A role for 3-O-sulfated heparan sulfate in cell fusion induced by herpes simplex virus type 1

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Membrane fusion induced by herpes simplex virus (HSV) is required for both entry and cell-to-cell spread. It is mediated by the viral glycoprotein gB, gD, gH–gL and gD receptors. Although 3-O-sulfated heparan sulfate (3-OH HS) is a receptor for HSV-1 entry, the requirement for heparan sulfate in the fusion process has been ruled out. Here, it is demonstrated that cells expressing 3-OH HS, generated by D-glucosaminyl 3-O-sulfotransferase isoforms-3 and/or -5 (3-OST-3 and 3-OST-5), fused with cells expressing the four glycoproteins. The cell fusion observed exhibited similar requirements but was independent of protein receptors, HVEM or nectin-1. Additionally, removal of 3-OH HS from the cell surface by heparinase-I treatment and, in separate experiments, the presence of soluble 3-OST-3 and 3-OST-5-modified HS, significantly inhibited fusion. Taken together, these results indicate that 3-OH HS can play a crucial role in virus entry and cell fusion.

Heparan sulfate (HS) glycosaminoglycans are unbranched polymers covalently attached to the protein cores of proteoglycans. The HS polymer is assembled by sequential addition of D-glucuronic acid (or iduronic acid) alternating with N-acetylgalactosamine. The chains are then modified heterogeneously, and in domains, by multiple enzymes. These modifications provide specific binding sites for a variety of proteins, including cell adhesion molecules, growth factors, chemokines, and factors regulating angiogenesis and blood coagulation (reviewed by Lindahl et al., 1998; Rosenberg et al., 1997). HS moieties on cell surface proteoglycans have also been implicated in pathogenesis induced by human herpesviruses and multiple other viruses (Liu & Thorp, 2002; Shukla & Spear, 2001). The ubiquitous and abundant expression of HS on mammalian cell surfaces makes it an ideal snare for the capture of viruses. In the case of herpes simplex virus type-1 (HSV-1), it was recently shown that 3-O-sulfated heparan sulfate (3-OH HS) generated by D-glucosaminyl 3-O-sulfotransferase (3-OST) isoforms-3 and -5, but not -1, can act as a receptor for HSV-1 envelope glycoprotein gD (Shukla et al., 1999; Xia et al., 2002). The demonstration of the binding sites within HS for gD, therefore, raises the possibility that properly modified HS can mediate both attachment and membrane fusion processes, including cell fusion, involved in HSV-1 entry and spread.

During entry, penetration proceeds by fusion of the viral envelope with the cell membrane. The current model of HSV entry suggests that viral envelope glycoprotein gC or glycoprotein gB makes the initial contact with HS (Herold et al., 1991; Shieh et al., 1992; WuDunn & Spear, 1989). After the attachment, interaction of gD with one of its receptors, in association with three other glycoproteins (gB, gH and gL), triggers the fusion between the viral envelope and cell membrane leading to penetration (Spear & Longnecker, 2003; Yoon et al., 2003). Several cellular receptors for HSV-1 gD are known. They belong to three different classes that include a member of the TNF-receptor family named HVEM (HveA) (Montgomery et al., 1996) and two members of the immunoglobulin superfamily, designated nectin-1 or HveC (Cocchi et al., 2000; Geraghty et al., 1998) and nectin-2 or HveB (Warner et al., 1998). Unique sites within HS chains, generated by 3-OSTs, give rise to the newest family of gD receptors, the 3-OH HS (Shukla et al., 1999; Xia et al., 2002). The 3-OSTs act to modify HS late in its biosynthesis (reviewed by Lindahl et al., 1998), and each isoform recognizes, as substrate, glucosamine residues in regions of the HS chain having specific, but different, prior modifications, including epimerization and sulfation at other positions (Liu et al., 1999; Xia et al., 2002). Thus, each 3-OST can generate potentially unique protein-binding sites within HS. To date, six different isoforms of 3-OSTs (3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4 and 3-OST-5) are known. All, except 3-OST-1, generate HSV-1 entry receptors (Shukla & Spear, 2001; Xia et al., 2002). Interestingly, only 3-OST-3A and 3-OST-3B generate structurally identical gD receptors (and hence are used
interchangeably throughout this study). The gD receptors generated by other isoforms are very similar, but likely not identical, in structure (Liu & Thorp, 2002; Shukla et al., 1999; Shukla & Spear, 2001; Xia et al., 2002). 3-OST-1 generates binding sites for antithrombin (Liu et al., 1999; Shworak et al., 1999) but fails to produce a receptor that binds to HSV-1 gD (Shukla et al., 1999). 3-OSTs (one or more) are expressed in human and mouse tissues relevant to HSV-1 infection examined thus far (Mochizuki et al., 2003; Shworak et al., 1999; Xia et al., 2002).

