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An unusual dependence of human herpesvirus-8 Glycoproteins-induced cell-to-cell fusion on heparan sulfate

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Abstract

Human herpes virus 8 (HHV-8) is known to interact with cell surface heparan sulfate (HS) for entry into a target cell. Here we investigated the role of HS during HHV-8 glycoproteins induced cell fusion. Interestingly, the observed fusion demonstrated an unusual dependence on HS as evident from following lines of evidence: 1) a significant reduction in cell-to-cell fusion occurred when target cells were treated with heparinase; 2) in a competition assay, when the effector cells expressing HHV-8 glycoproteins were challenged with soluble HS, cell-to-cell fusion was reduced; and, 3) coexpression of HHV-8 glycoproteins gH-gL on target cells resulted in inhibition of cell surface HS expression. Taken together, our results indicate that cell surface HS can play an additional role during HHV-8 pathogenesis.

Keywords

conjunctival epithelium; heparan sulfate; human herpes virus 8; glycoprotein

1. Introduction

Cell entry of herpesviruses is a highly coordinated two-step process that involves the initial attachment of the virus to the cell surface heparan sulfate (HS) and subsequent membrane fusion to deliver the viral genome into host cell cytoplasm [1]. HS glycosaminoglycans are unbranched sulfated polysaccharides that are found on the surface of most cells [2]. The binding of many viruses to cell surface HS is a critical step in the infection of a cell [3]. Although the requirements of fusion receptors vary among herpesviruses [4], however, the use of ubiquitously expressed HS during initial viral attachment/binding is a common feature shared by the herpesviridae [1,5] including human herpesvirus-8(HHV-8) [6,7]. This feature also implicates a common invasion strategy shared by many herpesviruses.

According to the current model of HHV-8 entry, the viral envelope glycoprotein B (gB) mediates the initial contact with cell surface HS [8]. After attachment, interaction of gB allows the virus to bind with its cellular receptors: $\alpha_3\beta_1$ integrin and a newly discovered cystine

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transporter xCT and DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) receptors for entry [8-10]. During entry, two other viral glycoproteins, gH-gL, together with gB mediate the membrane fusion of the viral envelope with host the cell membrane.

Normally HS is thought to be important during virus attachment to cells but not during herpesvirus-induced cell-to-cell fusion [1]. If at all, it normally has a negative influence on cell-to-cell fusion (O'Donnell and Shukla, unpublished results) unless a highly modified form, 3-O sulfated HS [5], is the major receptor for entry. The present study, however, provides evidence that HS may play an important positive role during HHV-8 glycoproteins mediated cell-to-cell fusion. It also suggests that the fusion may be pH-dependent. The unusual dependence on HS suggests a deeper role for it during HHV-8 entry and spread than previously appreciated.

2. Materials and methods

2.1. Cell cultures

Human embryonic kidney cells (293T), human conjunctival epithelial cells (HCjE) and Chinese hamster ovary (CHO-K1) cells were used [11-13]. 293T cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) and antibiotics. HCjE cells were provided by Ilene K. Gipson (Harvard University, Boston, MA) were grown in keratinocytes serum-free media (SFM; Gibco-Invitrogen Corp., Rockville, MD) supplemented with 25 µg/ml bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF), penicillin and streptomycin, and 0.4 mM calcium chloride and were cultured as previously described [11]. Dr. Patricia G Spear (Northwestern University, Chicago) kindly provided wild-type Chinese hamster ovary (CHO-K1) cells and CHO-745 cells that are deficient in heparan sulfate (HS) or GAG biosynthesis. Both wild-type CHO-K1 cells and CHO-745 cells were grown in Ham's F12 (Invitrogen Corp, Carlsbad, CA) supplemented with 10% FBS as described previously [5].

2.2. Plasmids

The plasmids used in this study were pPEP29 (gB), pPEP39 (gH), pPEP109 (gL) that express HHV-8 glycoproteins, plasmid pT7EMCLuc that express firefly luciferase gene under T7 promoter, plasmid pCAGT7 that expresses T7 RNA polymerase [14]. The control plasmid, pCDNA3, was purchased from Invitrogen (Carlsbad, CA, USA).

