

A role for heparan sulfate 3-*O*-sulfotransferase isoform 2 in herpes simplex virus type 1 entry and spread

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Received 9 September 2005; returned to author for revision 11 October 2005; accepted 2 November 2005

Available online 5 December 2005

Abstract

Heparan sulfate (HS) 3-*O*-sulfotransferase isoform-2 (3-OST-2), which belongs to a family of enzymes capable of generating herpes simplex virus type-1 (HSV-1) entry and spread receptors, is predominantly expressed in human brain. Despite its unique expression pattern, the ability of 3-OST-2 to mediate HSV-1 entry and cell-to-cell fusion is not known. Our results demonstrate that expression of 3-OST-2 can render Chinese hamster ovary K1 (CHO-K1) cells susceptible to entry of wild-type and mutant strains of HSV-1. Evidence for generation of gD receptors by 3-OST-2 were suggested by gD-mediated interference assay and the ability of 3-OST-2-expressing CHO-K1 cells to preferentially bind HSV-1 gD, which could be reversed by prior treatment of cells with HS lyases (heparinases II/III). In addition, 3-OST-2-expressing CHO-K1 cells acquired the ability to fuse with cells-expressing HSV-1 glycoproteins, a phenomenon that mimics a way of viral spread *in vivo*. Demonstrating specificity, the cell fusion was inhibited by soluble 3-*O*-sulfated forms of HS, but not unmodified HS. Taken together, our results raise the possibility of a role of 3-OST-2 in the spread of HSV-1 infection in the brain.

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Keywords: HSV-1; Entry; Heparan sulfate; Membrane fusion

Introduction

Herpes simplex virus-1 (HSV-1) is associated with multiple human diseases ranging from cold sores or fever blisters on mucosal layers of the skin to meningitis and encephalitis in the brain (Eisenstein et al., 2004; Whitley and Roizman, 2001). While the molecular details of the viral spread from skin to central nervous system are yet to be fully understood, it is likely that HSV-1 entry receptors play a key role in the spread process. In this regard, expression in the brain of heparan sulfate (HS) 3-*O*-sulfotransferase isoform-2 (3-OST-2), which is a member of the 3-*O*-sulfotransferase (3-OST) family of enzymes that can generate entry receptors for HSV-1, raises genuine curiosity about its ability to mediate HSV-1 infection (Shukla et al., 1999; Shukla and Spear, 2001; Shworak et al., 1999).

The virus, HSV-1, is a member of the herpesvirus family and alphaherpesvirus subfamily. According to the current model of HSV-1 entry, HS plays an important role in the attachment of the viral glycoproteins gC or gB to the target cell surface (Herold et al., 1991; Shieh et al., 1992; WuDunn and Spear, 1989). After attachment, fusion between the viral envelope and the target cell membrane is required for entry into cells. It is triggered by the binding of viral glycoprotein gD to one of its cell surface receptors in association with three other glycoproteins: gB, gL, and gH (Spear and Longnecker, 2003; Yoon et al., 2003). The gD receptors include nectin-1 and nectin-2, both of which are members of the immunoglobulin superfamily (Cocchi et al., 2000; Geraghty et al., 1998; Warner et al., 1998), HVEM, which is a member of the TNF-receptor family (Montgomery et al., 1996), and the modifications in HS triggered by some selective members of the 3-*O*-sulfotransferases (3-OSTs) family giving rise to the 3-*O*-sulfated HS (3-OS HS) gD receptor family (Shukla et al., 1999; Xia et al., 2002; Xu et al., 2005).

Physiologically, 3-OS HS is generated after multiple rounds of enzymatic modifications (including sulfations and

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epimerizations) within a newly synthesized HS chain that is normally composed of repeating disaccharide units (D-glucuronic or iduronic acid and N-acetylglucosamine) (reviewed by Lindahl et al., 1998; Rosenberg et al., 1997). The 3-O sulfation of glucosamine residues, which is the known function of 3-OSTs, is the last step in HS maturation process (Esko and Selleck, 2002; Rosenberg et al., 1997). Thus far seven different isoforms of 3-OST have been identified (3-OST-1, -2, -3A, -3B, -4, -5, and -6). These isoforms are able to recognize some specific monosaccharide sequences around the modification sites (Liu et al., 1999; Shworak et al., 1999; Xia et al., 2002; Xu et al., 2005). Despite the strong homology between the isoforms, 3-OS HS generated by individual enzymes appear to carry distinct “signatures” of the modifying enzymes. This is evident from the emerging data that modification by 3-OST-1 fails to generate HSV-1 entry receptor but strongly enhances anti-thrombin (AT) binding to HS, whereas 3-OST-3A and -3B isoforms, which are almost identical in structure and substrate specificities, do just the reverse (Liu and Rosenberg, 2002; Shukla et al., 1999; Shukla and Spear, 2001). In contrast, 3-OST-5 and 3-OST-6 each appear to perform both functions, but HSV-1 entry, like 3-OST-3, is limited to only the wild-type strains (Shukla et al., 1999; Tiwari et al., 2004; Xia et al., 2002; Xu et al., 2005). Interestingly, the ability of 3-OST-2 and 3-OST-4 isoforms to mediate HSV-1 entry and any unique features of 3-OS HS generated by the two enzymes have not been demonstrated.

