

# Epstein-Barr Virus Glycoprotein gB and gHgL Can Mediate Fusion and Entry in *trans*, and Heat Can Act as a Partial Surrogate for gHgL and Trigger a Conformational Change in gB

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## ABSTRACT

Epstein-Barr virus (EBV) fusion with an epithelial cell requires virus glycoproteins gHgL and gB and is triggered by an interaction between gHgL and integrin  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ , or  $\alpha v\beta 8$ . Fusion with a B cell requires gHgL, gp42, and gB and is triggered by an interaction between gp42 and human leukocyte antigen class II. We report here that, like alpha- and beta-herpesviruses, EBV, a gammaherpesvirus, can mediate cell fusion if gB and gHgL are expressed in *trans*. Entry of a gH-null virus into an epithelial cell is possible if the epithelial cell expresses gHgL, and entry of the same virus, which phenotypically lacks gHgL and gp42, into a B cell expressing gHgL is possible in the presence of a soluble integrin. Heat is capable of inducing the fusion of cells expressing only gB, and the proteolytic digestion pattern of gB in virions changes in the same way following the exposure of virus to heat or to soluble integrins. It is suggested that the Gibbs free energy released as a result of the high-affinity interaction of gHgL with an integrin contributes to the activation energy required to cause the refolding of gB from a prefusion to a postfusion conformation.

## IMPORTANCE

The core fusion machinery of herpesviruses consists of glycoproteins gB and gHgL. We demonstrate that as in alpha- and beta-herpesvirus, gB and gHgL of the gammaherpesvirus EBV can mediate fusion and entry when expressed in *trans* in opposing membranes, implicating interactions between the ectodomains of the proteins in the activation of fusion. We further show that heat and exposure to a soluble integrin, both of which activate fusion, result in the same changes in the proteolytic digestion pattern of gB, possibly representing the refolding of gB from its prefusion to its postfusion conformation.

Epstein-Barr virus (EBV), one of the two known human oncogenic gammaherpesviruses, primarily infects B lymphocytes and epithelial cells. An infection by the orally transmitted virus, which eventually occurs in more than 95% of the global population, is never cleared (1). Instead, a reservoir of latent infection is established in long-lived memory B cells with sporadic terminal differentiation of infected cells into plasma cells, leading to the reactivation of virus replication and cell death (2). Virus released from B cells is thought to be amplified in epithelial cells for the replenishment of the latent reservoir or shedding in saliva for transmission to a new host (3, 4).

As with all herpesviruses, EBV enters cells as its envelope fuses with a membrane of the cell. Fusion with B cells requires endocytosis and occurs in a low pH compartment, although low pH is not required (5). Fusion with epithelial cells occurs at neutral pH and, in at least some cell lines, is thought to take place at the cell surface (5). Both events require the activation of the herpesvirus core fusion machinery, comprised of the conserved herpesvirus glycoproteins gB and gHgL (6). The crystal structure of EBV gB, which is a type I single-pass membrane protein, reveals that it exists as a trimer (7). The trimer resembles the postfusion structure of the class III fusion proteins vesicular stomatitis virus glycoprotein G and baculovirus gp64, both of which are necessary and sufficient for fusion. A similar structure has been solved for herpes simplex virus glycoprotein gB (8), and the gB homologues of both viruses are thought to be the final executors of fusion. However, in contrast to the vesicular stomatitis virus and baculovirus fusion pro-

teins, none of the herpesvirus gB homologues can mediate fusion in the absence of gHgL.

Like gB, gH is a single-pass type I membrane protein, whereas gL is a peripheral membrane protein from which the signal peptide is cleaved. The crystal structure of the EBV gHgL dimer has a linear arrangement of 4 domains, and membrane distal domain I is formed by both gH and gL (9). The dimer resembles no known fusion protein, and its role generally is thought to be that of a regulator required to initiate the fusion-driving conversion of gB from a prefusion to a postfusion structure, although, to this point, there has been no experimental confirmation that EBV gB undergoes refolding (10).

The events responsible for the activation of a potential regulator such as gHgL differ for B cells and epithelial cells. The activation of fusion in the endosome of a B cell occurs as a result of an interaction between human leukocyte antigen class II (HLA class II) and a fourth gammaherpesvirus-specific glycoprotein, gp42 (11). Glycoprotein gp42, as a cleaved type II membrane protein,

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forms a tripartite complex with gHgL by virtue of its ability to bind to a region of gHgL which overlaps domain II (12, 13). The binding of gp42 to HLA class II changes its conformation, and this conformational change probably contributes to the activation of fusion (14). The activation of fusion with an epithelial cell, however, involves the direct interaction of gHgL with one of three integrins,  $\alpha\text{v}\beta\text{5}$ ,  $\alpha\text{v}\beta\text{6}$ , or  $\alpha\text{v}\beta\text{8}$  (15, 16). There is a KGD motif in domain II of gHgL which is responsible for this interaction. Integrin binding results in a conformational shift between domain I and domain II which, like the change in gp42 caused by HLA class II binding, may contribute to the activation of fusion (16, 17).

Because gp42 and integrins bind to overlapping sites in domain II of gHgL, the presence of gp42 in a complex with gHgL precludes the ability of the complex to interact with an integrin. Thus, EBV virions carry trimers of gHgLgp42 and dimers of gHgL in order to infect both B cells, which lack the necessary  $\alpha\text{v}$  integrins, and epithelial cells, which do not constitutively express HLA class II (12). The ratios of the two types of complex vary according to the cell in which the virus is made. Virus made in a B cell loses three-part complexes as a result of trafficking with HLA class II to the proteolytic antigen-processing compartment. Emerging virions are enriched for gHgL dimers and are approximately 5-fold better able to infect epithelial cells than virus made in an epithelial cell. In contrast, virions made in an HLA class II-negative epithelial cell are enriched for trimers of gHgLgp42 and can be as much as a hundred-fold more infectious for B cells than virus made in a B cell. This switching of tropism is thought to facilitate the cycling of virus between B cells and epithelial cells (18).