Multinucleated giant cells (polykaryocytes) resulting from virus-induced cell fusion are a hallmark of HSV-1 infection. Recently it was demonstrated that gB, gD, gH, gL (Browne et al., 2001; Muggeridge, 2000; Turner et al., 1998), and expression of HVEM or nectin-1, are required for cell fusion (Pertel et al., 2001). Interestingly, using cell lines deficient in glycosaminoglycan (GAG) biosynthesis but expressing gD protein receptors, it was also demonstrated that cell fusion did not require HS (Browne et al., 2001; Pertel et al., 2001). In contrast, the requirement for HS during cell fusion was previously suggested by showing that the syncytial mutant HSV-1 (MP) could induce the fusion of wild-type Chinese hamster ovary (CHO) cells but not of mutant cells defective for HS or GAG biosynthesis (Shieh & Spear, 1994).

The aim of this study was to determine whether the gD-binding form of HS, 3-O-ST HS, has a specific role in HSV-1-induced cell fusion, or not; and to dissect further the virus and cellular requirements of 3-O HS-mediated cell fusion. For that purpose, we decided to focus on three well-studied 3-OSTs: 3-OST-1, 3-OST-3 and 3-OST-5. While HS modified by the latter two mediate HSV-1 entry, 3-OST-1 failed to generate HSV-1 entry receptor (Shukla et al., 1999; Xia et al., 2002). In order to quantify HSV-1 glycoproteins and 3-O HS-induced cell fusion, a luciferase reporter gene activation assay was used (Pertel et al., 2001). Wild-type CHO-K1 cells express cell surface HS but lack functional gD receptors, including 3-O HS (Shukla et al., 1999). As a result, they are resistant to both HSV entry and virus-induced cell fusion (Montgomery et al., 1996; Shieh et al., 1992). In our experiments, the CHO-K1 cells designated 'effector' cells were co-transfected with plasmids expressing four HSV-1 (KOS) glycoproteins, pPEP98 (gB), pPEP99 (gD), pPEP100 (gH) and pPEP101 (gL), along with the plasmid pT7EMC1Luc that expresses the firefly luciferase gene under the T7 promoter (all plasmids described by Pertel et al., 2001). The other CHO-K1 cell population or 'target' cells were co-transfected with 3-OST-expressing plasmid (either 3-OST-1, 3-OST-3 or 3-OST-5) or HVEM plasmid (pBEC10) (Montgomery et al., 1996; Shukla et al., 1999; Xia et al., 2002) and pCAGT7, which expresses T7 RNA polymerase using chicken actin promoter and CMV enhancer (Pertel et al., 2001). The effector cells expressing pT7EMC1Luc and pCDNA3 (devoid of any glycoproteins) and the target cells expressing 3-OST-5 with T7 RNA polymerase were taken as the control. All cells were grown in six-well dishes containing F-12 Ham medium (Gibco) with 10% fetal bovine serum. At 18 h post-transfection, both the effector and the target cells were mixed together (1:1) and co-cultivated. The activation of the reporter luciferase gene as a measure of cell fusion was examined after 24 h. As shown in Fig. 1(A), CHO-K1 cells (control) in the absence of HSV-1 glycoproteins failed to fuse despite the presence of 3-OST-5 in the target cell, implying an essential role for glycoproteins during cell fusion. Interestingly, cells expressing 3-OST-3 but not 3-OST-1 induced cell fusion provided all four glycoproteins were co-expressed in the effector cells (Fig. 1A). The demonstration that 3-OST-1 expression did not result in fusion was very much in line with the fact that 3-OST-1-modified HS fails to bind HSV-1 gD, which is in turn required for the induction of fusion (Pertel et al., 2001; Shukla et al., 1999). Interestingly, cell fusion mediated by nectin-1 was higher than that mediated by 3-OST-3 and/or 3-OST-5 (Fig. 1A). This was not unexpected since it correlates well with higher entry signals reported with nectin-1 compared to 3-OST-3-expressing CHO-K1 cells (Shukla et al., 1999). One possibility for the observed higher entry and fusion with nectin-1 is enhanced receptor expression; higher than physiological levels of receptors are produced when nectin-1 is overexpressed in CHO-K1 cells. However, since 3-OST-3 and 3-OST-5 are HS-modifying enzymes, their activity is dependent on the availability of specific substrate sites within existing HS chains (Shukla et al., 1999; Xia et al., 2002). As shown in Fig. 1(B), a cell surface enzyme linked immunosorbent assay (cell-ELISA) did not detect any enhancement of cell surface HS levels by 3-OST-3 and/or 3-OST-5 overexpression in CHO-K1 cells compared to the wild-type cells. A CHO-K1 mutant cell line (pgsA-745) that does not express any cell surface HS was used as the negative control. Thus, overexpression of 3-OST-3 and/or 3-OST-5 does not enhance cell surface HS population, which, in turn, could partly be responsible for the lower HSV-1 entry and fusion compared to nectin-1 (Shukla et al., 1999).