The present study aims to identify a role for 3-OS HS modified by the 3-OST-2 isoform in HSV-1 entry and spread and at the same time look for evidence for any characteristic differences in HS generated by 3-OST-2. The results presented below demonstrate the ability of the enzyme to generate receptors for both wild-types and some mutant forms of HSV-1. The mutants, commonly known as Rid, carry a common point mutation at position 27 of gD (Dean et al., 1994; Montgomery et al., 1996). The ability to mediate entry of mutant virions with the Rid forms of gD appears to be a unique feature of 3-OS HS generated by 3-OST-2.

Results

Effect of 3-OST-2 expression on viral entry

Wild type Chinese hamster ovarian (CHO-K1) cells lack a functional HSV-1 gD receptor and are thus resistant to HSV-1 entry (Shieh et al., 1992). However, they can be made susceptible to entry through the transient transfection of plasmids expressing gD receptors (Montgomery et al., 1996). Thus, in order to determine the ability of 3-OST-2 to mediate HSV-1 entry, CHO-K1 cells were transiently transfected with 3-OST-2 expression and control plasmids. Reporter virus entry assays were performed to determine whether the expression of 3-OST-2 rendered CHO-K1 cells susceptible to entry of wild-type and a mutant, Rid1, forms of HSV-1 (Dean et al., 1994). The latter has a point mutation at position

27 (Q27P) of gD which prevents its entry via some receptors including HVEM and 3-OS HS generated by 3-OST-3 (Dean et al., 1994; Montgomery et al., 1996; Shukla et al., 1999). β -galactosidase-expressing recombinant HSV-1 (KOS) gL-86 and HSV-1Rid1tk12 respectively represented the wild-type and the mutant strains of HSV-1. In each assay, we compared the results with 3-OST-2 with that of a positive control: 3-OST-3 for HSV-1 (KOS) and nectin-1 for HSV-1 (Rid1). An empty vector, pCDNA3.1 (+) (Invitrogen), was used as the negative control. Clearly, as shown (Fig. 1), there was a significant increase in viral entry relative to those cells that were transfected with empty vector alone. For HSV-1 KOS (Fig. 1A), the response seen with 3-OST-2 expressing cells was comparable to that of the 3-OST-3 expressing cells, and, similarly, in entry of Rid1tk12 into 3-OST-2 cells and nectin-1 cells infected with the same virions (Fig. 1B). Thus, it appears that both HSV-1 (KOS) and Rid1 virions are able to use the 3-OS HS generated by 3-OST-2 as a receptor for entry into target cells. More specifically, the results also

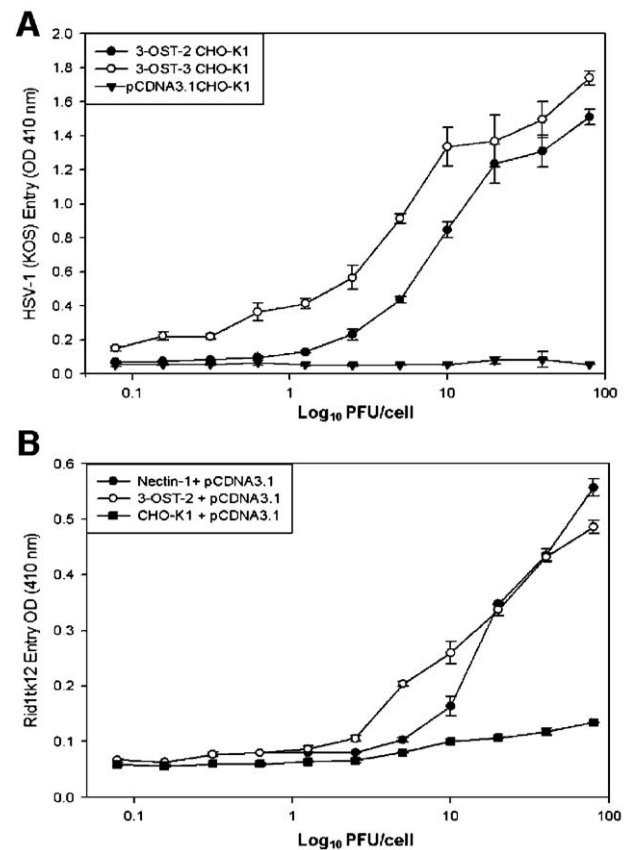


Fig. 1. Viral entry is dependent on the expression of 3-OST-2. (A) Entry of HSV-1(KOS). Chinese hamster ovarian (CHO-K1) cells were transfected with 3-OST-2, 3-OST-3-expressing plasmids or empty vector pCDNA3.1(+), as indicated. About 16 h after transfection, β -galactosidase-expressing HSV-1 KOS gL86 virus was added to the cells at the dosages indicated and incubated for 6 h. Viral entry was measured (as optical density, OD) using *o*-nitrophenyl- β -D-galactopyranoside (ONPG) substrate. (B) Entry of HSV-1 (Rid1). CHO-K1 cells were transfected with 3-OST-2, nectin-1-expressing plasmids or empty vector pCDNA3.1 and infected with recombinant β -galactosidase-expressing Rid1tk12 virus at the dosages indicated. Viral entry was measured as described above.