Recent studies of herpes simplex virus (HSV) (19) and human cytomegalovirus (HCMV) fusion (20, 21) have revealed that the gB and gHgL homologues of these viruses can mediate cell fusion (19, 21) and virus entry (20) if they are expressed in opposing membranes. Although the fusion in *trans* mediated by HSV proteins, which were expressed from plasmid vectors, was not as efficient as fusion in *cis*, fusion in *trans* mediated by HCMV proteins, which were expressed at high levels from nonreplicating adenovirus vectors, was equally as efficient as fusion in *cis*. This implies that the ectodomains of the proteins are of paramount importance to the execution of fusion. We report here that EBV gB and gHgL likewise can mediate not only fusion but also entry in *trans* into either an epithelial cell or into a B cell after exposure to a soluble integrin.

Heat, as a source of free energy, can act as a surrogate trigger of transitions from prefusion to postfusion conformations of the paramyxovirus F proteins (22–25) and the influenza virus hemagglutinin (26, 27). We similarly found that the exposure of cells expressing EBV gB to heat in the absence of gHgL resulted in fusion. The proteolytic digestion pattern of gB was changed in the same way when exposed to heat as when triggered by an integrin in the presence of gHgL, discriminating between what may be the prefusion and postfusion conformations of EBV gB and supporting the hypothesis that the protein undergoes a conformational change during fusion, possibly aided by the Gibbs free energy that would be released by high-affinity binding of gHgL to an integrin.

## MATERIALS AND METHODS

**Cells and virus.** Akata-GFP, a Burkitt lymphoma-derived cell line carrying a recombinant EBV in which the thymidine kinase gene is interrupted with a double cassette expressing neomycin resistance and green fluorescence protein (GFP), Akata-gH-null, carrying a recombinant EBV in

which the BXL2F2 open reading frame encoding gH is interrupted with the same cassette (28), and EBV-negative Akata cells, which have lost EBV episomes (29), were grown in RPMI 1640 (Sigma). AGS, human gastric carcinoma cells (American Type Culture Collection) that have been cured of parainfluenza type 5 infection by treatment with ribavirin, and CHO cells were grown in Ham's F-12 medium (Sigma). 293 cells, 293T14 cells expressing T7 RNA polymerase (30), and 293-B8 AVAP cells, which secrete truncated  $\alpha\text{v}\beta\text{8}$  conjugated to alkaline phosphatase (31), were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with nonessential amino acids (Gibco). HaCaT cells (American Type Culture Collection) were grown in DMEM-low glucose (HyClone). Media for all mammalian cells also were supplemented with 10% heat-inactivated fetal bovine serum (Gibco). Insect Sf9 cells were grown in Sf900 II medium (Invitrogen) and infected at a multiplicity of infection of 3 with baculovirus expressing a truncated form of gH together with gL (gHtgL) (32). Akata virus made in B cells was harvested from the spent culture media of cells that had been induced by treatment with anti-human immunoglobulin as described previously (28). Akata virus made in epithelial cells was harvested from spent culture media of AGS cells 5 days after induction with 12-*O*-tetradecanoylphorbol-13-acetate (Sigma) and 2.5 mM sodium butyrate (Calbiochem). In both cases, virus was concentrated by centrifugation as described previously (28).

**Antibodies.** Antibodies used included monoclonal antibodies CL59 to gH (28), CL55 to gB (33), 37E1 to  $\alpha\text{v}\beta\text{8}$  (31), 10D5 to  $\alpha\text{v}\beta\text{6}$ , and P1F6 to  $\alpha\text{v}\beta\text{5}$  (Chemicon), a rabbit polyclonal antibody (RgB) made to residues 754 to 857 of gB fused to glutathione-S-transferase, goat anti-rabbit IRDye 800 CW (LiCor Biosciences), goat anti-mouse antibody coupled to Alexa Fluor 647 (Life Technologies), sheep anti-mouse coupled to fluorescein isothiocyanate (FITC) (MP Biomedicals), and sheep anti-mouse coupled to phycoerythrin (Jackson ImmunoResearch). Antibodies CL59, CL55, 37E1, and RgB were purified by affinity chromatography on protein A coupled with agarose (RepliGen).

**Integrin and gHtgL purification.** Soluble truncated gHtgL and integrin  $\alpha\text{v}\beta\text{8}$  were purified from spent culture media as previously described (15, 16).

**Fusion assays.** Fusion was scored as previously described (15) either visually, in cells stained for glycoproteins by antibody CL55 or CL59, or in a luciferase-based assay in which one population of cells transfected with a plasmid expressing luciferase under the control of a T7 promoter was overlaid with 293T14 cells expressing T7 polymerase. To compare fusion in *trans* to fusion in *cis*, either cells were transfected with various combinations of plasmids pCAGGS, pCAGGS-gH, pCAGGS-gL, and pCAGGS-gB in *cis* or two separate populations of cells were transfected with combinations of plasmids. Twenty-four h later, cells were trypsinized and reseeded together. Fusion was measured after a further 24 h of incubation. Fusion of AGS cells supported by soluble gHtgL was obtained by transfecting cells with pCAGGS-gB for 24 h, adding 40 nM soluble gHtgL, a saturation concentration (34), or 40 nM soluble gHtgL preincubated with 6 nM soluble integrin  $\alpha\text{v}\beta\text{8}$ . Fusion was scored visually after an additional 24 h and expressed as a percentage of fusion achieved when gHgL and gB were expressed in *cis*. The induction of fusion by heat was done by transfecting AGS cells on culture slides with pCAGGS-gH and pCAGGS-gL or with pCAGGS-gB. Thirty-six h later, when cells reached approximately 90% confluence, the slides were placed in a preheated hybridization oven with the temperature maintained with a thermostat and additionally monitored with a digital thermometer (Fisher) with an accuracy of  $\pm 0.2^\circ\text{C}$ . Following exposure to heat for a set time, cool media were added to adjust the temperature back to  $37^\circ\text{C}$ , and the slides were incubated at  $37^\circ\text{C}$  for a further 16 h. Fusion was scored visually and compared to fusion achieved in cells transfected with pCAGGS-gH, pCAGGS-gL, and pCAGGS-gB and incubated at  $37^\circ\text{C}$  throughout.