Since multiple human tissues express more than one 3-OST isoform, and the possibility remains that co-expression of multiple isoforms could translate into additional gD-binding sites within HS chains, we decided to examine the effect of co-expression of 3-OST-3 and 3-OST-5 on the cell fusion process. As seen in Fig. 1(A), the combined effect of 3-OST-3 and 3-OST-5 co-expression showed about a twofold increase in fusion when compared to individual expression of 3-OST-3 or 3-OST-5 alone. The additive effect of 3-OST-3 and 3-OST-5 is clearly not due to any increase in cell surface HS population (Fig. 1B) and it is also not due to any changes in glycoprotein expression, since effector cells used in Fig. 1(A) were derived from a common pool of transfected cells. Therefore, enhanced luciferase activity suggests that possible co-expression of 3-OSTs in certain cells and tissues may cause higher susceptibility to HSV-1-induced cell fusion. It is also evident from Fig. 1(A) that the cell fusion was exclusively dependent on the expression of appropriate 3-OST and
O-sulfotransferase isoforms (3-OSTs) are not expressed naturally by CHO-K1 cells, independent of HVEM or nectin-1, since CHO-K1 cells do not express these protein receptors naturally (Spear et al., 2000). In order to verify the glycoprotein requirement for fusion mediated by 3-OS HS, we transfected CHO-K1 cells with various combinations of HSV-1 glycoproteins and performed the fusion assay using CHO-K1 with 3-OST-3B as target cells. As shown in Table 1, 3-OS HS-mediated fusion required co-expression of all four glycoproteins, gB, gD, gH and gL, which were also reported to be essential for fusion via HVEM and nectin-1 (Pertel et al., 2001; Turner et al., 1998). An identical glycoprotein requirement was also observed with target cells expressing 3-OST-5. Therefore, it is possible that all three gD receptors (3-OS HS, HVEM and nectin-1) mediate cell fusion via a common mechanism. Interestingly, the three receptors are already known to bind gD with almost identical affinities (Spear et al., 2000).

