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Zebrafish-Encoded 3-*O*-Sulfotransferase-3 Isoform Mediates Herpes Simplex Virus Type 1 Entry and Spread

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Abstract

Heparan sulfate proteoglycans modified by human glucosaminyl 3-*O*-sulfotransferase-3 (3-*O*ST-3) isoform generates the cellular receptor for herpes simplex virus type 1 (HSV-1). Interestingly, the ability of zebrafish (ZF)-encoded 3-*O*ST-3 isoform to modify heparan sulfate to mediate HSV-1 entry and cell–cell fusion has not been determined although it is predominantly expressed in ZF, a popular model organism to study viral infections. Here, we demonstrate that expression of ZF-encoded 3-*O*ST-3 isoform renders the resistant Chinese hamster ovary (CHO-K1) cells to become susceptible for HSV-1 entry. The following lines of evidence support the important role of ZF-encoded 3-*O*ST-3 isoform as the mediator of HSV-1 entry into CHO-K1 cells: (1) ZF 3-*O*ST-3–expressing CHO-K1 cells were able to preferentially bind HSV-1 glycoprotein D, and (2) CHO-K1 cells expressing ZF-encoded 3-*O*ST-3 acquire the ability to fuse with cells expressing HSV-1 glycoproteins. Finally, knocking down 3-*O*ST-3 receptor by siRNA in ZF fibroblasts cells significantly reduced HSV-1 entry and glycoprotein D binding to cells. Taken together, our results provide novel insight into the significance of ZF 3-*O*ST-3 isoform as an HSV-1 entry and fusion receptor and its potential involvement in the HSV-1 disease model of ZF.

Introduction

Heparan sulfate (HS) is a glycosaminoglycan of repeating disaccharide units that consist of *N*-acetylglucosamine and glucuronic/iduoronic acid. During the biosynthesis of HS, a long chain containing the alternate disaccharides is modified in a complex series of steps involving *N*-deactylation/*N*-sulfation of the glucosamine unit, C5 epimerization of the glucuronic acid to iduronic acid, and *O*-sulfation of both residues by multiple enzymes. The last *O*-sulfation step involves 3-*O*-sulfotransferases (3-*O*STs), which exist in multiple isoforms.[1–3] In each of these modification steps, only part of the substrate is modified, resulting in a high sequence diversity that is thought to give HS proteoglycans (HS proteoglycans) their functional specificity and versatility.[4] Thus, each 3-*O*ST can potentially generate unique protein-binding sites within the HS chain.[5,6] HS moieties prominently exposed on the cell surface have also been implicated as a primary receptor for human herpesviruses and many other viruses.[5–8]

In the case of herpes simplex virus type 1 (HSV-1), it was first shown that the HS chains can serve as an initial attachment site or binding receptor for the virions.[9] Later, Shukla *et al.* suggested that modification of HS by 3-*O*-sulfotransferase-3 (3-*O*ST-3) generates a unique entry receptor for HSV-1.[6] It was further demonstrated that 3-*O* sulfated HS (3-*O*S HS) generated by multiple 3-*O*STs can be an important receptor during cell fusion induced by HSV-1.[10–14] Enzymes that modify HS proteoglycans play essential roles during zebrafish (ZF) embryo development.[15–18] Recently, a combinatorial expression pattern of 3-*O*ST gene family was shown in the ZF model.[19] In addition, adult ZF was presented as a new model system for HSV-1 infection of the nervous system.[20] This study aims to identify a role for the ZF-encoded 3-*O*ST-3 isoform in HSV-1 entry and spread. The results presented below demonstrate that the resistant CHO-K1 cells expressing ZF 3-*O*ST-3 isoform together with ZF fibroblast cells expressing endogenous 3-*O*ST-3 receptors are susceptible to HSV-1 entry and spread. The significance of the functional 3-*O*ST-3 receptor for HSV-1 in ZF will likely develop a new area of investigations

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pertaining to targeted gene disruption, structure–function analysis of the 3-*O*STs gene, and screening for 3-*O*ST-3 inhibitors targeted to block viral entry in the ZF model.