**Cell infection.** Five hundred thousand 293 cells were seeded in 6-well plates, and 5 h later they were transfected with pCAGGS-gH and pCAGGS-gL or with pCAGGS-gB. After a further 36 h, cells were infected with equal genome copy numbers of either Akata-GFP virus or Akata-gH-

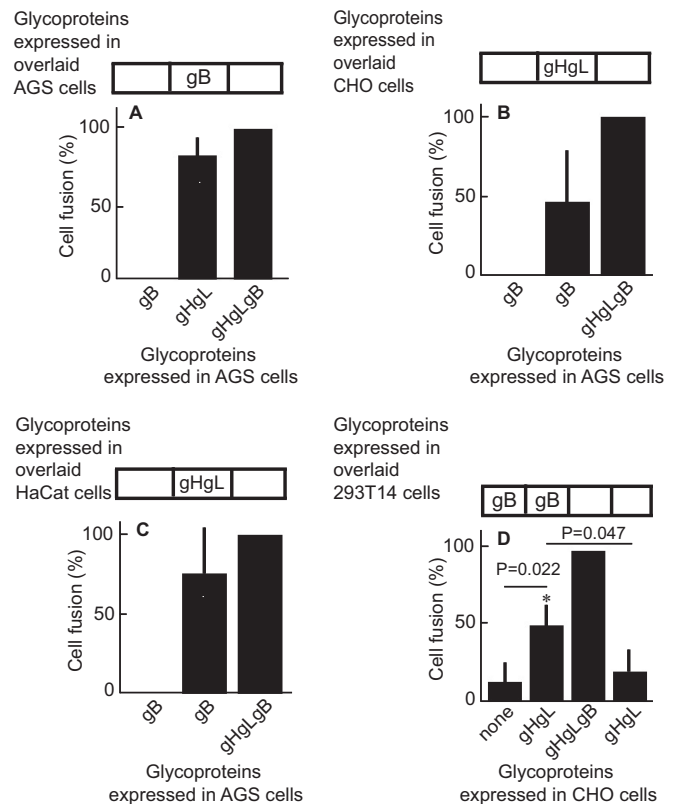
null virus. Both viruses were bound at 37°C 1 h. Unbound virus was removed, and cells were washed and incubated at 37°C for 48 h. Cells were stained with CL59 to gH or CL55 to gB and anti-mouse antibody coupled to Alexa Fluor 647. Glycoprotein expression and infection were measured by flow cytometry. CHO cells were transfected with pCAGGS-CR2 (35) or with pCAGGS-CR2, pCAGGS-gH, and pCAGGS-gL. Twenty-four h later they were infected with Akata-GFP virus or Akata-gH-null virus as described above. At 72 h postinfection, cells were examined visually for GFP expression. Two million EBV-negative Akata cells were nucleofected with pCAGGS-gH and pCAGGS-gL or with pCAGGS alone using an Amaxa nucleofactor 4-D and program CA137. Thirty-six h later, cells were infected with Akata GFP virus or Akata-gH-null virus. Virus was bound for 30 min in the cold, unbound virus was removed, and cells and bound virus were overlaid with 100  $\mu$ l media or media containing soluble  $\alpha$ v $\beta$ 8 at 37°C. Two h later, medium was added and cells were incubated for 48 h. Cells were stained with CL59 to gH or CL55 to gB and anti-mouse antibody coupled to Alexa Fluor 647. Glycoprotein expression and infection were measured by flow cytometry.

**EBV digestion with proteinase K and Western blotting.** Concentrated Akata-GFP virus was either preincubated at temperatures between 37°C and 45°C for 30 min, preincubated with 0.6 nM to 6 nM  $\alpha$ v $\beta$ 8 at room temperature, or kept at room temperature without treatment. Samples were left undigested or were digested with proteinase K (10  $\mu$ g/ml) for 10 min at room temperature before analysis by SDS-PAGE on a 12.5% reducing gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and reacted with RgB to the cytoplasmic tail of gB and goat anti-rabbit IRDye 800 CW secondary antibody. Fluorescence was read using an Odyssey infrared imager (LiCor Biosciences).

## RESULTS

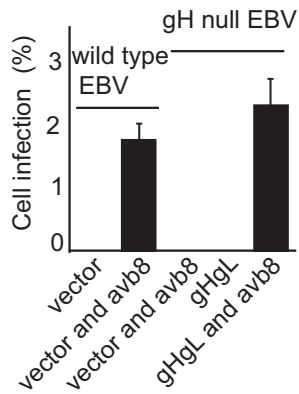
**Epithelial cell fusion occurs when gB and gHgL are expressed in trans.** To determine whether EBV gB and gHgL can mediate fusion *in trans*, we first used a cell-based fusion assay in which fusion is scored visually. Three separate dishes of AGS cells were transfected with plasmids expressing gB, gHgL, or both gB and gHgL. Twenty-four h later, cells were overlaid or trypsinized and reseeded in groups that consisted of either 50% of cells expressing gB and gHgL and 50% of cells transfected with empty vector or 50% of cells expressing gB and 50% of cells expressing gHgL. More than 80% of the level of fusion seen if gHgL and gB were expressed *in cis* was seen when proteins were expressed *in trans* (Fig. 1A). Similar experiments were done in which AGS cells were transfected with plasmids expressing gB or gHgL and gB and reseeded with CHO cells (Fig. 1B) or HaCaT cells (Fig. 1C) transfected with empty vector or with plasmids expressing gHgL. Once again, fusion was seen if glycoproteins were provided *in cis* or *in trans*, although the levels were lower, particularly in the case of the CHO cells, than those seen when all cells were AGS cells. This may reflect in part the fact that CHO cells do not express a human integrin with which EBV gHgL can productively interact. Fusion *in trans* also was achieved in a luciferase assay in which plasmids expressing gHgL were expressed in CHO cells and CHO cells were overlaid with 293T14 cells expressing gB (Fig. 1D).