Since expression of 3-OSTs results in efficient cell fusion, it is very likely that the 3-OS HS generated by them is the actual mediator of fusion. Therefore, in order to confirm that possibility, we examined the effect of the HS-degrading enzyme heparinase-I on cell fusion. This enzyme selectively cleaves both heparin and HS chains containing 1→4 linkages between glucosamines and O-sulfated iduronic acid residues (Ernst et al., 1995). CHO-K1 cells transiently transfected with 3-OST-5 expression plasmid were divided into two equal pools. Heparinase-I (1.5 U ml⁻¹, Sigma) was added to one pool while the other pool was compensated with an identical volume of phosphate-buffered saline (PBS) (Shukla et al., 1999). Both treated and untreated pools were mixed with equal amounts of HSV-infected cells to form multinucleated polykaryocytes. To verify that 3-OS HS-mediated fusion also results in polykaryocyte formation, we either mock transfected CHO-K1 cells with empty vector (pCAGGS), 3-OST3H-expression plasmid alone, or in combination with 3-OST-5-expression plasmid, as target cells. These cells were then mixed and co-cultivated with the effector cells expressing the four glycoproteins for 24 h and then stained with Giemsa solution. The polykaryocytes observed with cells co-expressing 3-OST-3B and 3-OST-5 were larger and more numerous than cells expressing 3-OST-3B alone (data not shown). This result, taken together with the data shown in Fig. 1(A), suggests that the co-expression of 3-OST-5 and 3-OST-3B enhances both fusion and polykaryocyte formation.

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**Fig. 1.** (A) Cell fusion is dependent on the expression of appropriate 3-O-sulfotransferase(s). Chinese hamster ovary (CHO)-K1 cells were used as effector and target cells. Effector cells were transfected with plasmids expressing HSV-1 gB, gD, and gH–gL and luciferase reporter plasmid. Target CHO-K1 cells were transfected with plasmids expressing T7 RNA polymerase and 3-O-sulfotransferase isoforms (3-OST-1, 3-OST-3B, 3-OST-5 as indicated) or nectin-1. Effector cells without glycoproteins were considered the control. Luciferase activity was measured 24 h after co-cultivating the effector and target cells. Relative luciferase units (RLUs) were determined using a Sirius luminometer (Berthold detection systems) and are from one experiment performed in triplicate. (B) Overexpression of 3-OSTs does not result in enhanced cell surface heparan sulfate (HS). Cell surface expression of HS was determined by cell-ELISA. Chinese hamster ovary (CHO)-K1 cells transfected with the pCDNA3 plasmid were used as a positive control while a glycosaminoglycans (GAGs)-deficient cell line (pgsA-745) was used as a negative control. CHO-K1 cells were transfected with plasmid expressing 3-OST-3A, 3-OST-5, and both 3OST-3 and 3-OST-5, or with empty vector (pCDNA3). The cells were grown in 96-well culture dishes. Twenty-four hours later, cell-ELISA assays were performed. Live cells were exposed to a 1:100 dilution of anti-HS monoclonal antibody (10E4 epitope) (US Biological) for 30 min and then washed and fixed with PBS containing 2% (v/v) formaldehyde and 0.2% glutaraldehyde. Binding of the primary antibodies was detected by use of biotinylated secondary antibodies and streptavidin-conjugated horseradish peroxidase (AMDEX, Amersham). The peroxidase activity was assayed as a measure of HS expression on the cell surface.

**Table 1.** HSV-1 glycoproteins required for cell fusion mediated by 3-OS HS

<table>
<thead>
<tr>
<th>Glycoproteins expressed in effector cells</th>
<th>Fusion*</th>
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</thead>
<tbody>
<tr>
<td>gB, gH, gL</td>
<td>No</td>
</tr>
<tr>
<td>gD, gH, gL</td>
<td>No</td>
</tr>
<tr>
<td>gB, gD, gH, gL</td>
<td>Yes</td>
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*Fusion was determined by luciferase reporter gene activation assay (Pertel et al., 2001).
Fig. 2. Enzymic removal of cell surface HS results in detectably reduced fusion. The effector cells expressing viral glycoproteins (gB, gD, gH–gL) were treated with heparinase-I (white bar) or left untreated (black bar), prior to co-cultivation with target cells expressing 3-O-sulfotransferase isoform 5 (3-OST-5). Control effector cells were devoid of glycoproteins. Percentage values of luciferase activity are shown from one experiment performed in triplicate.