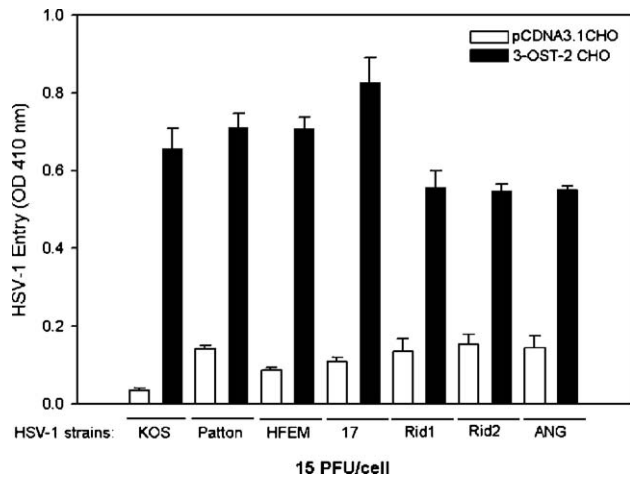


Fig. 2. Entry of various HSV-1 wild type and mutant strains in 3-OST-2 expressing cells. Entry of HSV-1 wild type strains (KOS, Patton, HFEM, 17) and mutant strains (Rid1, Rid2, ANG). Chinese hamster ovarian (CHO-K1) and β -galactosidase-expressing IE β 8 cells were transfected with 3-OST-2 expressing plasmids or empty vector pCDNA3.1 (+), as indicated. Cells were also transfected with 3-OST-3 or nectin-1 expressing plasmids to serve as a positive control (data not shown). Viral entry assay was performed as described in Fig. 1. Each bar represents entry at a viral concentration of 15 pfu per cell for cells expressing 3-OST-2 and those transfected with empty vector.

suggest that HSV-1 glycoprotein gD (both the wild-type and the mutant Rid1 form) could interact with the 3-OS HS generated by 3-OST-2, which was verified through further experiments.

After testing entry with HSV-1 (KOS) and Rid1 alone, we decided to test the susceptibility of 3-OST-2 expressing cells to a variety of HSV-1 wild type and mutant strains. Since none of these new strains tested have β -galactosidase activity, IE β 8 cells were used because this cell line is able to express β -galactosidase upon HSV-1 entry (Montgomery et al., 1996). Fig. 2 shows that each wild type strain (KOS, Patton, HFEM, 17) and each mutant strain (Rid2 and ANG) were able to use 3-OS HS generated by 3-OST-2 as an entry receptor. The mutant strains used have a common point mutation at position 27 in gD (Montgomery et al., 1996).

gD-mediated interference assay with 3-OST-2 expressing cells

In order to generate evidence that the 3-OS HS generated by 3-OST-2 can also interact with gD, a gD-mediated interference assay was performed. It is based on the observation that cells co-expressing gD and one of its receptors become resistant to HSV-1 entry due to sequestration of the entry receptor by cellular gD (Geraghty et al., 2000; Shukla et al., 1999). Since the process of interference is dependent on the relative ratio of gD and the receptor, one effective way of controlling this in vitro, is by co-transfecting cells with gD to receptor plasmids ratio of 4:1 (Geraghty et al., 2000). Thus, using the same ratio, cells were transfected with gD and 3-OST-2 expression constructs, and changes in viral entry were observed compared to the control cells transfected with 3-OST-2 and the empty vector pCDNA3.1 (+). As shown in Fig. 3, for 3-OST-2 cells co-expressing gD (wild-type or Rid1) there was a

significant reduction in viral entry when infected with either HSV-1 KOS (Fig. 3A) or Rid1 (Fig. 3B) compared to those that were not expressing the glycoproteins. For Rid1 (Fig. 3B), the response seen with 3-OST-2 in either case was very similar to that seen with nectin-1 used as a positive control. Thus, it can be assumed then that the reduction in viral entry is due to the interaction of both forms of gD with 3-OS HS generated by 3-OST-2.

Cell ELISA with soluble gD:Fc

To generate more direct evidence for the generation of gD receptors by the action of 3-OST-2 enzyme on HS, a previously defined soluble gD-cell binding assay was used (Shukla et al., 1999). This assay is based on the principle that a recombinant soluble form of HSV-1 gD fused with the Fc portion of rabbit IgG (gD:Fc) can preferentially bind to cells expressing 3-OS HS on their surfaces. As a way to demonstrate the involvement of 3-OS HS as the gD receptor,