2.3. Cell fusion assays and estimation of cell fusion

Cell fusion assays were performed as described previously [15,16]. Briefly, the CHO-K1 cells designated "effector" cells were co-transfected using lipofectamine (Gibco/BRL) with plasmids expressing three HHV-8 glycoproteins glycoproteins, pPEP29 (gB) or gB mutant, pPEP39 (gH), pPEP109 (gL), along with the plasmid pT7EMCLuc (0.5 µg DNA of each plasmid). The cultured "target" cells (HcJE or 293T or CHO-K1 cells) grown up to 50-60% confluency were co-transfected with pCAGT7 that expresses T7 RNA polymerase using chicken actin promoter and CMV enhancer [14]. The effector cells expressing pT7EMCLuc and pCDNA3.1 (devoid of any glycoproteins) co-cultured with the target cells expressing T7 RNA polymerase were considered as controls. The 293T, CHO-K1 and HCjE cells designated as "target cells" were transfected with a vector expressing T7 RNA polymerase. At 18 h post transfection, both the effector and the target cells were mixed together (1:1 ratio) and co-cultivated. The activation of the reporter luciferase gene as a measure of cell fusion was examined after 30 hours. A group of fused cells (8-10 joint cells) were scored positive for cell fusion.

2.4. Microscopic visualization of HHV-8 glycoproteins induced cell fusion

Effector CHO-K1 cells expressing HHV-8 glycoproteins (gB, gH-gL) were co-expressed with green fluorescent protein (GFP) expression plasmid, similarly the target 293T cells were transfected with red fluorescent plasmid (RFP). Before co-culture, one pool of target 293T cells was pre-treated with heparinase I (1.0 µg/ml), while other pool was treated with PBS as a control. Both cultured cells were co-cultured in chamber polystyrene vessels (BD Falcon) for 30 hours. The cells were fixed and stained with giemsa.

2.5. Effect of pH on HHV-8 glycoproteins induced cell fusion

Monolayers of target cells (293T and HCjE cells) were either mock treated or treated with 0.08 µM concentration of lysosomotropic agent bafilomycin [BFLA-1] before co-culture with effector CHO-K1 cells expressing HHV-8 glycoproteins. Relative luciferase unites (RLUs) were measured using luminometer from four independent experiments performed in triplicate.

3. Results and Discussion

We begin our investigation to determine the ability of a natural target HcJc cell to fuse with HHV-8 glycoprotein expressing cells and the significance of HS in this process. In our experiments, the CHO-K1 cells designated “effector” cells were co-transfected with individual HHV-8 glycoproteins; pPEP29 (gB), pPEP39 (gH), pPEP109 (gL) or in combinations (gH-gL and gB, gH-gL) along with the plasmid pT7EMCLuc that expresses firefly luciferase gene [14]. The gB mutant used in this study had a deletion in the last 58 amino acids of the cytoplasmic tail to enhance to cell surface expression of gB [14]. The 293T, CHO-K1 and HCjE cells designated as “target cells” were transfected with a vector expressing T7 RNA polymerase. As shown in Fig.1 (panels A-C), maximum luciferase activity occurred when the effector cells were expressed with the three HHV-8 glycoproteins gB, gH-gL irrespective of cell type tested. The presence of a correct set of viral glycoproteins in one set of effector cells together with the presence of cellular receptors on target cells are the prerequisites for efficient cell fusion. Interestingly, the set of viral glycoprotein requirements for cell fusion varies among herpesviruses [17-20]. The demonstration that expression of three HHV-8 glycoproteins (gB, gH-gL) results in cell fusion in natural target HCjE cells of ocular origin was in line with previously published finding with 293T cells [14]. Of note, co-cultivating CHO effector cells expressing HHV-8 glycoproteins with CHO target cells had the lowest luciferase activity (Fig. 1B and 1E). It is possible that processing and expression of HHV-8 glycoproteins could be cell-type dependent [14]. It is also possible that HHV-8 receptor(s) expressed in CHO cells are at lower level as compared to 293T and HCjE cells.

