5 epitope (gL_{V5}), $gB_{\Delta 867\text{-}5E1}$, or one of the $gB_{H1/H8}$ chimeras, in the presence or absence of gD and HVEM, essentially as described previously (14). gH_{st} (which also carries the V5 epitope) and gL_{V5} were described previously (14). In the negative control mixture, gH_{V5} replaced gH_{st}. Plasmid encoding epidermal growth factor receptor 2 was used to make the amounts of transfected DNAs equal. In all cases, two T25

TABLE 1. Oligonucleotides and primers used in this study to generate $gB_{H1/H8}$ chimeras

Chimera	Oligonucleotide pair for site-directed mutagenesis of HSV-1 gB	Inserted restriction sites	Position of replaced HSV-1 gB fragment (aa)	Primer pair for generation of HHV-8 gB amplimer	Position of inserted HHV-8 gB fragment (aa)
$gB_{1-3(H8)}$	5′-GAACACCGATGCAAGCTT TTACGTGTGCCC-3′	HindIII, XbaI	112–217	5′-TTTTAAAGCTTCAGAGTGTGTAGTGC-3′	65–167
	5′-GTACGTGCGCAACAATCT AGAGACCACCGCGTT-3′			5′-AGTAAATGTGTTTCTAGACCGTTG-3′	
$gB_{3-4(H8)}$	5′-GTACGTGCGCAACAATCT AGAGACCACCGCGTT-3′	XbaI, HpaI	217–270	5′-CAACGGTCTAGAAAACACATTTACTGAC-3′	169–221
	5′-GTACGGGACGACGGTTA ACTGCATCGTCG-3′			5′-AACGGTGGTCCAACTCTGTATATGCC-3′	
$gB_{4-5(H8)}$	5′-GTACGGGACGACGGTTA ACTGCATCGTCG-3′	HpaI, Asp718	271–361	5′-CCGTTAACTGCGAGATAGTCGACATGAT-3′	223–311
	5′-TCATGGTGCAGAGGTACC GGCGCTTTGGCAC-3′			5′-CACAGGTACCTGGTCTTGCTCTCGGA-3′	
$gB_{5-6(H8)}$	5′-TCATGGTGCAGAGGTACC GGCGCTTTGGCAC-3′	Asp718, XhoI	364–412	5′-GACCAGGTACCTGTGTCCGCTAGCACTGT GG-3′	316–361
	5′-CCTGGGGGACTGCCTCGA GAAGGACGCCCGCGA-3′			5′-CCGACTCGAGACAAGAGTACGTGTCGGTA AA-3′	
$gB_{6-7(H8)}$	5′-CCTGGGGGACTGCCTCGA GAAGGACGCCCGCGA-3′	XhoI, EcoRV	365–485	5′-CTCTTGTCTCGAGTCGGATATCAACAC-3′	412–529
	5′-CGCGTGGTGCGATATCCA GAATCACGAGCTG-3′			5′-GTCCCTGACCTGAGGCCTACACCAT-3′	

flasks of 293T cells (~2.8×10^6 cells/T25 flask) were transfected for each sample by means of Arrest-in. Each plasmid was transfected in the amount of 1.5 μg of DNA/T25 flask (3 μg for HVEM). Eighteen to 24 h after transfection, cells were solubilized without freezing in EA1plus buffer (250 mM NaCl, 50 mM HEPES, and 0.1% Nonidet P-40, pH 8) (14, 35) containing the protease inhibitors Nα-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride and Nα-*p*-tosyl-L-phenylalanine chloromethyl ketone (0.3 mM each) and centrifuged at 14,000 rpm for 1 h. The supernatants were cleared by absorption to protein A-Sepharose resin (Sigma Aldrich, Milan, Italy) for 1 h at 4°C. The protein complexes present in the cleared supernatant were allowed to absorb to Strep-Tactin Sepharose (IBA GmbH, Gottingen, Germany) for 1 h at 4°C with gentle mixing. The beads were washed five times with buffer W (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA), and the absorbed proteins were eluted by means of sample buffer containing 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 50 mM Tris-HCl, pH 7, and 2.5% sucrose. The eluted proteins were separated by denaturing polyacrylamide gel electrophoresis (PAGE) and transferred to Hybond ECL (enhanced chemiluminescence) nitrocellulose membrane (GE Healthcare, Milan, Italy). The membranes were blocked for 1 h at room temperature with ECL Advance Blocking Agent (GE Healthcare, Milan, Italy) and allowed to react overnight with the indicated antibody, diluted 1:20,000, followed by anti-mouse peroxidase-conjugated antibody (1:80,000) (GE Healthcare). The blot was developed by an ECL Advanced Western Blotting Detection Kit (GE Healthcare), according to manufacturer's instructions.