Materials and Methods

Plasmid

ZF-encoded 3-*O*ST-3 gene (1203 bp) was cloned into pDream2.1 plasmid. Positive clones were selected by PCR and further verified by DNA sequencing (Genscript). Human 3-*O*ST-3 (H 3-*O*ST-3)–expressing plasmid (pDS43) was a generous gift from Dr. Shukla (University of Illinois at Chicago).[6] The HSV-1 (KOS)–glycoprotein expressing plasmids used were pPEP98 (gB), pPEP99 (glycoprotein D [gD]), pPEP100 (gH), and pPEP101 (gL).[21] Other plasmids used in this study include nectin-1 (pBG38), pCAGT7 (T7 RNA polymerase), and pT7EMCLuc (luciferase gene).

Cell culture and viruses

Wild-type CHO-K1 cells were kindly provided by P.G. Spear (Northwestern University, Chicago). All CHO-K1 cells were grown in Ham's F-12 medium (Gibco/BRL) supplemented with 10% fetal bovine serum, and penicillin and streptomycin (Gibco/BRL). African green monkey kidney (Vero) cells were grown as previously described.[22] The β-galactosidase-expressing recombinant HSV-1 (KOS) gL86[6] and green fluorescent protein (GFP)–expressing HSV-1 (K26GFP) were provided by P.G. Spear (Northwestern University, Chicago) and P. Desai (Johns Hopkins University).[23] ZF fibroblasts cells (ZF4) obtained from American Type Culture Collection were grown in recommended the medium (DMEM:F12) and culture conditions.

HSV-1 entry assay

The entry assay previously described was used.[22] CHO-K1 cells were grown in six-well plates to subconfluence and transfected with 2.5 μg of human or ZF-encoded 3-*O*ST isoform (3-*O*ST-3), or with pDream2.1 empty vector using LipofectAMINE (Gibco/BRL). At 16 h posttransfection, the cells were replated into 96-well dishes for infection with recombinant virus HSV-1 (KOS) gL86 at the plaque-forming units (pfu) indicated. After 6 h postinfection, β-galactosidase assays were performed using either a soluble substrate *o*-nitrophenyl-β-D-galactopyranoside (at 3.0 mg/mL; ImmunoPure, Pierce) or X-gal (Sigma). For the soluble substrate, the enzymatic activity was measured at 410 nm using a microplate reader (ELX808 absorbance microplate reader; BioTek Instruments Inc.).

Fluorescent microscopy of viral entry

Cultured monolayers of CHO-K1 cells expressing H 3-*O*ST-3 and ZF 3-*O*ST-3 (approximately 10^6 cells/well) were grown overnight in F-12 media containing 10% fetal bovine serum on chamber slides (Lab-Tek). Wild-type CHO-K1 cells expressing pDream2.1 served as a negative control. The cells were infected with GFP-tagged HSV-1 (VP26-GFP) at 25 plaque-forming units (pfu)/cell in serum-free media Opti-MEM (Invitrogen). The GFP was fused with the HSV-1 UL35 open-reading frame that encodes a 12-kDa capsid protein designated VP26.[23] VP26 is located on the outer surface of the capsid, specifically on the tips of the hexons that constitute the capsid shell. At 90 min postinfection, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde and stained with 10 nm rhodamine-conjugated phalloidin (Invitrogen) for F-actin. Images of immunofluorescent-labeled cells were acquired using a confocal microscope (Nikon D-Eclipse-C1) with software EZ-C1.

Enzyme-linked immunosorbent assay

As previously described, an enzyme-linked immunosorbent assay with entire cells and a chimeric form of HSV-1 gD with rabbit IgG Fc (gD1:Fc) was used.[6] Briefly, cells were transfected with identical amounts of 3-*O*ST-3, or nectin-1-expressing plasmids. After the monolayers were 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 (1:5000 in phosphate-buffered saline [PBS] with 3% bovine serum albumin) (Sigma) and a horseradish peroxidase detection system (AMDEX streptavidin-conjugated horseradish peroxidase from Amersham) diluted 1:20,000. A slow kinetic form of 3-, 30-, 5-, 50-tetramethylbenzidine (Sigma) was used as the substrate. The optical density was read immediately after adding the substrate at 650 nm using a microplate reader (ELX808 absorbance microplate reader; BioTek Instruments Inc.).