In order to determine whether the cell surface HS has a specific role in HHV-8 induced cell fusion, the effect of HS degrading enzyme, heparinase-I, on cell fusion was examined. Heparinase I selectively cleaves both heparin and HS chains [21]. The target cells (293T, CHO-K1 and HCjE) were divided into two equal pools. Heparinase-I (1.5 units/ml) was added to one pool while the other pool was compensated with an identical volume of PBS [5]. Both treated and untreated pools were mixed with equal amounts of effector CHO-K1 cells expressing HHV-8 glycoproteins. After incubation there was nearly 70% reduction of the fusion in the heparinase-I treated cells as compared to their corresponding untreated 293T and HCjE cells (Fig. 1, panels D and F). Therefore, removal of HS from cell surface results in significantly reduced fusion. A similar effect of heparinase was also reported during HSV-1 induced cell to cell fusion when 3-O sulfated HS is used as the major receptor for its entry glycoprotein, gD [15,16].

We next microscopically visualized the HHV-8 glycoprotein induced cell fusion in presence and absence of heparinase. Pre-treatment of target cells (293T, CHO-K1 and HCjE) with

heparinase resulted in a significant decrease in the number of fused cells both visually and quantitatively (Fig. 2A; panels: c, g, k and d, h, l, respectively), compared to PBS treated target cells (Fig. 2A; panels: a, e, and i). In addition, polykaryocytes tended to be larger and contained more nuclei in heparinase untreated target cells (Fig. 2A; panels: a, e and i).

Confocal imaging to visually demonstrate the HHV-8 glycoproteins-induced fusion was next performed. Effector CHO-K1 cells expressing HHV-8 glycoproteins (gB, gH-gL) were co-expressed with green fluorescent expression plasmid, similarly the target 293T cells were transfected with red fluorescent plasmid. Before co-culture, one pool of target 293T cells was pre-treated with heparinase, while other pool was treated with PBS as a control. The PBS treated 293T cells showed the co-localization (yellow) after co-cultivation with effector (red) and target (green) cells (Fig. 2B; panels a, b). In contrast, minimal cell fusion was observed between effector and target cells in the presence of heparinase (Fig. 2B; panel c). Interestingly, we also noticed intense co-localization in a cup-like structure formed by the target cell in close contact with HHV-8 glycoproteins expressing effector cell (Fig. 2B; panels a, b). The ability of target cells to form a cup-like structure was not observed in heparinase-treated target cells. This observation is interesting in the light of the fact that herpesvirus-cell interaction leads to rearrangement of actin cytoskeleton to facilitate viral entry [22,23]. Notably, a recent study has shown that HHV-8 gH-gL complex has ability to adhere at lamellipodium-like structures [24].

To further verify the results obtained from heparinase I treatment and the specific role of cell surface HS in fusion, a competition assay was performed using a soluble form of HS. Soluble HS from bovine kidney (ICN; 1.0 µg/ml) was added to CHO-K1 effector cells expressing viral glycoproteins prior to mixing with 293T target cells. The glycoproteins expressing effector cells with mock HS treatment (buffer alone) was used as a control. As shown in Fig. 3 (panel A with white bars), soluble HS inhibited fusion between 293T target cells and effector CHO-K1 expressing HHV-8 gB, gH-gL or gB (mut), gH-gL, while HS untreated cells had fusion activity (Fig. 3, panel A with black bars). Previously it has been shown that HHV-8 glycoprotein B (gB) and MHV-68 glycoprotein H (gH) interacts with cell surface HS [6,25, 26]. Therefore, it was expected that pretreatment of HHV-8 glycoproteins expressing effector CHO-K1 cells with soluble HS would prevent binding to target 293T cells. The demonstration that HHV-8 induced cell-to-cell fusion was significantly impaired either by enzymatic removal of target cell surface HS by heparinase treatment, or by pretreatment of soluble HS to the effector cells expressing viral glycoproteins suggest a strong possibility for direct interactions between cell surface HS and HHV-8 glycoproteins for fusion.

To assess whether co-expression of HHV-8 glycoproteins in target 293T cells had any effects on the cell surface expression of HS, a cell surface enzyme-linked immunosorbent assay (cell-ELISA) was used. As shown in Fig. 3 (panel B), cell-ELISA detected a significant reduction in the expression of cell surface HS when 293T target cells were co-expressed with HHV-8 glycoproteins H and L (gH-gL). In this experiment, wild-type CHO-K1 cells that express HS were used as a positive control and CHO-K1 mutant cell line (pgsA-745) that does not express cell surface HS was used as the negative control. In parallel experiments, we also confirmed the expression of HHV-8 glycoproteins in effector CHO-K1 cells. The detection of HHV-8 gB, gH alone or gH-gL in combination was observed, while no gL was detected in absence of gH (data not shown). These results were in agreement with previous reports [24].