RESULTS

Construction and characterization of HSV glycoproteins carrying YFP fragments. The N-terminal (aa −1 to 157) or C-terminal (aa 158 to 238) fragments of YFP (31) were cloned 1 to 10 aa upstream of the stop codon of gB, gH, gC, or gB_{Δ}. The resulting chimeras were designated gB_C, gH_N, gC_C, $gB_{\Delta C}$. Briefly, the site for N or C insertion in the glycoprotein endodomain was the same as that used previously to insert the EGFP fragments (2). Previous studies indicated that insertion at these sites did not interfere with the glycoprotein activities (2), a feature documented also in this study as the ability of the transfected glycoproteins to form syncytia (see Fig. 2). gB_{Δ} has a deletion of endocytosis motifs and of a US3 phosphorylation site located in the endodomain downstream of aa 867 to maximize its persistence in the exocytic compartment and plasma membranes (3). The electrophoretic mobility of the chimeric $gB_{\Delta C}$, gB_C, gH_N, and gC_C glycoproteins in 293T cells is shown in Fig. 1A and was clearly decreased relative to that of the respective wt forms. The cellular localization of the chimeric glycoproteins was analyzed by IFA. The example shown in Fig. 1B for COS-7 cells (which provide better microscopic images than 293T cells) was typical of glycoproteins. All chimeric glycoproteins reached the plasma membrane, as detected in paraformaldehyde-fixed cells (Fig. 1B). gB_C was present at the plasma membrane only in small amounts, in accordance with its ability to be retrieved to endosomal vacuoles.

Construction and characterization of $gB_{H1/H8}$ chimeras. To carry out preliminary mapping studies of gB regions critical to the interaction with gH/gL, we engineered a panel of $gB_{H1/H8}$ chimeras. The rationale for the construction relied on the consideration that the gB structure is influenced overall by cysteines and that cysteine spacing is conserved across gB orthologs. In the $gB_{H1/H8}$ chimeras, the region of HSV-1 gB bracketed by consecutive cysteines was replaced with the corresponding region from HHV-8 gB. The backbone for the $gB_{H1/H8}$ chimeras was HSV-1 gB_{Δ} in which the lysine-rich tract spanning aa 68 to 77 (amino acid numbering includes the signal sequence) was replaced with the epitope recognized by MAb 5E1 (40). In order to replace the HSV-1 gB portion bracketed by a cysteine pair with the corresponding region from HHV-8 gB, restriction sites were engineered adjacent to the selected pair of cysteines. The HSV-1 gB sequence bracketed by the two engineered restriction sites was removed and replaced with an amplimer generated on the corresponding region of HHV-8 gB. Thus, in the constructs $gB_{1-3(H8)}$, $gB_{3-4(H8)}$, $gB_{4-5(H8)}$, $gB_{5-6(H8)}$, and $gB_{6-7(H8)}$, the HHV-8 gB sequence replaced the corresponding portion of HSV-1 gB between paired cysteines (cysteines 1 and 3, 3 and 4, 4 and 5, 5 and 6, 6 and 7, respectively)