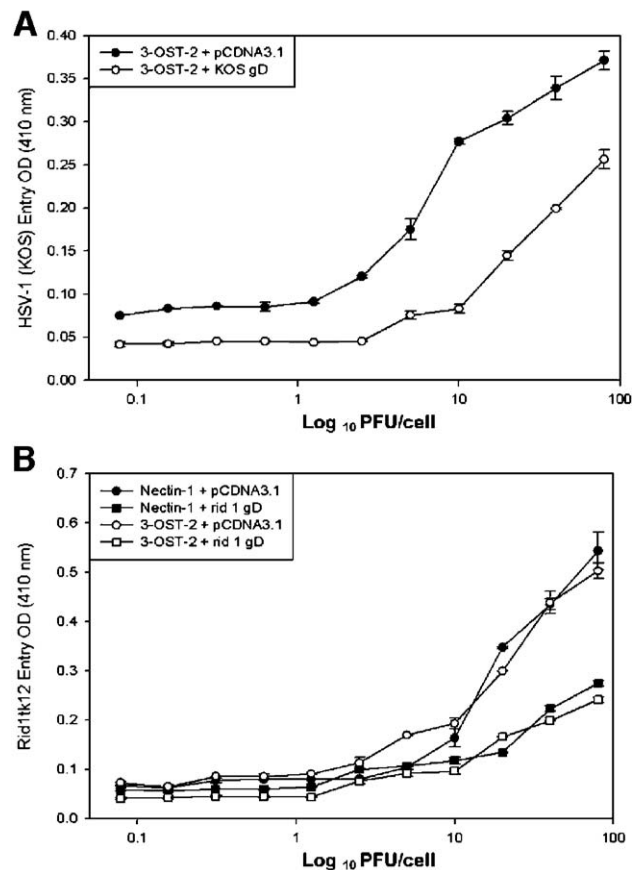


Fig. 3. Co-expression of gD with 3-OST-2 renders resistance to HSV-1 entry. (A) Effects of HSV-1 glycoprotein gD when coexpressed with 3-OST-2 on entry of HSV-1(KOS). CHO-K1 cells were co-transfected with 3-OST-2 expression plasmid and gD expression plasmid or pCDNA3.1 (+) as indicated. The cells were then infected with HSV-1 KOS gL86 at the doses indicated. After 6 h, *o*-nitrophenyl- β -D-galactopyranoside (ONPG) substrate was added, and viral entry was measured spectrophotometrically (OD₄₁₀). (B) Effects of HSV-1 Rid1 gD on HSV-1(KOS)Rid1 entry when coexpressed with either 3-OST-2 or nectin-1. CHO-K1 cells were co-transfected with 3-OST-2 expression plasmid and Rid1 gD expression plasmid or pCDNA3.1 (+) as indicated and infected with HSV-1 Rid 1. Viral entry was measured as described in panel A.

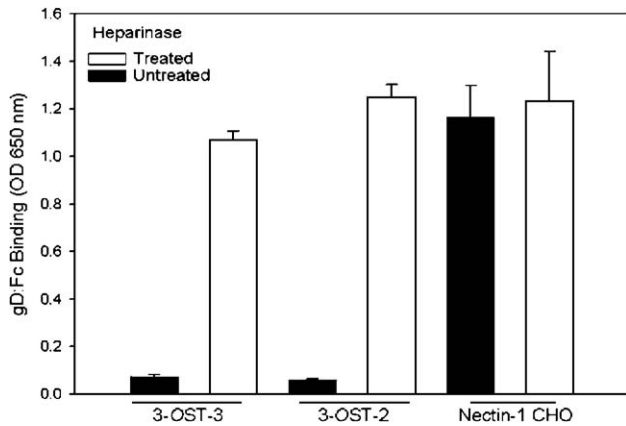


Fig. 4. Enzymatic removal of 3-OST-2 modified HS from cells also removes gD-binding receptors. A soluble recombinant form of HSV-1 gD (gD:Fc) was allowed to bind cells expressing the 3-OST isoforms as indicated. The cells were either treated with a mixture of heparinase II/III (treated) or mock treated (untreated). Binding of gD:Fc was detected by use of a secondary antibody against rabbit IgG:Fc and a horseradish peroxidase detection system (Amersham). The values shown represent the amount of reaction product detected spectrophotometrically (OD at 650 nm).

the preferentially binding to cells can be reversed by prior treatment of cells with heparin lyases (heparinases) (Shukla et al., 1999; Yabe et al., 2001). For this experiment, CHO-K1 cells were transiently transfected with 3-OST-2, 3-OST-3, or nectin-1 and each divided into two groups. While one group was treated with a mixture of heparinase II and III, the other was mock treated with the buffer alone for the same amount of time. If gD does bind to 3-OS HS generated by 3-OST-2 then we would expect to see a reduction in gD binding to the cell surface of the heparinase-treated cells due to loss of 3-OS HS by heparinase action. As shown in Fig. 4, there was a significantly higher amount of gD:Fc bound to mock-treated cells compared to those cells that were treated with heparinase-II and -III. The use of nectin-1 in this experiment shows the specificity of this assay for HS based receptors since the binding of gD to nectin-1-expressing cells was not significantly compromised by heparinase treatment. Thus, based on this and the results from the gD-mediated interference assay, it is very likely that expression of 3-OST-2 in CHO-K1 cells results in generation of gD receptors for HSV-1.