To assess whether co-expression of HHV-8 glycoproteins in target 293T cells also negatively affect cell fusion, we next performed the cell fusion assay. Our hypothesis was that expression of HHV-8 glycoproteins should lead to down regulation of receptors, including HS. As can be seen in Fig 3 (panel C), co-expression of HHV-8 gH-gL in 293T cells significantly inhibited not only HS expression but significantly reduced cell fusion. Similar down-regulation of HS

and cell fusion were observed in the human conjunctival cells (data not shown). The result that HHV-8 gH-gL renders target cells more resistant to fusion mimics the inhibition of HHV-8 infection by the expression of gH-gL [24].

We next examined the role of pH during HHV-8 glycoproteins-mediated cell fusion. It has been shown that HHV-8 entry into a cell is mediated by endocytosis and therefore is pH dependent [27]. Thus, the effect of lysosomotropic agent bafilomycin A1 (BFLA-1), an inhibitor of the vacuolar type H⁺ ATPase, was tested on HHV-8 glycoproteins-mediated cell fusion. BFLA-1 blocks the lowering of pH within acidic compartments in the cell, including the endosomal lumen [28]. Monolayers of 293T or HCjE target cells were pretreated with BFLA-1 before co-culturing with HHV-8 glycoproteins expressing effector cells. As shown in Fig. 3 (panels D, E), BFLA-1 significantly blocked HHV-8 cell fusion in both 293T and HCjE cells suggesting the role of pH during the fusion event.

Taken together our results provide convincing evidence in support of a potential role of HS in HHV-8 induced cell-to-cell fusion in 293T cells and cells of human ocular origin. Our results correlate well with a recent finding that HS proteoglycans, syndecans, can act as receptors for HHV-8 gH/gL and enhance HHV-8 infection into 293T cells [24]. Similarly, over expression of Ext1 enzyme, which is critically required in HS biosynthesis, seems to increase the infection of a closely related murine gammaherpesvirus, MHV-68 [29]. Future studies will need to address whether interaction with HS/HS proteoglycans marks an independent but essential step during fusion, or it is part of a cascade of interactions required to optimize the fusion? In any case, it appears likely that HS has a deeper and perhaps more direct role in HHV-8 induced membrane fusion than previously appreciated.

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Abbreviations

HS	heparan sulfate
HCjE	human conjunctival epithelial cells
HSV-1	human herpes virus 8

Fig. 1.

(A-C). HHV-8 glycoproteins [gB, gH-gL] are required for cell fusion in various cell lines. Effector CHO-K1 cells (Ec) were transfected with plasmids expressing the indicated individual HHV-8 glycoproteins or in combination along with the luciferase reporter plasmid. In parallel 293T, CHO, and human conjunctival epithelial cell (HCjE) target cells (Tc) were transfected with T7 RNA polymerase. Both Ec and Tc were then co-cultured in three different combinations as shown in panel A (CHO + 293T); panel B (CHO + CHO-K1) and panel C (CHO-K1 + HCjE). After co-cultivation of the Ec and Tc, relative luciferase units (RLUs) were measured by using luminometer. Error bars represent standard deviations. * $P < 0.05$, analysis of variance (ANOVA).

(D-F). Enzymatic removal of cell surface heparan sulfate [HS] results in significant reduction in HHV-8 induced cell-to-cell fusion in various cell lines. The target cells 293T (panel D), CHO (panel E), and HcJE (panel F) were separately treated with heparinase I (white bar) or left untreated (black bar) prior to co-cultivation with CHO-K1 effector cells expressing HHV-8 glycoproteins [gB, gH-gL or gBmut, gH-gL]. Effector CHO-K1 cells devoid of glycoproteins was considered as negative control (grey bar). RLUs were measured 30 h after co-cultivating the effector and target cells. The significant difference was noticed in heparinase treated target cells compared to untreated cells. * $P < 0.05$, ANOVA.