HSV-1 glycoprotein induced cell fusion assays

A cell-to-cell fusion assay described previously was used.[10,24] CHO-K1 cells were grown in six-well plates to subconfluent levels. The so-called target cells were transfected with plasmids expressing 3-*O*ST-3 isoform and the luciferase gene. The effector or virus-like cells were cotransfected with plasmids expressing HSV-1 glycoproteins gB, gD, gH, and gL, and T7 RNA polymerase. In either case, the total amount of DNA used for transfection was kept constant. After 16 h, target and effector cells were mixed in a 1:1 ratio and then replated in 24-well dishes. The activation of the reporter luciferase gene as a measure of cell fusion was examined after 24 h. Luminosity readings were obtained using a Sirius luminometer (Berthold Detection Systems). Cell fusion was observed by microscopy following Giemsa (Fluka) staining or quantified using a luciferase reporter gene activation assay as previously described.[24]

Heparinase treatment

ZF4, CHO-K1 expressing human-encoded 3-*O*ST-3 receptor, and HeLa cells were pretreated with heparinase-II and -III (4 U/mL; Sigma) or with 1×PBS alone and incubated for 90 min at room temperature. The cells were then washed with PBS and used for the viral entry.

Si-RNA interference of ZF-encoded 3-OST-3

SiRNA that downregulate ZF 3-*O*ST-3 RNA expression (Invitrogen) was tested in ZF fibroblast cells (ZF4) to interfere with receptor formation. ZF4 cells were plated onto a six-well tissue culture dish and were transfected with appropriate RNA duplexes along with control duplex. After 24 h, cells were loosened with cell dissociation buffer (Invitrogen) and replated onto 96-well tissue culture dishes. Viral entry assays were performed as previously described with serial dilutions of HSV-1 (KOS) gL86.[6] As stated earlier, a spectrophotometer

at an optical density of 410 nm was used to measure β-galactosidase activity.

Results

Wild-type CHO-K1 cells expressing ZF-encoded 3-OST-3 isoform is susceptible to HSV-1 entry

Wild-type CHO-K1 cells are resistant to HSV-1 entry due to lack of a functional HSV-1 gD receptor and thus provide a very useful reagent for the discovery of gD receptors.[9] Therefore, to determine the ability of ZF-encoded 3-*O*ST-3 isoform to mediate HSV-1 entry, CHO-K1 cells were transiently transfected with the ZF 3-*O*ST-3 expression plasmid. Entry assays were performed using reporter HSV-1 (KOS) gL86 virus to determine whether expression of ZF 3-*O*ST-3 leads to viral entry in resistant CHO-K1 cells. In this assay, we compared the viral entry in CHO-K1 cells expressing ZF-encoded 3-*O*ST-3 with that of a human-encoded 3-*O*ST-3 (positive control). CHO-K1 cells expressing an empty vector pDream2.1 was used as the negative control. As shown (Fig. 1A), there was a significant increase in viral entry in ZF 3-*O*ST-3-expressing cells relative to those cells that were transfected with empty vector alone. For HSV-1 entry, the dosage response seen with ZF-encoded 3-*O*ST-3-expressing CHO-K1 cells were very similar to that of the H 3-*O*ST-3-expressing cells. This result was further confirmed by X-gal assay. As shown in Figure 1B, panel a, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) activity was found to be positive for H 3-*O*ST-3 cells similar to ZF 3-*O*ST-3 cells (Fig. 1B, panel b). Wild-type CHO-K1 cells expressing pDream2.1 empty vector remained resistant to HSV-1 entry (Fig. 1B, panel c).