Effect of 3-OST-2 expression on viral induced cell fusion

After establishing the ability of 3-OST-2 in HSV-1 entry, we next examined whether the receptor generated by this enzyme could also facilitate cell-to-cell fusion. Once again CHO-K1 cells were used since due to the absence of a gD receptor, CHO-K1 cells are resistant not only to HSV-1 entry, but virus-induced cell fusion as well (Pertel et al., 2001; Shieh et al., 1992). A standard luciferase reporter gene assay was performed to quantify the induced cell fusion between 3-OS HS cells modified by 3-OST-1, 3-OST-2, or 3-OST-3 and HSV-1 glycoproteins (Pertel et al., 2001). The “effector” CHO-K1 cells were transiently transfected with each of four glycoprotein plasmids: pPEP98 (gB), pPEP99 (gD) pPEP100 (gH), and

pPEP101 (gL), as well as, the plasmid pT7EMCLuc that expresses a luciferase reporter gene. The “target” cells were transfected with a 3-OST plasmid expressing 3-OST-1, 3-OST-2, or 3-OST-3 and the plasmid pCAGT7, which expresses T7 RNA polymerase to induce expression of the luciferase gene. For a negative control, cells were transfected with T7 RNA polymerase and 3-OST-1 because 3-OS HS modified by this isoform has been shown not to interact with HSV-1 (Shukla et al., 1999; Tiwari et al., 2004; Xu et al., 2005). The cells expressing 3-OST-3 and T7 RNA polymerase served as a positive control. Fig. 5A shows a very similar high amount of fusion occurring in both 3-OST-2 and 3-OST-3 expressing cells. Clearly, the 3-OS HS generated by 3-OST-2 is capable of mediating cell fusion as well.

Basically to verify the results obtained by the luciferase assay we decided to look at polykaryocyte formation by mixing of 3-OST-2-expressing target cells with the effector cells. This phenomenon mimics multinucleated cell (polykaryocyte) formation during an actual HSV-1 infection whereby infected cells fuse with uninfected neighboring cells allowing the spread of the virions to the neighboring cells. Since it is possible for polykaryocytes to spontaneously form in some instances, a blue plaque assay was performed to show the

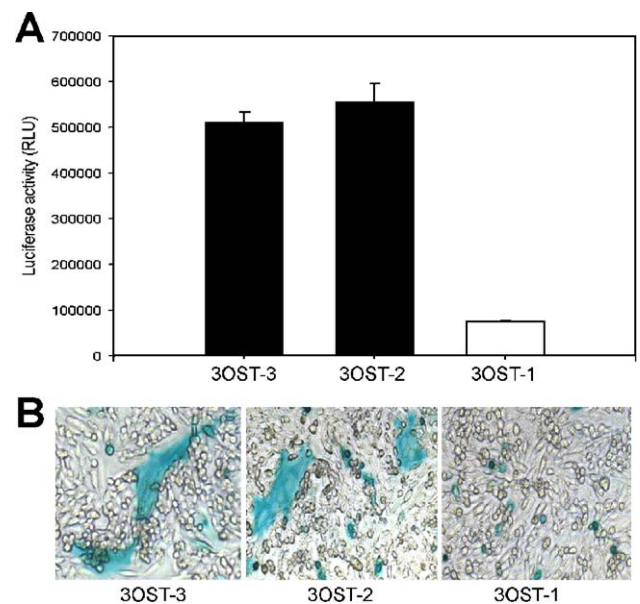


Fig. 5. 3-OST-2-expressing CHO-K1 cells gain the ability to fuse with cells co-expressing HSV-1 glycoproteins gB, gD, gH and gL. The target cells were transfected with plasmids expressing 3-OST-1, 3-OST-2, or 3-OST-3 (as indicated) and luciferase reporter gene. The effector cells were transfected with HSV-1 glycoproteins gB, gD, gH and gL, and T7 RNA polymerase. A luciferase reporter assay was performed 24 h after the two cell populations were mixed together. Cell fusion was measured in relative luciferase units (RLUs). 3-OST-3 and 3-OST-1 were used as the positive and negative controls respectively. (A) Expression of 3-OST-2 results in the fusion of CHO-K1 cells with glycoprotein-expressing cells as measured by a reporter assay. (B) Multinucleated cells or polykaryocytes were observed with cells expressing 3-OST-2. CHO-K1 cells were transfected with plasmids expressing 3-OST cDNAs as indicated and mixed with the effector cells and stained with gH and gL monoclonal antibodies followed by detection with a biotinylated secondary anti-mouse antibody, streptavidin conjugated with β -galactosidase and X-gal at 72 h post-mixing.

polykaryocytes were specifically expressing the viral glycoproteins. A monoclonal antibody for gH and gL was used to identify HSV-1 viral glycoprotein expression. Our results (Fig. 5B) show formation of polykaryocytes in the cell populations expressing 3-OST-2, which were similar in size to those cells expressing 3-OST-3. Clearly, virtually none were seen in the 3-OST-1 expressing cells. The background seen in each well is due to effector cells expressing gH and gL that did not fuse with target cells. Transfection is not 100% efficient so it is not uncommon for glycoprotein cells to fail to come into contact with target cells expressing HSV-1 viral receptors. However, clearly a difference can be seen between plaques and the individual cells that have been stained with β -galactosidase. A similar polykaryocyte formation study was done, except the cells were stained with Giemsa, instead of β -galactosidase. Similar results were seen with the 3-OST-2 and 3-OST-3 cells showing large polykaryocytes similar in size, and the 3-OST-1 expressing cells with virtually no polykaryocyte formation (data not shown). These results reinforce our finding that cells expressing 3-OST-2 allow cell fusion to occur, and thus potentially could facilitate spread of HSV-1 in an actual infection.