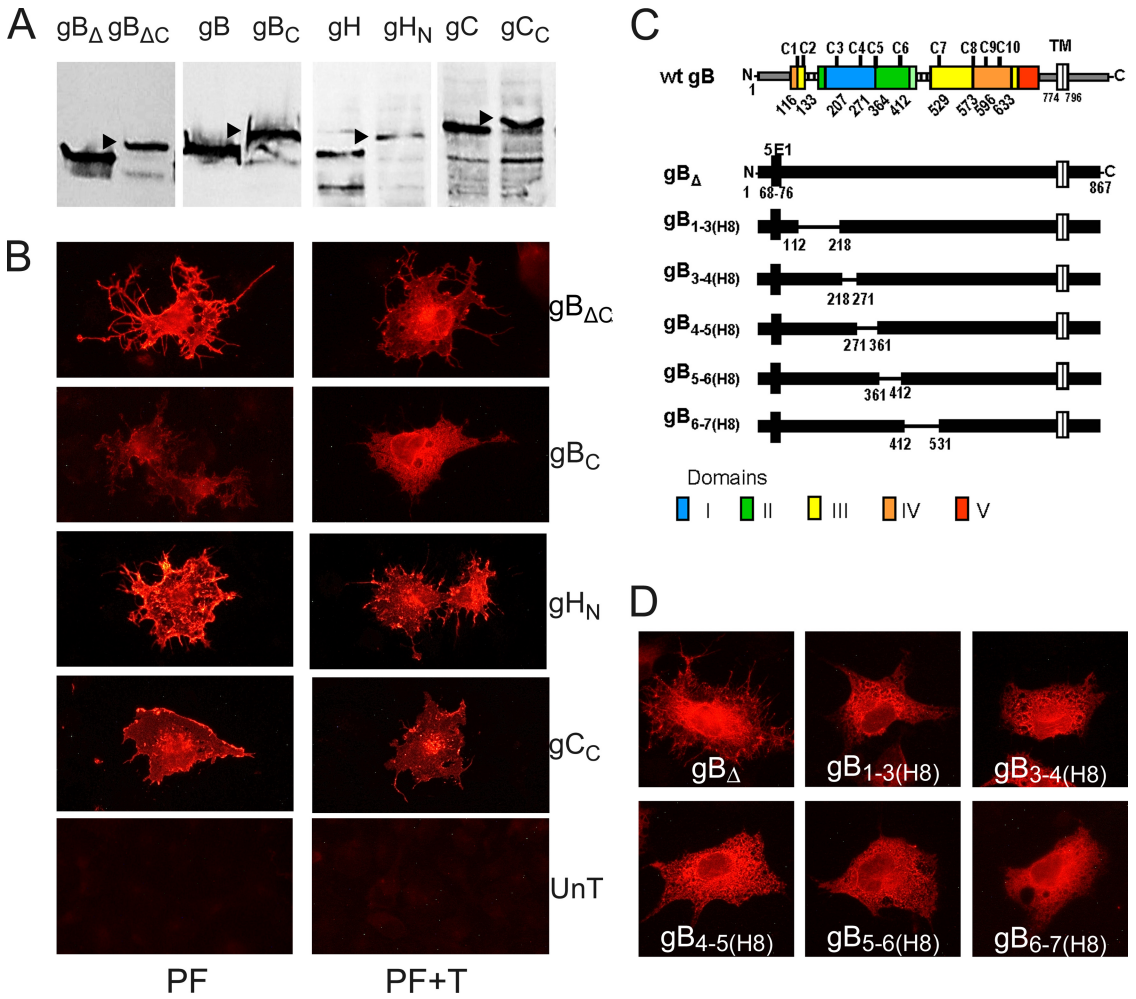

FIG. 1. Characterization of glycoprotein constructs generated in this study. (A and B) Characterization of gB_{Δ}, gB, gH, and gC glycoproteins fused to YFP N or C fragments. (A) Electrophoretic mobility of $gB_{\Delta C}$, gB_C, gH_N, gC_C, relative to their wt counterparts. 293T cells were transfected with a plasmid encoding the indicated glycoprotein (gH was cotransfected with gL). At 20 to 24 h after transfection cells were harvested, and proteins were separated by denaturing PAGE. Glycoproteins were detected by Western blotting with MAbs H1817 to gB, H12 to gH, and H633 to gC. Arrowheads point to the glycoproteins fused to the indicated YFP N or C fragments, all of which exhibit a decreased electrophoretic mobility relative to their wt counterparts. (B) Cellular localization of $gB_{\Delta C}$, gB_C, gH_N, gC_C detected by IFA in paraformaldehyde-fixed cells (PF) or in paraformaldehyde- and Triton X-100-treated cells (PF + T). It can be seen that all glycoproteins reach the plasma membrane (to a lesser extent in the case of gB_C, as expected). Immunoreactivity was detected with MAbs H233 to gB, 53S to gH, and H633 to gC. UnT, mock-transfected cells immunostained with MAb H233. (C and D) Schematic linear maps of $gB_{H1/H8}$ chimeras (C) and their intracellular distribution (D). In panel C the top line represents the linear map of HSV-1 wt-gB. Indicated are color-coded structural domains I to V (19) and the positions of cysteines (C) 1 to 10. Bottom figures indicate cysteine coordinates. TM, transmembrane domain. gB_{Δ} as well as all the $gB_{H1/H8}$ chimeras carries a stop codon at residue 867 and the 5E1 epitope (40) in place of the segment of aa 68 to 76. The thin line in each $gB_{H1/H8}$ chimera indicates the HSV-1 gB portion that was replaced by the homologous HHV-8 gB portion. Figures denote boundaries of the substituted portions. Intracellular distribution of $gB_{H1/H8}$ chimeras is shown in panel D. COS-7 cells were transfected with the indicated plasmid, fixed at 24 h after transfection, and immunostained with MAb H1817. gB localization was mainly intracellular and reticular in cells expressing the chimeras, whereas it was clearly in thin elongations at the plasma membrane in cells expressing gB_{Δ}.

(Fig. 1C). Overall, the portion of ectodomain covered by the chimeras represents about three-fourths of the solved structure. Efforts to generate chimeras spanning the regions downstream of aa 529 (cysteine 7) were unsuccessful as the chimeric proteins did not accumulate in appreciable quantities, which is in agreement with other observations (26, 32).