Fig. 3. Soluble 3-O-sulfated heparan sulfate (3-OS HS), modified by 3-OST-3 and 3-OST-5, inhibits cell fusion. The effector cells were treated with soluble 3-OS HS (modified in vitro by 3-OST-1, 3-OST-3 and 3-OST-5), or mock treated (none), prior to co-cultivation with target cells. Luciferase activity was measured 24 h post co-cultivation. Percentage increases in luciferase activity, relative to the control from one experiment performed in triplicate, are shown.

effector cells and co-cultivated. As shown in Fig. 2, a nearly 40% reduction in fusion was observed with heparinase-I-treated cells as compared to untreated cells. Therefore, removal of HS from the cell surface, which includes 3-OS HS, by heparinase-I results in detectable reduced fusion.

To further verify the specific role of 3-OS HS in fusion, a competition assay was performed using soluble forms of HS. Approximately 1·0 μg ml⁻¹ of the soluble HS from bovine kidney (ICN) modified in vitro either by purified 3-OST-1, 3-OST-3α, or 3-OST-5 (procedures described in Liu et al., 1999; Chen et al., 2003) was added to the glycoprotein-expressing effector cells, prior to mixing with 3-OST-5-expressing target cells. The extent of 3-O-sulfation to the HS was monitored by determining the incorporation of [³⁵S]sulfate into the polysaccharide after in vitro modification by 3-OSTs (procedures described in Shukla et al., 1999). The estimated numbers of 3-O-sulfate groups per HS polysaccharide chain was 1·1 sulfate per chain for 3-OST-1-modified HS, while for both 3-OST-3- and 3-OST-5-modified HS the values were 1·3 sulfate per chain. The concentration of unlabelled HS was determined using alcian blue (Bjornsson, 1998). Glycoprotein-expressing effector cells, with mock HS treatment (buffer alone), were taken as a control. As shown in Fig. 3, the in vitro 3-OST-1-, 3-OST-3α- and 3-OST-5-modified forms of HS inhibited fusion. Clearly, the impairment of fusion was more pronounced when HS was 3-O-sulfated in vitro by either 3-OST-3α or 3-OST-5 compared to 3-OST-1. This result indicates the specificity of different forms of in vitro modified, soluble 3-OS HS in blocking fusion. It is interesting that blocking by 3-OS HS was more pronounced than with soluble heparin. Browne et al. (2001) have previously found that at least 50 μg heparin ml⁻¹ was required to effectively block the fusion of COS 7 cells expressing the glycoproteins with Vero cells. Some possible explanations for our result include the use of a different cell line (CHO-K1), use of a specific gD receptor (3-OS HS), and soluble HS (instead of heparin).

Taken together, our results provide convincing evidence in support of a potential role of 3-OS HS in cell fusion. Clearly HS, including 3-OS HS, can play multiple roles in HSV-1 pathogenesis. It initially helps with attachment to cell surfaces (Shieh et al., 1992), provides a receptor for gD binding and entry (Shukla et al., 1999; Xia et al., 2002), and now we demonstrate that it also mediates cell fusion, which is a means of HSV-1 spread. These findings are important given the fact that HS proteoglycans, along with one or more HSV-1 entry receptor-generating 3-OSTs, are expressed widely in human cell types that are the natural hosts to HSV-1 infection. Although a naturally susceptible cell type in which 3-OS HS is the sole mediator of HSV-1 entry and fusion has yet to be found, it is very realistic to assume that in many cell types 3-OS HS, in association with other known receptors (HVEM and nectin-1), facilitates HSV-1 entry and spread. Future studies designed to determine the relative significance of HSV-1 entry receptors in human cell types, hopefully, would provide more information on the physiological significance of 3-OS HS and other entry receptors in entry and cell spread.

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