Next, to gain evidence that cell surface expression of 3-OS HS, and not the enzyme itself, was responsible for the induction of the cell fusion, the effect of enzymatic removal of 3-OS HS on cell fusion by treatment with a mixture of heparinase II and III was studied. These enzymes selectively degrade HS chains by cleaving them. For this experiment, 3-OST-2 and 3-OST-3-expressing CHO-K1 cells (as a positive control) were separated into two distinct pools, one treated with heparinase-II/III and the other untreated and mixed with the effector cells expressing HSV-1 glycoproteins. Nectin-1 expressing cells were used as a control to show that the effect of heparinase treatment was specific to 3-OST-2 (3-OST-3) cells. As seen in Fig. 6A, untreated cells expressing 3-OST-2 and 3-OST-3 show fusion occurring, with 3-OST-2 cells showing a slight increase in luciferase activity, while the cells treated with heparinase-II/III showed about a 90% reduction in fusion. The results indicate that it is the 3-OS HS on the cell surface of the target cells modified by 3-OST-2 that HSV-1 is binding with to induce fusion and not the enzyme itself or any other receptor.

To verify further that 3-OS HS is the mediator of the fusion, a competition assay was performed using soluble forms of 3-OS HS generated *in vitro* by purified 3-OST-1, 3-OST-3, or 3-OST-5 enzymes (Tiwari et al., 2004). Approximately 1.0 μ g/ml of the soluble HS of each kind were added to the glycoproteins expressing effector cells, prior to mixing with 3-OST-2-expressing target cells. The glycoprotein expressing effector cells with mock HS treatment (buffer alone) was considered a control. We hypothesized that 3-OST3B and 3-OST-5 generated 3-OS HS should competitively inhibit fusion whereas 3-OST-1 generated should not, as it fails to bind gD (Shukla et al., 1999; Tiwari et al., 2004). Results shown in Fig. 6B confirm our hypothesis. This figure shows a significant reduction in fusion in the cell populations that received either soluble 3-OS HS generated by 3-OST-3 or 3-OST-5 compared

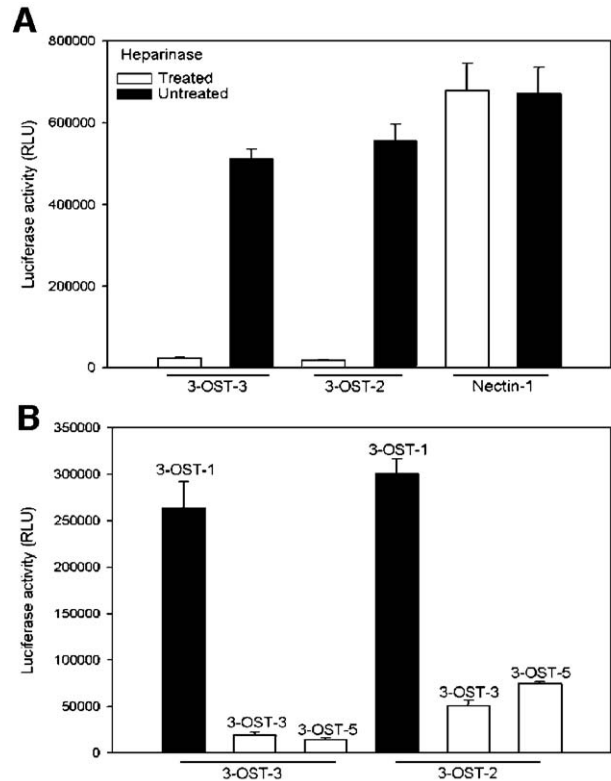


Fig. 6. 3-OS HS is likely the mediator of the cell fusion. (A) Degradation of HS by the addition of heparinase-II/III reduces cell fusion. Cell expressing the four viral glycoproteins (gB, gD, gH and gL) were treated with heparinase-II/III (black bar) or left untreated (white bar) prior to mixing with cells expressing either 3-OST-2, 3-OST-3 or nectin-1. Luciferase activity was measured 24 h after co-cultivation in relative luciferase units (RLUs) using a Sirius luminometer. (B) 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 generated by the isoform indicated above bars. The treatments were performed prior to mixing with the target cells expressing 3-OST-2 or 3-OST-3 plasmids. Luciferase activity was measured in RLUs.

to the cells that received the non-gD binding soluble 3-OS HS generated by 3-OST-1. These results indicate that 3-OS HS generated by 3-OST isoforms capable of mediating entry can specifically block the fusion induced by cells-expressing 3-OST-2. It is worth mentioning here that Browne et al. (2001) have previously found that significantly higher concentration (at least 50 μ g/ml) of heparin was required to effectively block the fusion of COS 7 cells expressing HSV-1 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) as opposed to an unknown combination of gD receptors in the cell lines used by them, and use of specifically modified soluble HS (instead of heparin).