Immunofluorescence of cells transfected with the chimeras showed a predominantly intracellular, partly reticular localization in contrast with that of gB_{Δ}, which localized in part to the plasma membrane, suggesting that the chimeras were hampered in cell surface transport (Fig. 1D). Accordingly, all $gB_{H1/H8}$ chimeras were defective in cell-cell fusion activity (data not shown), most likely in consequence of defective cell surface localization. The electrophoretic mobility of each chimera is shown in Fig. 3.

gB-gH/gL interaction detected by split YFP complementation assay. The first analysis of gB-gH/gL interaction was performed in whole 293T and COS-7 cells by means of a split YFP complementation assay. Cells were transfected with plasmid mixtures containing either the full-length gB or the truncated

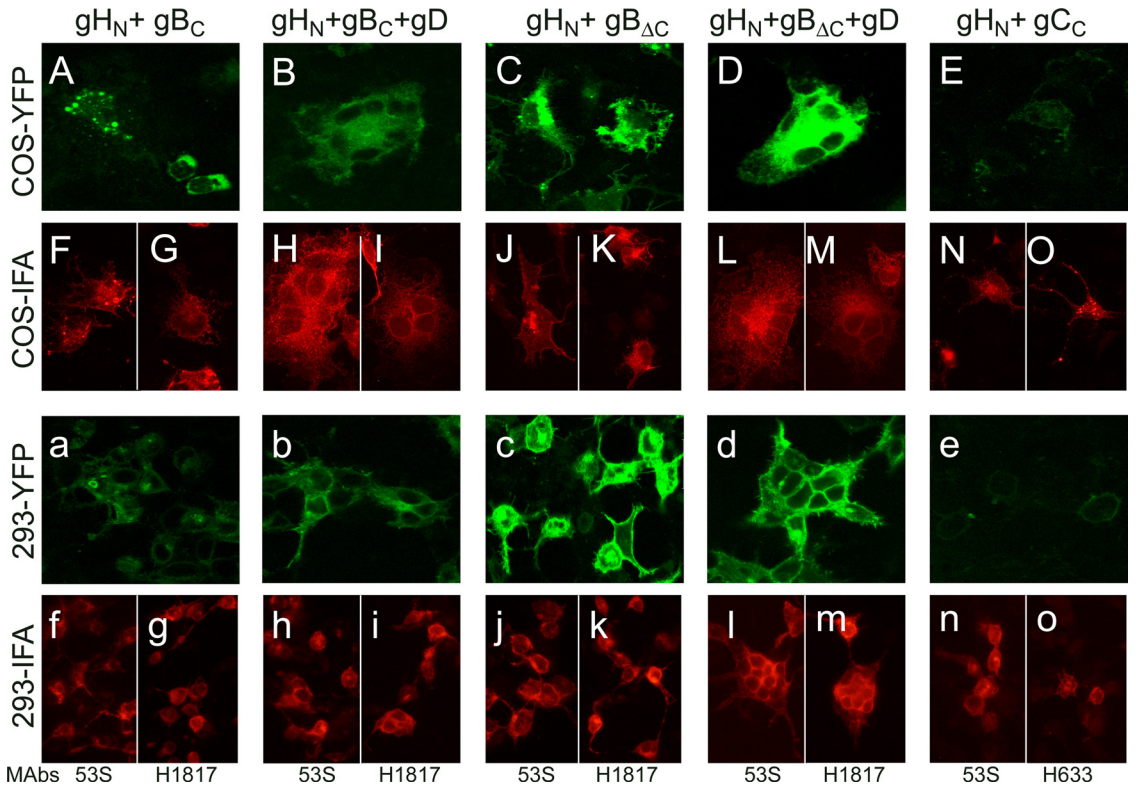

FIG. 2. gB-gH/gL interaction detected by means of a split YFP complementation assay (green panels) and gH, gB, gB$_\Delta$, and gC expression detected by immunofluorescence (red panels). COS-7 (COS) or 293T (293) cells were transfected with mixtures containing five plasmids (expressing gH, gB, gL, and gC with or without gD), two of which encoded glycoproteins fused to N- or C-terminal YFP fragments, as specified at the top of each column. When gD was omitted, it was replaced with epidermal growth factor receptor 2 with a deletion of signaling sequences (37). At 20 h (293T) or 40 h (COS-7) after transfection, cells were observed unfixed (green panels) or after IFA with MAbs 53S to gH, H1817 to gB, and H633 to gC, as specified below the panels. The first specimen to be observed in each series (row A to E or a to e) was the gC$_C$-containing one, and it was employed to adjust the confocal microscope settings, which were not modified thereafter. Cells transfected with mixtures containing gB$_{\Delta C}$ exhibited a stronger YFP fluorescence than those containing gB$_C$ (compare C to A, D to B, c to a, and d to b). It can be observed that cells transfected with mixtures that contained the quartet of gB, gD, and gH/gL formed syncytia, indicating that chimeric glycoproteins carrying N- or C-terminal YFP fragments were not hampered in their ability to mediate cell-cell fusion.