**Cells expressing gHgL can be infected with a gH-null virus.** Cell-based fusion assays are subject to the criticism that the transfer of membrane from one cell to another might occur and allow transfer of glycoproteins, putting them back *in cis* instead of *in trans*. This concern is somewhat ameliorated by the observation that in HCMV cell-based fusion assays, fusion is as efficient when proteins are expressed *in trans* as in *cis* (21). Even more compelling, however, is the finding that entry of HCMV virions lacking gB can be effected if gB is expressed in target cells (20). An EBV



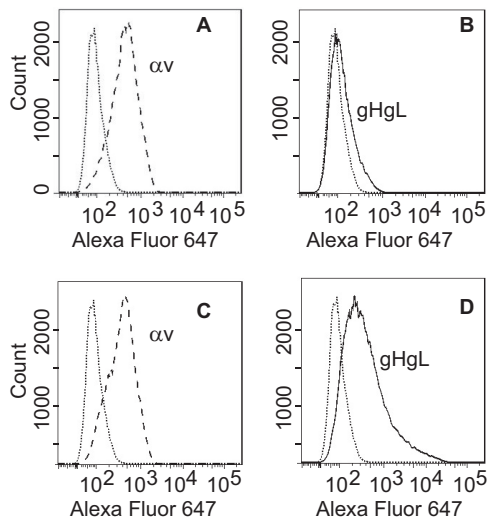
**FIG 1** Glycoproteins gHgL and gB can trigger fusion of epithelial cells when expressed *in trans*. (A to C) AGS cells were transfected with plasmids expressing gHgL and gB *in cis* or with gHgL alone or gB alone and overlaid with AGS (A), CHO (B), or HaCaT (C) cells transfected with empty vector, gHgL, or gB as indicated. Fusion was measured 24 h after cells were overlaid by staining with monoclonal antibody CL55 to gB and counting the percentage of stained cells that contained 4 or more nuclei. Fusion *in trans* was expressed as the percentage of fusion *in cis*. The values for fusion *in cis* were 24%  $\pm$  1.8% (A), 28%  $\pm$  5.3% (B), and 25%  $\pm$  2.7% (C). (D) CHO cells were transfected with a plasmid expressing luciferase under the control of a T7 promoter and with either empty vector, gHgL and gB *in cis*, or gHgL or gB alone and overlaid with 293T14 cells transfected with empty vector or gB as indicated. Fusion was measured in terms of luciferase activity. The error bars are the standard deviations from 4 experiments.

virus lacking gB is not currently available. Therefore, we explored whether Akata-gH-null virus, which is phenotypically null for gHgL and gp42 (28), could infect cells expressing gHgL. The first experiments were done with CHO cells transfected with plasmids expressing the EBV receptor CR2 together with gHgL. These cells were chosen because, as noted above, they do not express any of the human integrins to which EBV gHgL can bind; thus, they presumably would not be subject to the receptor interference, which has been reported to occur when cells are transfected with HCMV gHgL (20). Fusion was triggered by the addition of soluble integrin  $\alpha$ v $\beta$ 8, which can trigger cell-to-cell fusion of CHO cells transfected with gB and gHgL *in cis* (15). Although only low levels of infection were seen, they were as high as those seen with Akata-GFP virus, which carries a full complement of glycoproteins (Fig. 2). To improve on this result, we attempted the infection of 293 cells, which naturally express CR2 and can be infected to higher levels with Akata-GFP virus than can CHO cells. 293 cells do express  $\alpha$ v $\beta$ 5 and  $\alpha$ v $\beta$ 8, although very little  $\alpha$ v $\beta$ 6; thus, they might

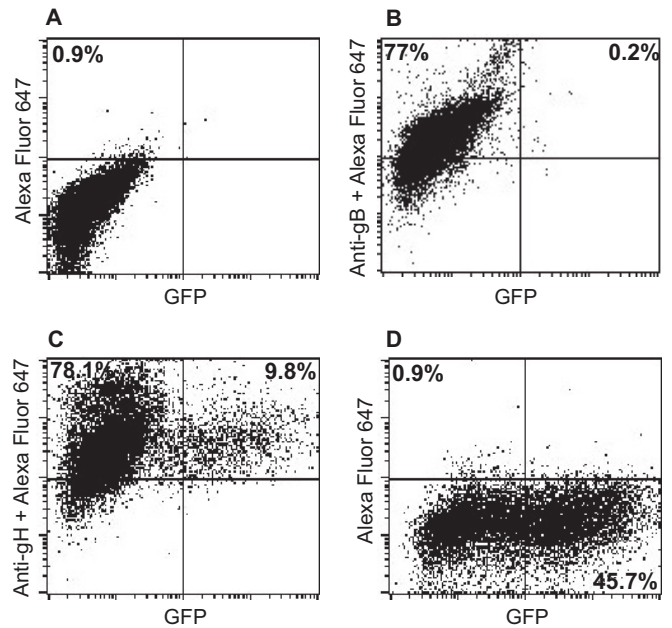


**FIG 2** Integrins can induce infection of CHO cells expressing CR2 and gHgL with a gH-null virus. CHO cells were transfected with a plasmid expressing CR2 and empty vector or plasmids expressing CR2 and gHgL. Twenty-four h later, cells were infected with Akata-gH-null virus. Virus was bound for 2 h, and cells and virus then were overlaid with soluble  $\alpha\text{v}\beta 8$  as indicated. Two hours later additional medium was added, and GFP expression was determined visually at 3 days. The errors bars are the standard deviations from 6 experiments.