Fig. 2.

Microscopic visualization and quantification of HHV-8 glycoproteins mediated cell fusion in presence of heparinase. (A) In this experiment, effector CHO-K1 cells (Ec) were transfected with HHV-8 glycoproteins [gB, gH, gL]. The corresponding target cells (Tc; 293T, CHO and / or HCjE) were either pre-treated with heparinase I (1.0 μ g/ml) or left untreated before co-cultivation for 30 hrs. The cells were fixed and stained with giemsa. The boxed region in heparinase untreated cells (shown in panels a, e and i) indicating fused cells are highlighted in panels b, f and j. The effects of heparinase treated cells are shown in panels c, g and k. The fused cells were counted for both heparinase treated and untreated cells (panels d, h and l). Error bars represent standard deviations. * $P < 0.05$, ANOVA.

(B) Effector CHO-K1 [Ec] cells were co-transfected with red fluorescent protein (RFP-expressing Ds-Red N1) construct along with HHV-8 gB, gH-gL expressing plasmids, while target 293T cells [Tc] were transfected with green fluorescent protein (GFP) expressing plasmid and viewed using confocal microscope (Nikon eclipse TE2000-E) at $60\times$ objective. The fusion [co-localization] between Ec and Tc are shown in panels a, b. The enlarged box region in panel b shows the formation of a cup shaped structure (regions highlighted by the arrows) during fusion between Ec and Tc cells. Panel c demonstrates the effect of heparinase treatment on HHV-8 glycoprotein mediated cell fusion. Bar scale 20 μm .

Fig. 3.

(A). Soluble HS inhibits HHV-8 induced cell fusion. The effector [Ec] CHO-K1 cells expressing HHV-8 glycoproteins [gB, gH-gL] were treated with soluble HS (white bar), or mock treated (black bar), prior to co-cultivation with target 293T cells. RLUs were measured 24 h post cocultivation. * $P < 0.05$, ANOVA.

(B) Co-expression of HHV-8 glycoproteins gH-gL in 293T cells result in significant inhibition of cell surface heparan sulfate (HS). Cell surface expression of HS was determined by cell-ELISA. CHO K-1 cells transfected with pCDNA3 plasmid were used as a positive control (black bar) while a glycosaminoglycans [GAGs] deficient cell line [pgsA-745] (white bar) along with heparinase treated (HS+) CHO-K1 cells (dark grey bar) was used as negative

control. 293T cells co-transfected with individual glycoprotein plasmid expressing HHV-8 gB, gH, gH-gL and K8.1, or with empty vector [pCDNA3.1]. Live cells were exposed to 1:100 dilution of anti-HS monoclonal antibody [10E4 epitope] for 30 min and then washed and fixed. Binding of the primary antibodies was detected by use of biotinylated secondary antibodies and streptavidin-conjugated horseradish peroxidase [AMDEX]. The peroxidase activity was assayed as a measure of HS expression on cell surface. * $P < 0.05$; ** $P < 0.01$, ANOVA.

(C) Expression HHV-8 glycoproteins gH-gL in 293T target cells result significant inhibition of cell fusion. The target 293T [Tc] cells transfected with individual HHV-8 glycoproteins [gB, gH, gH-gL, K8.1 or pCDNA3.1 control plasmid] were co-cultivated with CHO-K1 effector cells [Ec] expressing HHV-8 glycoproteins [gB, gH-gL]. The data shown in the above experiments are the means of triplicate measures and are representative of three independent experiments. * $P < 0.05$, ANOVA.

(D-E). Lysosomotropic agents negatively affect HHV-8 glycoprotein-mediated cell fusion in various cell lines. Monolayers of target cells (293T cells in panel D and HCjE in panel E) were either mock treated (black bars) or treated with indicated concentration of bafilomycin [BFLA-1] (white bars) prior to mixing with effector cells expressing HHV-8 glycoproteins [gB, gH-gL]. Effector cells devoid of HHV-8 glycoproteins co-cultured with target 293T cells and HCjE cells were used as negative control (grey bars). RLUs were measured using luminometer from four independent experiments performed in triplicate. * $P < 0.05$, ANOVA.