gB$_\Delta$, each one chimeric with the C fragment. A typical transfection mixture contained the combination of plasmids expressing gH$_N$, gB$_C$, wt-gL, and wt-gC with or without gD. When gD plasmid was omitted, transfection mixtures were made equal in DNA by the addition of a plasmid encoding epidermal growth factor receptor 2 carrying a deletion of the signaling sequences (37). This control further ensured that exocytic membranes were loaded with comparable amounts of membrane proteins. To detect the extent of nonspecific fluorescence emission, gB$_C$ was replaced with gC$_C$ (in the combination gH$_N$, gC$_C$, wt-gL, and gB with or without gD). We considered gC a proper negative control in that gC is present in the same subcellular compartments as the other glycoproteins, it participates in virus attachment but not in virus entry, and it does not interact with any members of the glycoprotein quartet. As detailed elsewhere (2), we made use of the negative gC$_C$-containing sample to set the confocal microscope. Specifically, the first analyzed sample in a series was always the gC$_C$-containing one; any specimen belonging to the same series was analyzed without further changes to the confocal microscope settings. Immunofluorescence analysis verified that all the relevant glycoproteins were expressed. This control ruled out the possibility that the lack of YFP fluorescence in gC$_C$-containing samples (Fig. 2, frames E and e) was due to lack of expression of one or more glycoproteins. The main results were as follows. First, in both cell 293T and COS-7 cells observed 20 and 40 h after transfection, respectively, the combination of gH$_N$/gL with gB$_C$ and of gH$_N$/gL with gB$_{\Delta C}$ resulted in readily detectable YFP fluorescence (Fig. 2, frames A, C, a, and c). This result contrasts with previous reports in which the interaction of gH$_N$/gL with gB was detected only in cells coexpressing gD or exposed to soluble gD (1, 2). Second, cells transfected with mixtures containing gB$_{\Delta C}$ exhibited a stronger YFP fluorescence than those containing gB$_C$ (Fig. 2, compare frames C to A, D to B, c to a, and d to b). Third, YFP fluorescence was readily detected in cells transfected with the glycoprotein quartet, i.e., in the presence of gD, in agreement with previous findings (1, 2). Cells transfected with the quartet formed syncytia, documenting that the chimeric gH$_N$, gB$_C$, and gB$_{\Delta C}$ were not impaired in cell-cell fusion activity.

gB-gH/gL complex detected by pull-down experiments by means of gH$_{st}$. To ascertain whether the gB-gH/gL interaction detected by split YFP complementation assay gave rise to a biochemically detectable complex, we made use of a pull-down