be subject to receptor interference. However, after transfection of plasmids expressing gHgL, the expression levels of  $\alpha\text{v}$  integrin were essentially unchanged (Fig. 3). Therefore, cells were transfected with plasmids expressing gHgL or gB and infected with Akata-gH-null virus. Glycoprotein expression and infection were measured by flow cytometry (Fig. 4). No infection by Akata-gH-null virus was seen in cells transfected with empty vector (Fig. 4A) or cells expressing gB (Fig. 4B). However, almost 10% of cells transfected with gHgL were infected with Akata-gH-null virus (Fig. 4C). This represented slightly more than 20% of the untreated cells that could be infected with Akata-GFP virus (Fig. 4D), which carries all virus glycoproteins.



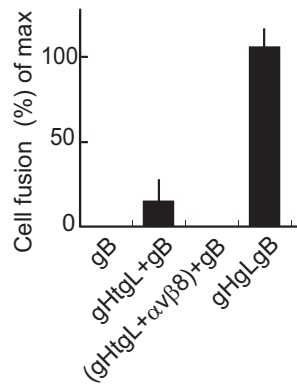
**FIG 3** Expression of gHgL does not reduce  $\alpha\text{v}$  integrin expression. 293 cells were transfected with an empty vector (A and B) or with plasmids expressing gHgL (C and D), and 48 h later they were stained either with monoclonal antibody CL59 to gH (B and D) or with monoclonal antibody L230 to  $\alpha\text{v}$  integrin (A and C), followed by goat anti-mouse antibody coupled to Alexa Fluor 647. Isotype controls are indicated by dotted lines.



**FIG 4** gH-null virus can infect 293 cells expressing gHgL but not cells expressing gB. 293 cells were transfected with empty vector (A and D) or with plasmids expressing gB (B) or gHgL (C), and 36 h later they were infected with Akata-gH-null virus (A, B, and C) or Akata-GFP virus (D). At 48 h postinfection, glycoprotein expression was detected using monoclonal antibody CL55 to gB (B) or monoclonal antibody CL59 to gHgL (C) and goat anti-mouse antibody conjugated to Alexa Fluor 647 (A to D), and infection was monitored in terms of GFP expression.

Since fusion and virus entry could be mediated by the expression of gB and gHgL in opposing membranes, we also asked whether soluble EBV gHgL mediates fusion, as can, at low levels, soluble herpes simplex virus gHgL (19). AGS cells were transfected with plasmids expressing gB, and soluble gHgL was added 24 h later. Only around 14% of fusion achieved by expressing gB and gHgL in *cis* was seen (Fig. 5). However, this low level was eliminated if the gHgL were preincubated with soluble  $\alpha\text{v}\beta 8$ , supporting its specificity.

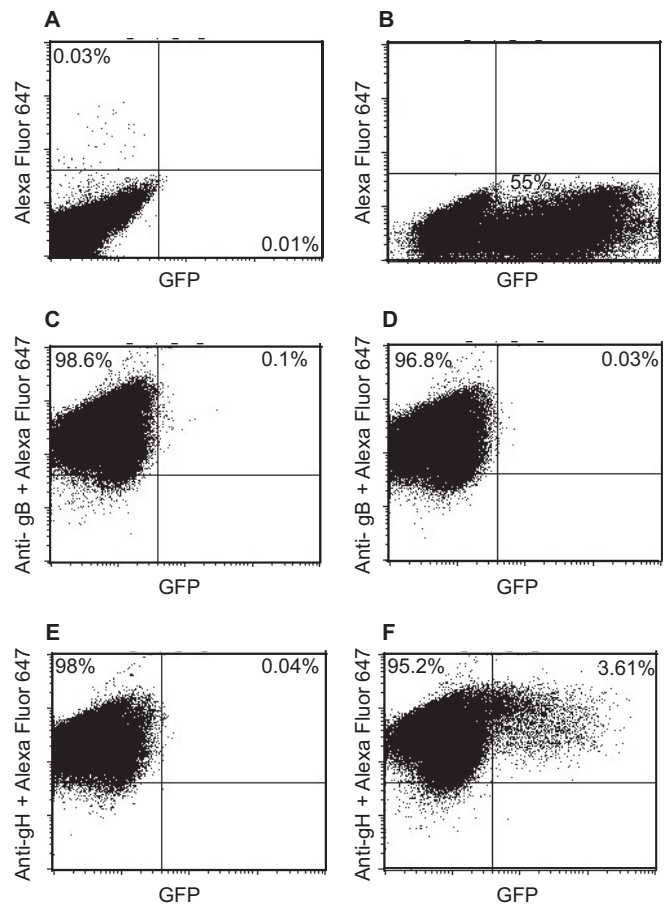
Mutations in EBV gH have been shown to alter fusion with epithelial cells and B cells differentially (17, 36), raising the possibility that there is a fundamental difference between the two events. The availability of soluble integrins and the knowledge that fusion can proceed with proteins expressed in *trans* gave us the opportunity to confirm that elements of the process are shared. EBV fusion and entry of a B cell ultimately requires gB and gHgL, although the triggering of the core fusion machinery here requires an interaction between gp42 and HLA class II. We asked whether the Akata-gH-null virus, lacking gp42, could be triggered by a soluble integrin to enter a B cell expressing only gHgL. As expected, no gHgL-null virus could enter cells transfected with empty vector (Fig. 6A), no gHgL-null virus could enter cells expressing gB with or without the addition of a soluble integrin (Fig. 6C and D), and no gHgL-null virus could enter cells expressing gHgL in the absence of a soluble integrin (Fig. 6E). However, low levels of infection of cells expressing gHgL were clearly seen in the presence of a soluble integrin (Fig. 6F), reaching approximately 7% of the number of untransfected cells that could be infected with the same DNA copy number of wild-type Akata virus (Fig. 6B).