Discussion

Among the major achievements of our study are the demonstration of the ability of 3-OST-2 isoform to generate HSV-1 entry and also cell-to-cell fusion receptors, both of which could play important roles in the spread of the viral infection *in vivo*. Similarly, the finding that Rid1 and related mutant virions (Rid2 and ANG) can use 3-OS HS generated by

3-OST-2 for entry provide evidence for the existence of some potentially unique structural features of the final products generated by 3-OST-2 compared to the other members of the family. This finding is also a testament to the theory that different 3-OST isoforms are capable of generating very specific and somewhat unique structures within HS chains generating protein binding sites for regulating many important cellular processes (Rosenberg et al., 1997). Clearly, HS is already known for its ability to activate certain ligands including, anti-thrombin, chemokines, cell adhesion molecules, and a variety of growth factors (Lindahl et al., 1998; Rosenberg et al., 1997).

It is very interesting that 3-OST-2 isoform is predominantly expressed in the brain (Xu et al., 2005; Shworak et al., 1999) raising curiosity about its normal biological functions and also its ability to mediate HSV-1 entry. Obviously, the enzyme plays some important regulatory functions since loss of its expression is associated with many types of cancers (Miyamoto et al., 2003). Although our study does not address any issues related with its normal biological functions, we do, however, provide some clues about the final products generated by this enzyme. It is likely that 3-OS HS generated by this enzyme contains a disaccharide-IdoA2S-GlcNH₂3S-(or-IdoA2S-GlcNH₂3S6S-), which is required for HSV-1 wild-type gD binding activity (Shukla et al., 1999; Xia et al., 2002; Xu et al., 2005). Other disaccharide products generated by this enzyme could very well be unique to it, since no other 3-OST isoform is currently known to generate receptors for the Rid1 form of gD. Our findings will, hopefully, guide future efforts to carefully identify the final products generated by this enzyme.

Regarding the ability of 3-OST-2 to mediate HSV-1 entry, it is indeed a serious matter that HSV-1 is capable of spreading to and infecting human brain causing deadly diseases like encephalitis and meningitis (Eisenstein et al., 2004; Whitley and Kimberlin, 2005). Although the *in vivo* spread of the virus infection is a complicated process determined by multiple host–pathogen factors, the role of entry receptors in this process cannot be ruled out. In fact, we believe it is likely that the entry receptors play a crucial role in the spread of the infectious virions. Among the well-characterized gD receptors, nectin-1, appears to be the prime candidate for mediating viral spread to and within the brain (Simpson et al., 2005; Shukla et al., 1999). Our findings present a new possibility of an additional receptor playing a role in HSV-1 infection of the brain. Quite interestingly, this finding could help resolve any receptor related issues associated with the differences seen in the spread patterns (or tissue tropism) of HSV-1 and HSV-2 in the brain tissue, especially since HSV-2 is frequently associated with meningitis but not encephalitis in adults (Eisenstein et al., 2004; Whitley and Roizman, 2001; Whitley and Kimberlin, 2005). If receptors have any role in this, it could not be explained if nectin-1 were the only receptor in the brain since it is a receptor for both HSV-1 and -2 (Geraghty et al., 1998). Since like any other 3-OSTs, the receptor generated by 3-OST-2 fails to mediate HSV-2 entry (data not shown), it is possible that the existence of a HSV-1 specific receptor like the one generated by 3-OST-2 in the brain could potentially provide

some explanation for the inability of HSV-2 to cause meningitis in the adult brain. Of course, it is premature to make any such conclusions yet, but this study does however, provide a good starting point to further understand the role of the entry receptors, their age related expression patterns, and other molecular determinants of the viral spread in the brain.

Materials and methods

Materials

Human 3-OST-2 and 3-OST-3-expressing plasmids were received as a gift from Dr. Jian Liu (University of North Carolina, Chapel Hill) (Shworak et al., 1999). The HSV-1 (KOS) glycoprotein expressing plasmids used were pPEP98 (gB), pPEP99 (gD), pPEP100 (gH), and pPEP101 (gL) (Pertel and Spear, 1997). Other plasmids used include pMW13 (RID1 gD), pCAGT7 (T7 RNA polymerase), pT7EMCLuc (luciferase gene) for the luciferase assay, and a control empty vector pCDNA3.1 (+) from Invitrogen (Carlsbad, CA, USA). Heparinase-II and -III were obtained from Sigma chemical company, while the *o*-nitrophenyl- β -D-galactopyranoside (ONPG) reagent was from ImmunoPure.

Cell culture and viruses

Wild type Chinese hamster ovarian-K1 (CHO-K1) cells and IE β 8 cells were provided by P.G. Spear (Northwestern University). All CHO-K1 cells were grown in Ham's F-12 medium (Gibco/BRL), Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin (Gibco/BRL). The β -galactosidase expressing recombinant HSV-1 (KOS) gL86 and HSV-1 (KOS) Rid1tk12 viruses were provided by P.G. Spear (Northwestern University). The other HSV-1 wild type (Patton, HFEM,17) and mutant strains (ANG and Rid2) were also provided by P.G. Spear (Northwestern University).