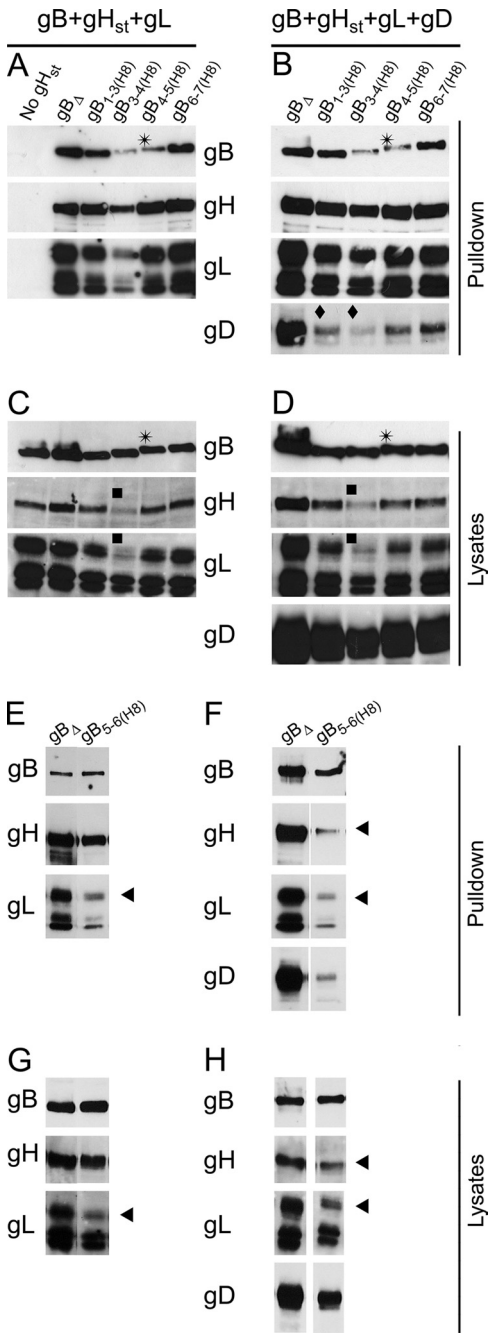

FIG. 3. Ability of gH_{st} to pull down gB_{Δ} or the indicated $gB_{H1/H8}$ chimeras. 293T cells were cotransfected with mixtures containing three glycoproteins, gB_{Δ} or $gB_{H1/H8}$ with gH_{st} and gL_{V5} (gB+gHst+GL), or four glycoproteins, gB_{Δ} or $gB_{H1/H8}$ with gH_{st}, gL, and gD (gB+gH_{st}+gL+gD), as indicated at the top of the panels. The sample indicated as No gH_{st} carried gH_{V5} instead of gH_{st} (which includes the V5 epitope). Cells were harvested 16 to 20 h after transfection. Following cell lysis, gH_{st} was allowed to react with Strep-Tactin resin. The proteins in complex with gH_{st} were retained by the resin, separated by PAGE, and identified by Western blotting with MAb 1817 to gB, MAb V5 to gH and gL, and H170 to gD (Pull-down). A small aliquot of the lysates was separated by PAGE (Lysates), and glycoproteins were identified by WB. Plasmids encoding gH_{st}, gH_{V5}, and gL_{V5} were described previously (14). Asterisks in panels A and B identify $gB_{4-5(H8)}$ which was present in smaller amounts than gB_{Δ} in the pull-down assays but not the in lysates (C and D). Squares in panels C and D indicate that gH and gL accumulated in smaller amounts in cells coexpressing $gB_{3-4(H8)}$. Diamonds identify small amounts of gD present in the pull-down fraction (B) in cells coexpressing $gB_{1-3(H8)}$ and $gB_{3-4(H8)}$. Triangles identify small amounts of gH/gL, in both the pull-down and lysate fractions in cells coexpressing $gB_{5-6(H8)}$ (E to H).

assay applied recently in our laboratory, which rests on the ability of One-Strep-tagged proteins to specifically absorb to Strep-Tactin resin and thus retain any protein in complex with them. Here, we employed gH_{st} (14). 293T cells were transfected with plasmids encoding gH_{st}, gL_{V5}, and gB_{Δ} with or without gD. To detect background proteins not specifically retained by Strep-Tactin resin, in a replicate sample gH_{st} was replaced with gH_{V5}. At 16 to 20 h after transfection, proteins were solubilized and allowed to absorb to Strep-Tactin resin; any protein retained with gH_{st} was separated by PAGE and analyzed by Western blotting. Figure 3A and B show that gH_{st}/gL pulled down gB not only in the presence (Fig. 3B) but also in the absence of gD (Fig. 3A). This result confirms the above finding with the split YFP complementation assay. In a study describing mapping of the gD region involved in the interaction with gB and gH/gL, we recently reported that gH_{st}/gL pulls down full-length gB (14). Thus, both in the pull-down assay and in the YFP complementation assay, the full-length gB interacted with gH/gL in a detectable manner, and removal of the gB C-terminal tail endocytosis motifs simply enhanced complex detection.

Pull-down of $gB_{H1/H8}$ chimeras by gH_{st}/gL in the absence or presence of gD. The ability of gH_{st}/gL to pull down the $gB_{H1/H8}$ chimeras in the absence or presence of gD was assessed by transfecting 293T cells with plasmids encoding gH_{st}, gL_{V5}, and one of the chimeric gB proteins (or gB_{Δ} as a positive control) with or without gD. To detect background proteins unspecifically retained by the resin, a replicate sample contained gH_{V5} instead of gH_{st} (which also carries the V5 epitope) (Fig. 3). At 16 to 20 h after transfection proteins were solubilized, absorbed to Strep-Tactin resin, and analyzed by Western blotting. The experiment was repeated three times, with essentially similar results. Although the assay is not quantitative, we interpret the consistently lower amount of pulled-down gB (or gD) as indicative of a weaker or less stable interaction, provided that the amounts of gB (or gD) present in the respective lysate were not grossly reduced.

The results are shown in Fig. 3. Several observations can be made. (i) All chimeras were pulled down by gH_{st}/gL although in various amounts (Fig. 3A and B). This result implies that there are multiple contact sites between gH/gL and gB. (ii) In cells transfected with three (gH_{st}/gL and chimeric gB) (Fig. 3A, C, E, and G) or four (gH_{st}/gL with gD and chimeric gB) (Fig. 3B, D, F, and H) glycoproteins, the amount of $gB_{3-4(H8)}$ and $gB_{4-5(H8)}$ pulled-down by gH_{st}/gL was much lower than the amount of gB_{Δ} (Fig. 3A and B). Analysis of the lysates (Fig. 3C and D) showed that in cells cotransfected with $gB_{4-5(H8)}$, there was no major decrease in gB accumulation. This result implies a defect in the interaction of gH_{st}/gL with $gB_{4-5(H8)}$. (iii) Unexpectedly, in cells cotransfected with $gB_{3-4(H8)}$, we observed a reduction in gH_{st}/gL accumulation (Fig. 3C and D), implying that altered forms of gB affected the accumulation, and likely the stability, of gH_{st}/gL. Because of this effect, it was unclear