**FIG 5** Soluble gHtgL is capable of supporting low levels of fusion. AGS cells were transfected with plasmids expressing gB or gB and gHtgL, and 24 h later they were overlaid as indicated with soluble gHtgL or gHtgL that had been preincubated with soluble  $\alpha v\beta 8$ . After a further 24 h, cells were fixed and stained with the monoclonal antibody CL55 to gB and FITC-conjugated goat anti-mouse antibody. Fusion was calculated as the percentage of stained cells containing 4 or more nuclei and is expressed as a percentage of that seen in cells transfected with gB and gHtgL. The errors bars are standard deviations from 4 experiments.

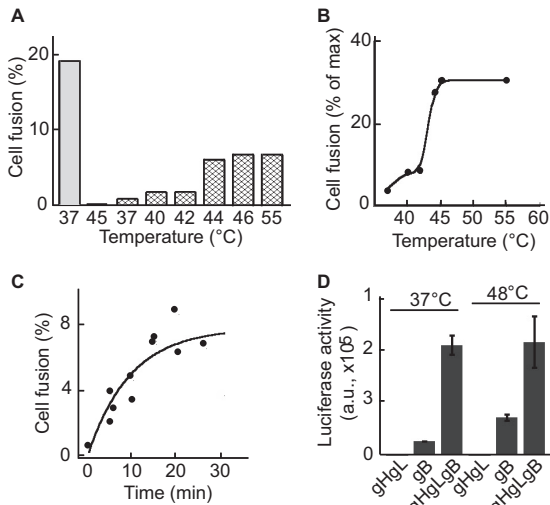
**Exposure of cells expressing gB to transient heat shock can trigger fusion.** The ability of gB and gHtgL to mediate fusion *in trans* implicates the ectodomains of the proteins in the process. Integrins bind gHtgL with very high affinity. The thermodynamic  $K_D$  (equilibrium dissociation constant) values for the interactions are between  $2 \times 10^{-9}$  and  $6 \times 10^{-9}$  M (16). Therefore, we considered whether the Gibbs free energy released by such a high-affinity interaction contributes to the process, possibly by helping to overcome an activation energy barrier needed to support the large conformational change proposed to occur as the prefusion form of gB adopts its postfusion conformation (10). Heat has been shown to act as a surrogate form of energy capable of destabilizing metastable virus fusion proteins (22–27). Therefore, we looked at the effect of heat on the ability of gB to mediate fusion. Cells expressing gB alone or gHtgL alone were exposed for 20 min to different temperatures. Cells expressing gHtgL alone showed no evidence of fusion (Fig. 7A), but cells transfected with gB alone supported low levels of fusion that plateaued at a temperature of 45°C and at approximately 30% of that seen in control cells transfected with gB and gHtgL and maintained at 37°C throughout (Fig. 7B). Holding the cells for longer than 30 min at 45°C did not lead to any further increase in fusion (Fig. 7C). Cells expressing gB together with gHtgL fused maximally at 37°C. Exposing such cells to 48°C for 20 min did not increase fusion (Fig. 7D).

**Exposure of virus to heat or triggering by exposure to soluble integrins changes the proteolytic digestion pattern of gB in the same way.** Proteolysis has been used to monitor conformational changes in protein structure (25). When plasmids expressing gB are transfected into cells, a significant portion of the protein that is made is found within the cell as well as at the cell surface; thus, it is unavailable to receive a trigger of conformational change via an integrin and gHtgL. Therefore, the proteinase K digestion patterns of gB were analyzed in untreated virions, virions heated to 45°C, and virions exposed to 6 nM soluble integrin  $\alpha v\beta 8$ . CL55, the only available monoclonal antibody to gB, does not react in Western blotting, and the only robust polyclonal antibody that we had available was raised against the gB cytoplasmic tail. This antibody,



**FIG 6** gHtgL null virus can infect B cells expressing gHtgL if triggered with soluble  $\alpha v\beta 8$ . EBV-negative Akata B cells were nucleoporated with empty vector (A and B), with a plasmid expressing gB (C and D), or with plasmids expressing gH and gL (E and F). After 36 h, cells were bound to Akata-GFP virus (B) or Akata-gH-null virus (A and C to F) on ice for 30 min and then overlaid with medium alone (A, B, C, and E) or medium containing soluble  $\alpha v\beta 8$  (D and F). At 48 h postinfection, cells were stained with monoclonal antibody CL55 to gB (C and D), monoclonal antibody CL59 to gH (E and F), and goat anti-mouse antibody conjugated to Alexa Fluor 647 (A to F). Glycoprotein and GFP expression were measured by flow cytometry.

however, could recognize by Western blotting both the full-length uncleaved gB, which is found in virions (37), and the carboxy-terminal fragment of gB, which is cleaved by furins in the cell in which it is made (38) (Fig. 8A, lane 1). After proteolytic digestion, only a few small fragments of gB could be seen (Fig. 8A, lane 2), presumably because only these fragments contained pieces of the cytoplasmic tail. However, in virions exposed to heat, an additional larger fragment was visible, indicating a change in accessibility of protease digestion sites (Fig. 8A, lane 3). The same fragment was seen in virions that had been exposed to the soluble integrin  $\alpha v\beta 8$  (Fig. 8A, lane 4). Pre- and postfusion forms of the class III fusion proteins baculovirus gp64, vesicular stomatitis virus G protein, and HSV gB have been proposed to exist in an equilibrium, as at least some conformational changes induced by low pH can be reversed if the pH is raised (39–42). Therefore, we determined whether the change in the proteolytic digestion pattern seen after heating could be reversed by returning the protein to 20°C. Although the larger fragments were slightly less promi-