To generate more direct and visual evidence for viral entry, we used GFP-tagged capsid of HSV-1 (VP26GFP)[22,23] to infect ZF 3-*O*ST-3-expressing CHO-K1 cells. The image of infected CHO-K1 cells expressing human- and ZF-encoded 3-*O*ST-3 with HSV-1VP26GFP virus showed maximum green viral capsid accumulation around the cell membrane and its colocalization with F-actin (red)–stained cells (Fig. 1C, panel b) similar to CHO-K1 cells expressing H 3-*O*ST-3 receptor (Fig. 1C, panel a), whereas CHO-K1 cells expressing pDream2.1 empty vector showed little viral capsid binding and no colocalization with cell membrane (Fig. 1C, panel c).

CHO-K1 cells expressing ZF 3-OST-3 isoform preferentially binds to soluble HSV-1 gD

To provide evidence for the generation of gD receptors within HS by the action of ZF-encoded 3-*O*ST-3 enzyme, a

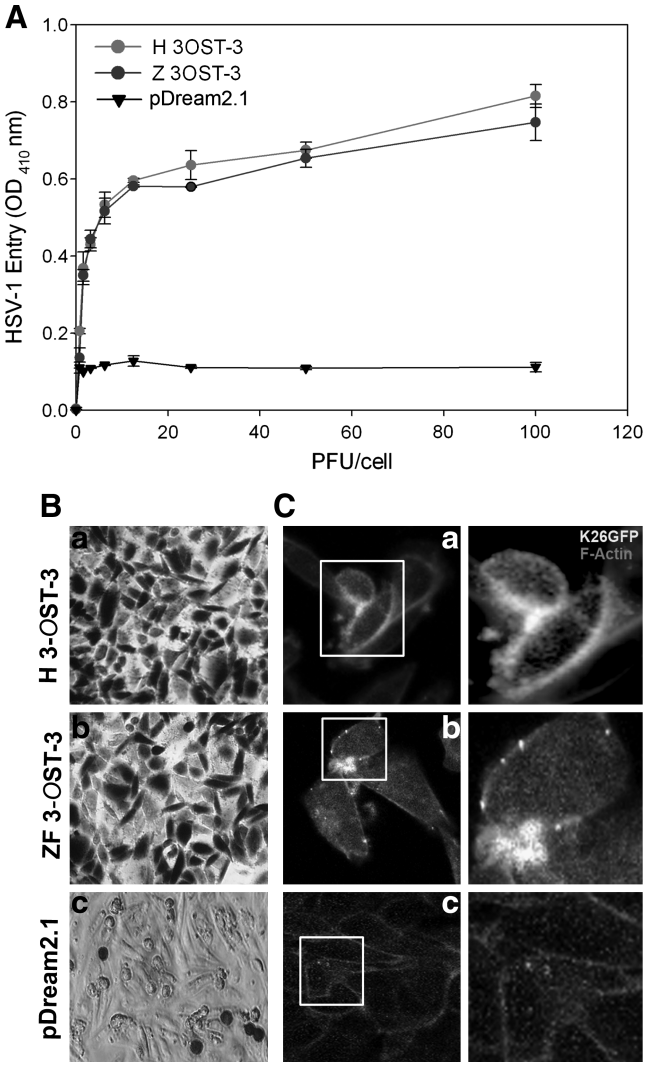

FIG. 1. Expression of zebrafish (ZF) 3-*O*-sulfotransferase-3 (3-*O*ST-3) isoform in wild-type Chinese hamster ovary (CHO-K1) cells results in herpes simplex virus type 1 (HSV-1) entry. (**A**) Dose–response curve of HSV-1 entry into CHO-K1 cells expressing ZF 3-*O*ST-3 isoform. Resistant wild-type CHO-K1 cells were transfected with ZF 3-*O*ST-3 at 2.5 μg DNA, which in resulted HSV-1 KOS (gL86) entry, similar to human 3-*O*ST-3 (H 3-*O*ST-3) expression. Cells transfected with empty vector pDream2.1 at 2.5 μg DNA was used as a negative control. The enzymatic activity was measured at an optical density of 410 nm (OD_{410}). Each value shown is the mean of three or more determinations (±standard deviation). (**B**) HSV-1 entry into ZF 3-*O*ST-3 isoform expressing CHO-K1 cells was confirmed by X-gal staining. CHO-K1 cells (4×10^6 cells) expressing H 3-*O*ST-3 (panel **a**) and ZF 3-*O*ST-3 (panel **b**) were challenged with β-galactosidase-expressing recombinant HSV-1 KOS (gL86) at 25 pfu/cell. Wild-type CHO-K1 cells expressing pDream2.1 (panel **c**) were also infected in parallel as a negative control. After 6 h of infection at 37°C, cells were washed with phosphate-buffered saline, fixed, permeabilized, and incubated with X-gal (5 bromo-4 chloro-3-indoyl-β-D-galactosidase) at 1.0 mg/mL, which yields an insoluble blue product upon hydrolysis by β-galactosidase. Blue cells (representing viral entry) were identified. Microscopy was performed using a 20×objective of Nikon D-Eclipse-C1. (**C**) Observation of HSV-1 entry in cultured CHO-K1 expressing ZF 3-*O*ST-3 isoform. Confluent monolayers of ZF 3OST-3 CHO-K1 cells (panel **b**) were infected with green fluorescent protein–tagged capsid of HSV-1 (VP26-green fluorescent protein) at 25 pfu/cell for 90 min. In parallel CHO-K1 cells expressing pDream2.1 (panel **c**) and H 3-*O*ST-3 (panel **a**) were used as negative and positive controls, respectively. At 90 min postinfection, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde, and stained with 10 nm rhodamine-conjugated phalloidin (red) for F-actin. The images were taken using a fluorescent confocal microscope (Nikon D-Eclipse-C1) at 40× objective.