whether the interaction between $gB_{3\text{-}4(H8)}$ and gH_{st}/gL was defective. (iv) Also in cells cotransfected with $gB_{5\text{-}6(H8)}$ (Fig. 3E to H) there was a reduction in the accumulation of gH_{st}/gL in lysates (Fig. 3H) and an even greater reduction in the pull-down fraction (Fig. 3F). Inasmuch as gH_{st}/gL pulled down $gB_{5\text{-}6(H8)}$ at quantities comparable to the amount of gB_{Δ} (Fig. 3F), the results rule out major defects in the interaction between gH_{st}/gL and this form of gB. (v) Again unexpectedly, some of the $gB_{H1/H8}$ chimeras affected the amount of gD pulled down by gH_{st}/gL (Fig. 3B) while they did not grossly affect the amount of gD accumulated in the cell (Fig. 3D). Such an effect was particularly evident with $gB_{1\text{-}3(H8)}$ or $gB_{3\text{-}4(H8)}$. Overall, the results underscore that the amount/stability of gB in complex with gH_{st}/gL is affected by the form of gB itself and, furthermore, that the form of gB affects the amount/stability of gD recruited to gH_{st}/gL. Given that gD can independently interact with both gB and gH/gL, one interpretation of this finding is the formation of a quaternary (gD-gB-gH/gL) complex and the possibility that gB contributes to its stabilization and/or half-life.

gB_{Δ}-gH/gL interaction takes place in the exocytic membranes. As shown in Fig. 2, the $gB_{H1/H8}$ chimeras preferentially accumulate in the exocytic membranes, raising the possibility that the interactions observed between gB_{Δ} and gH/gL occur in this compartment. To verify this point further, cells transfected with gH_N/gL and $gB_{\Delta C}$ in the absence or presence of gD were exposed to BFA, an inhibitor that causes Golgi complex disassembly and its redistribution into the endoplasmic reticulum and consequently halts glycoprotein transport (7). As shown in Fig. 4 exposure of transfected cells to BFA did not abrogate the YFP fluorescence emitted by $gH_N/gL\text{-}gB_{\Delta C}$ both in the absence or presence of gD, providing evidence that interaction takes place in an early compartment of the exocytic pathway, most likely in the endoplasmic reticulum.

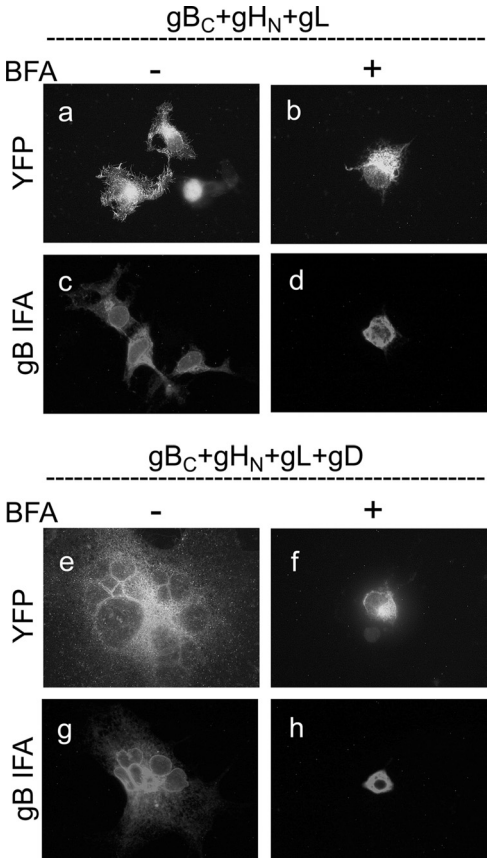

FIG. 4. Effect of BFA on the gB-gH/gL interaction detected by means of a split YFP complementation assay. COS-7 cells were transfected with the indicated plasmid mixtures and exposed (+) or not (−) to BFA. BFA treatment did not abrogate YFP fluorescence (panels b and f) but prevented syncytia formation in cells transfected with the glycoprotein quartet (compare e to f and g to h) because glycoprotein transport along the exocytic pathway was halted.

DISCUSSION

We document the ability of gH/gL and gB to interact with each other in the absence of gD. The interaction was detected by two independent assays, i.e., in whole cells (293T and COS-7) by means of a split YFP complementation assay and biochemically by means of a pull-down assay that made use of One-Strep-tagged gH (gH_{st}). The form of gB employed in most of the current experiments carried a deletion of the endocytosis and phosphorylation motifs (gB_{Δ}) and localized preferentially to the exocytic and plasma membranes (3). This localization contrasts with that of wt-gB, which is sorted to large endocytic vacuoles. Inasmuch as gH/gL also localize predominantly to exocytic and plasma membranes, a longer-lasting colocalization of gH/gL and gB in the same subcellular compartments likely augmented the amount of complexes accumulated in the cells and increased their detection. In the current split YFP complementation assay, fluorescence exhibited by gB_{Δ}-containing samples was stronger than that exhibited by the corresponding full-length gB-containing samples. This difference may account in part for the failure to detect the gB-gH/gL complex in earlier studies where full-length gB was employed. Cells exhibiting a higher efficiency of transfection and expression (like the COS-7 and 293T cells employed in this study) may have further contributed to increased sensitivity of the current assays (1). In previous studies from our laboratory, failure was likely due to the use of EGFP instead of the stronger YFP (2). The criteria adopted here to authenticate the gB-gH/gL interaction were the same as those discussed in a previous study (2) and included gC as a negative control. gC is an HSV virion glycoprotein that accumulates in the exocytic and plasma membrane compartment and enables virus attachment but not virus entry. When gC_C replaced gB_C in the split YFP complementation assay, it gave rise to a weak background fluorescence exhibited by a small number of cells. Evidence of complex formation between gB_{Δ}-gH/gL was further provided biochemically in pull-down assays, in which gH_{st}/gL absorbed to the specific Strep-Tactin resin and thus retained any associated gB. Inasmuch as we made use of overexpressing transfected cells and as the YFP complementation assay tends to stabilize even weak interactions, the interaction between gB and gH/gL may be transient or may involve a minor fraction of the glycoproteins. The results cumulatively underscore the ability of gB and gH/gL to interact specifically with each other in the absence of gD.