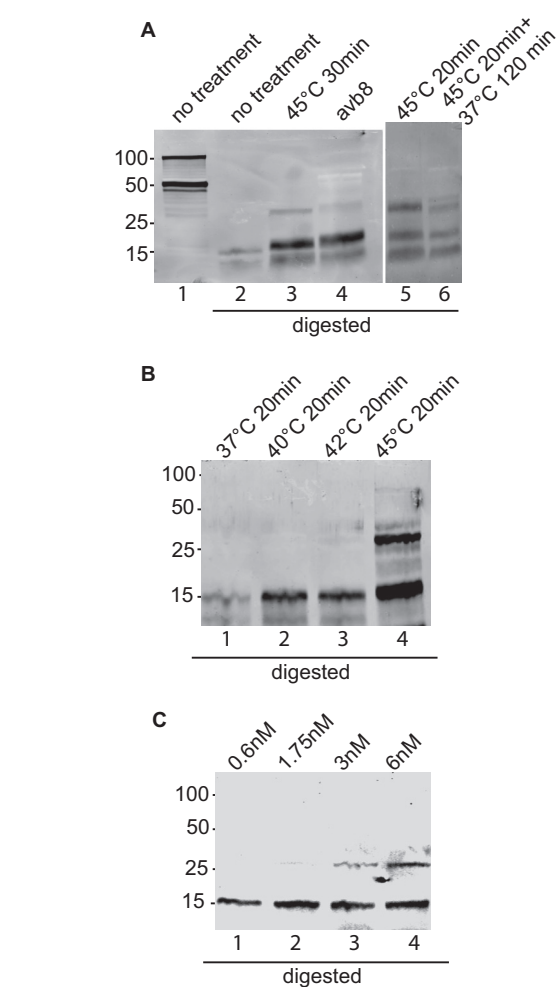


**FIG 7** Increase of temperature triggers fusion of AGS cells expressing gB. (A) AGS cells were transfected with plasmids expressing gB, gH, and gL (gray bar), gH and gL alone (second bar from the left), or gB alone (hatched bars). Twenty-four h later, cells were incubated at the indicated temperature for 20 min, cooled, and returned to 37°C. After a further 16 h, cells were fixed and stained with monoclonal antibody CL55 to gB and FITC-conjugated goat anti-mouse antibody. Fusion was calculated as the percentage of stained cells containing 4 or more nuclei. (B) Graphical representation of data shown in panel A, where the maximum fusion (max) is that seen in cells transfected with plasmids expressing gB, gH, and gL and maintained at 37°C. (C) Kinetics of fusion achieved by exposing cells to 45°C for different times as indicated. (D) CHO cells were transfected with plasmids expressing gH and gL, gB alone, or gB, gH, and gL as indicated together with a plasmid expressing luciferase under the control of the T7 promoter. Twenty-four h later, cells were trypsinized and mixed with an equal number of 293T14 cells for 150 min. Cells then either were maintained at 37°C or warmed to 48°C for 20 min before the addition of cool medium and a return to 37°C. Twenty-four h later, cells were lysed and luciferase activity was measured in a luminometer. Error bars represent the standard deviations from 4 replicates, and the experiment is representative of three independent experiments. a.u., arbitrary units.

ment in the sample that had been returned to 37°C, there was no convincing indication of reversibility in this or repeat experiments (Fig. 8A, lanes 5 and 6). We also determined at what temperature changes in the digestion pattern become apparent. The larger fragments seen when virus was exposed to 45°C could not be seen at lower temperatures but were faintly apparent at 42°C (Fig. 8B). Likewise, a titration of the soluble integrin used to trigger the conformational change indicated that whereas no change in gB could be elicited with 0.6 nM  $\alpha\text{v}\beta\text{8}$ , a small fraction of gB changed conformation in response to 1.75 nM and more again at 3 and 6 nM (Fig. 8C).

## DISCUSSION

The initial observations that EBV glycoproteins gB and gHgL can mediate fusion when expressed in *trans* in opposing cells were not unexpected, given previous work done with HSV and HCMV (19, 21). Fusion between several different epithelial cell lines was quite robust; in some cases, at least 80% of that obtained when glycoproteins were expressed in *cis*. These values are almost as high as those reported for the expression of HCMV gB and gHgL, where fusion in *trans* in cell-based assays was as efficient as fusion in *cis* (21), and they are considerably higher than those reported for HSV, where cells transfected with plasmids expressing gB were



**FIG 8** Proteolytic digestion pattern of gB in virus incubated with soluble  $\alpha\text{v}\beta\text{8}$  or treated with heat. (A, left) Aliquots of virus were held at room temperature, incubated at 45°C for 30 min, or incubated at room temperature with 6 nM soluble  $\alpha\text{v}\beta\text{8}$ . All but one of the aliquots held at room temperature were digested with 10  $\mu\text{g}/\text{ml}$  proteinase K for 10 min, and all aliquots were analyzed by SDS-PAGE and Western blotting with antibody to gB. The blot is representative of those obtained in three independent experiments. (Right) Two aliquots of virus were incubated at 45°C for 20 min. One was digested immediately, and the other was incubated at 37°C for 120 min before digestion. The blot is representative of 5 independent experiments. (B) Four aliquots of virus were incubated for 30 min at the temperatures indicated, digested with proteinase K, and analyzed by SDS-PAGE and Western blotting with antibody to gB. The blot is representative of 4 independent experiments. (C) Four aliquots of virus were incubated at room temperature with soluble  $\alpha\text{v}\beta\text{8}$  at the concentrations indicated, digested with proteinase K, and analyzed by SDS-PAGE and Western blotting with antibody to gB. The blot is representative of 2 independent experiments.

mixed with those transfected with plasmids expressing gHgL, gD, and nectin (19). However, fusion of cells in which HSV gB and gHgL were expressed in *cis* and only gD was expressed in *trans* also were lower, reaching levels less than half of those seen when all proteins were expressed in *cis*, so this may at least in part reflect the greater complexity of the HSV than the EBV and HCMV models.