HSV-1 viral entry assay

Standard entry assays were used as described previously (Shukla et al., 1999). Briefly, CHO-K1 (or IE β 8 cells) cells were transferred to 6-well plates, grown to a subconfluent level, and transfected with 1 μ g of 3-OST isoforms (3-OST-2, 3-OST-3), nectin-1, gD or pCDNA3.1 (+) using LipofectA-MINE (Gibco/BRL). After about 16 h, the cells were replated into 96-well dishes for infection with recombinant viruses (or wild-type viruses of IE β 8 cells). At 6-h post-infection, β -galactosidase assay were performed using either a soluble substrate *o*-nitrophenyl- β -D-galactopyranoside (ONPG) or X-gal. For the soluble substrate, the enzymatic activity was measured at 410 nm using a micro-plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA). For X-gal assay, the cells were fixed (2% formaldehyde and 0.2% glutaraldehyde) and permeabilized (2 mM MgCl₂, 0.01% deoxycholate, 0.02% nonidet NP-40 (Sigma)). Finally, 1 ml of β -galactosidase reagent (1.0 mg/ml X-Gal in

ferricyanide buffer) was added to each well and incubated at 37 °C for 90 min before the cells were examined using bright field microscopy under the 20× objective (Zeiss, Axiovert 100 M).

For the gD-mediated interference assay (Geraghty et al., 2000; Shukla et al., 1999, 2000), cells were co-transfected with 3-OST expression plasmid and either gD-expressing or empty vector pCDNA3.1 (+) in a 1:1 ratio, with each plasmid added at a final concentration of 1 µg/µl. Thus, the total amount of transfected DNA was kept constant. After transfection, the cells were infected, and the infection was quantitated by the ONPG assay as described above. The enzymatic activity was measured at 410 nm using a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA).

Preparation of soluble forms of HS

Soluble HS from bovine kidney (ICN) was modified in vitro either by purified 3-OST-1, 3-OST-3A, or 3-OST-5 (Dr. Jian Liu, University of North Carolina, Chapel Hill) as described elsewhere (Shukla et al., 1999; Tiwari et al., 2004). The extent of 3-O-sulfation to HS was monitored by determining the incorporation of [³⁵S] sulfate into the polysaccharide after in vitro modification by 3-OSTs. The estimated numbers of 3-O-sulfate group per HS polysaccharide chain was 1.1 sulfate /chain for 3-OST-1 modified HS; 1.3 sulfate /chain each for 3-OST-3A and 3-OST-5 modified HS. The concentration of unlabeled HS was determined using alcian blue dye (Bjornsson, 1998).

Cell fusion assays

A standard cell-to-cell fusion assay was used as described previously (Pertel et al., 2001; Tiwari et al., 2004). Briefly, CHO-K1 cells were grown in 6-well plates and grown to a subconfluent level. The “target” cells were transfected with plasmids expressing a 3-OST isoform and the luciferase gene. The “effector” cells were transfected with plasmids expressing HSV-1 glycoproteins gD, gB, gH, and gL, and T7 RNA polymerase. In either case, the total amount of transfected DNA was kept constant. After 16 h, target and effector cells were mixed in a 1:1 ratio and then replated in 24-well dishes. Luciferase activity was measured 24 h later.

To demonstrate sensitivity to heparinase treatment target cells were treated with a 1:1 mixture of heparinase-II/III for 2 h prior to mixing with the effector cells. Some cells were mock treated with the buffer alone to serve as a control. Luciferase activity was measured as described above. For competition assay, 1 µg/µl of HS generated in vitro by soluble forms of 3-OST-1, 3-OST-3, and 3-OST-5 (Jian Liu, University of North Carolina, Chapel Hill) were added to the effector cells 2 h prior to mixing with the target cells. Luciferase activity was measured 24 h later. For polykaryocyte study, a blue plaque assay was performed using a monoclonal antibody for gH and gL detected by a biotiny-

lated secondary anti-mouse antibody, streptavidin conjugated with β-galactosidase and X-gal. The effector and target cells were incubated for 72 h at 37 °C and 5% CO₂ and observed under the microscope for multinucleated giant cell (polykaryocyte) formation.

Cell ELISA

A cell ELISA protocol using a recombinant form of a HSV-1 gD chimera with rabbit IgG Fc (gD1:Fc) was used as described elsewhere (Geraghty et al., 2000; Shukla et al., 1999). Briefly, the cells were transfected with identical amounts of 3-OST-2, 3-OST-3, or nectin-1-expressing plasmids. After the monolayers have grown to confluence, soluble gD1:Fc was added for 1 h. After multiple rounds of washing, gD1:Fc binding was detected by biotinylated anti-rabbit IgG (1:5,000 in PBS with 3% BSA) (Sigma) and a horseradish peroxidase detection system (AMDEX streptavidin-conjugated horseradish peroxidase, from Amersham, Arlington Heights, IL, USA) diluted 1:10,000. The slow kinetic form of 3,3', 5,5'-tetramethylbenzidine (Sigma) was used as the substrate. The optical density was read at 650 nm using a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA).

Acknowledgments

The authors would like to thank Dr. Jian Liu (University of North Carolina, Chapel Hill) and Patricia Spear (Northwestern University, Chicago) for reagents. Tibor Valyi-Nagy, Christian Clement and Perry Scanlan for helpful discussions. This work was supported by National Institutes of Health grant RO1 AI057860 to D. Shukla. V. Tiwari is supported by a postdoctoral fellowship (0525768Z) from the American Heart Association.

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