Two lines of evidence argue that the gB-gH/gL interaction occurs in the membranes of the exocytic compartment. Thus,

chimeric gB glycoproteins were not hampered in their interaction with gH/gL despite their preferential accumulation in a reticular, endoplasmic reticulum-like compartment. Second, exposure of glycoprotein-expressing cells to BFA, an inhibitor of glycoprotein transport, did not result in abrogation of the gB-gH/gL interaction. We interpret these results to mean that members of the HSV glycoprotein quartet do not require transport to and localization at the plasma membrane in order to interact with each other. This feature is shared with other membrane protein systems, e.g., human immunodeficiency virus gp120 and its CD4 receptor (27).

In order to obtain preliminary information on the gB regions(s) involved in the interaction with gH/gL, we engineered five $gB_{H1/H8}$ chimeras, in which portions of HSV-1 gB bracketed by consecutive cysteines were replaced with the corresponding portion of HHV-8 gB. Cumulatively, the replaced portions spanned aa 112 to 529; the chimeras covered about three-fourths of the gB solved structure (19). The region downstream of residue 529 could not be investigated since it is involved in trimerization, and mutants generated in this study (C. Forghieri and G. Campadelli-Fiume, unpublished results) or elsewhere (26, 32) did not yield forms of gB able to accumulate at sufficient quantities. The homologous replacement strategy applied here, in particular, the maintenance of cysteines and cysteine spacings, together with the possibility to model HHV-8 gB structure on that of HSV-1 gB, ensured that the overall structure would be preserved in the chimeras and yielded forms of gB that accumulated at sufficient quantities for the current assays. The ability of the $gB_{H1/H8}$ chimeras to interact with gH/gL was analyzed in pull-down experiments by means of gH_{st}/gL. Although this assay is not quantitative, we interpreted a consistently smaller amount of pulled-down gB as indicative of a weaker or less stable interaction. The main results of this series of experiments were as follows. (i) None of the gB chimeras was completely hampered in its ability to interact with gH/gL. Nonetheless, some of them, in particular $gB_{4–5(H8)}$, exhibited a reduced ability to be pulled down by gH_{st}/gL in either the absence or the presence of gD. The results suggested that the interaction of gB with gH/gL involved multiple residues and was not completely eliminated in any of the chimeras. The result with $gB_{4–5(H8)}$ was interesting since the replaced portion encodes part of the pleckstrin-like domain 1, which carries the bipartite fusion loop (19). Since this region in the crystal structure of vesicular stomatitis virus glycoprotein G in the prefusion conformation is highly exposed on the surface, it may constitute a site for gB interaction with gH/gL.

The gB-gH/gL interaction in the absence of gD adds to the number of interactions that were detected in earlier studies (1, 2, 14, 35) and highlights an unforeseen complexity in the cross talk among the members of the HSV glycoprotein quartet. The interactions include all possible permutations, i.e., gD-gH/gL in the absence and presence of gB, gD-gB in the absence and presence of gH/gL, and, finally, gB-gH/gL in the absence and presence of gD. With respect to the significance of the last interaction, it is worth noting that cells transfected with the HSV glycoprotein quartet (gD, gB, and gH/gL) undergo cell-cell fusion (41), a condition that mimics the virion-cell fusion that takes place at virus entry. Because absence of gD precludes fusion from taking place, the gB-gH/gL complex in the absence of gD likely mirrors a complex that exists prior to fusion. The data favor the view that certain complexes among the HSV glycoprotein quartet are assembled prior to the onset of fusion. In contrast with the model hypothesized in earlier works (8, 11, 23), we propose that the trigger to fusion exerted at the gD encounter with one of its receptors consists of an intramolecular signaling in gD, which is then transmitted to the precomplexed executors of fusion, gB and gH/gL. Subsequent events remain largely speculative and may include one or more of the following: changes in gB and/or gH/gL conformations, changes in complex stoichiometry, temporary assembly of a quaternary complex, or, more likely, disassembly of the preassembled complexes such that gB and/or gH/gL execute fusion as single entities rather than in complex with gD.

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