Of perhaps greater interest were the observations on entry in *trans* made with the gH-null virus, phenotypically negative for gHgL and gp42 (28), which contrasted with those made for both HSV and HCMV. Infection with HSV lacking either gHgL or gB

could not be achieved with either of the missing proteins expressed *in trans* (20), and although HCMV lacking gB could enter a cell expressing gB, a virus lacking gHgL was unable to enter a cell expressing gHgL. It is not clear why HSV entry was not seen with proteins expressed *in trans*. However, the failure of the HCMV particles lacking gHgL to infect a cell expressing gHgL was interpreted as reflecting the need for the gHgL complex, but not gB, to be oriented toward a cell membrane carrying receptors. The identities of such putative receptors are not yet known, but experimental evidence for their existence has been provided by interference studies (43, 44). Receptors for EBV gHgL have been definitively identified as  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ , and  $\alpha v\beta 8$  (15, 16), but the observation that soluble forms of these proteins can trigger EBV fusion (15) implies that the importance of the integrin-gHgL interaction, at least during the initial stages of entry, does not depend on a canonical receptor engagement, including additional signaling or coordinated events. Thus, CHO cells lacking human integrins but expressing gHgL could be infected, albeit at low levels, with the EBV gH-null virus in the presence of a soluble integrin. The infection of human 293 cells expressing both integrins capable of interacting with gHgL and gHgL was substantial. Receptor interference, reported to occur in cells expressing HCMV gHgL, was not an issue, perhaps because in contrast to the HCMV studies, where adenovirus vectors were used to transduce glycoproteins at high levels, the work described here used plasmid vectors which do not express high levels of protein. Certainly no loss of the expression of  $\alpha v$  integrins was observed. Beyond this, however, there is a need to explain how gHgL opposing the virus and not another cell could have been triggered to initiate a fusion cascade, as is currently envisioned for herpesviruses. The EBV that was used was made in a B cell that lacks expression of  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ , and  $\alpha v\beta 8$  and expresses no other gHgL receptors capable of triggering fusion (45, 46), so gHgL could not have been triggered by any cell molecules incorporated in the virus envelope. Thus, we assume that the trigger came either from an integrin expressed in the same cell as gHgL or from an integrin expressed on an adjacent cell. We cannot currently distinguish between these two possibilities.

The ability of the gH-null virus to infect a gHgL-expressing B cell in the presence of a soluble integrin is perhaps easier to envision spatially, although it is interesting that, to this point, it has never been certain that a combination of gHgL and an integrin could substitute functionally for gHgLgp42 and HLA class II for B cell entry. There are clear differences between B cell and epithelial cell fusion that are reflected in the differential behavior of gH mutants (17, 33, 36), and the levels of infection seen here were not high. However, they are consistent with at least some of the events involving gHgL and fusion being common to both cell types. In our hands, a soluble form of gHgL, gHtgL, also was able to support low but reproducible levels of epithelial cell fusion, which contrasts with work done by others with EBV (47, 48). The reasons for these discrepancies at the limits of detection are not clear but may reflect protein expression levels and variability in cell types and assays used. In one case soluble gHgL was made, as in the current study, from a baculovirus construct, but the cells used were CHO cells and not AGS (47). In another case the soluble gHgL was expressed from a plasmid vector in CHO cells. A significant amount of the protein formed aggregates, and fusion was measured only in a B cell context of fusion, with gp42 coexpressed with the soluble gHgL and CHO cells expressing HLA class II as the target (48). The presence of soluble gHgL also decreased the

amounts of gp42 expressed, a characteristic that the authors suggested contributed to a reduced level of fusion seen in its presence. The fusion achieved here with soluble gHtgL is, however, consistent with work done with HSV (19), where fusion levels also were reproducible, although, at 2.1% of fusion mediated by full-length membrane anchored protein, the levels were even lower than those seen here. Function is clearly only residual when gHgL is not membrane anchored, perhaps in part reflecting a spatial flexibility inconsistent with optimal functional interactions.

Current models of herpesvirus entry favor gB as the primary fusogen, and the crystal structures of HSV gB and EBV gB (7, 8) generally are interpreted as representing the postfusion forms which have already undergone the large conformational change thought likely to be required for fusion to occur (10). Such a conformational change would require activating energy to allow refolding. Consistent with previous demonstrations that heat can provide such activation energy for a virus fusogen (22–27), we found that as much as 30% of the fusion seen when gHgL and gB were expressed *in cis* could be achieved by heating cells expressing gB alone to 45°C. This temperature is lower than the 62°C reported to be optimal for a pre- to postfusion conformation change in the class I fusion protein, influenza virus hemagglutinin, where low pH and elevated temperature can be used interchangeably as triggers of fusion (26, 27). It is closer to the 50°C optimum reported for the class II fusion proteins of many paramyxoviruses (23–25). Of even greater interest was the fact that the proteolytic digestion patterns of gB in virus to which heat was applied and in virus triggered by exposure to soluble integrins were the same. This is consistent with a change representing a refolding of gB from a prefusion to a postfusion conformation and, together with observations made with liposomes that HSV gB can directly interact with lipids via its fusion loops while gHgL cannot (49), provides support for the model in which gB is the primary executor of fusion. Somewhat disappointingly, we were unable to reverse the conformational change by returning virus to 37°C and mirror the reversibility of other class III fusion proteins, which undergo a pH-dependent conformational change (50). Whether this reflects a fundamental difference in the thermodynamic stability of gB or simple experimental failure will need further exploration. However, to a limited extent we were able to monitor the appearance of the conformational change by increasing the temperature in small increments or by exposing virus to gradually increasing amounts of soluble integrin. We speculate that at least part of the contribution that the gHgL integrin interaction makes to activation of fusion by gB is by providing Gibbs free energy to facilitate its refolding.

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