previously defined soluble gD-cell binding assay was used.[6] 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-*O*S HS. Interestingly, in the case of 3-*O*S HS, but not for nectin-1, the preferential binding of gD to cells can be reversed by prior treatment of cells with heparin lyases (heparinases).[6] Thus, CHO-K1 cells were transiently transfected with ZF and H 3-*O*ST-3 isoforms, 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. As shown in Figure 2, there was a significantly higher amount of gD:Fc bound to the mock-treated 3-*O*ST-3-expressing cells than those cells that were treated with heparinase-II and -III. The same pattern, however, was not seen with nectin-1-expressing cells. Taken together with the results from the gD-mediated interference assay, it is very likely that expression of ZF 3-*O*ST-3 in CHO-K1 cells results in the generation of HSV-1gD receptors.

ZF-encoded 3-OST-3 mediates HSV-1 glycoprotein-induced cell-to-cell fusion

After establishing the ability of 3-*O*ST-3 to serve as a receptor for HSV-1 entry, we next examined whether the same receptor could also facilitate cell-to-cell fusion. Once again CHO-K1 cells were used that are also resistant to virus-induced cell fusion due to the absence of a gD receptor.[10] A luciferase reporter gene assay was performed to quantify the induced cell fusion between 3-*O*S HS cells modified by 3-*O*ST-3 and HSV-1 glycoproteins.[10] 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-*O*ST plasmid expressing ZF-encoded 3-*O*ST-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-*O*ST-1 because 3-*O*S HS modified by this isoform has been shown not to interact with HSV-1.[10] The cells expressing H 3-*O*ST-3 and T7RNA polymerase served as a positive control. Figure 3A shows a high amount of fusion occurring in both human- and ZF-encoded 3-*O*ST-3-expressing cells. Next to verify the results obtained by the luciferase assay, we looked at polykaryocyte formation by mixing ZF-encoded 3-*O*ST-3 expressing target cells with the effector cells. This phenomenon mimics multinucleated cell (polykaryocyte) formation during an *in vivo* HSV-1 infection when infected cells fuse with uninfected cells, allowing the spread of the virions to the neighboring cells. Our results show that the polykaryocytes of similar number (Fig. 3B) and sizes were formed in both the cell populations expressing H 3-*O*ST-3 (Fig. 3C, panel b) and ZF 3-*O*ST-3 (Fig. 3C, panel c). Virtually no polykaryocytes were seen in the negative control 3-*O*ST-1-expressing cells (Fig. 3C, panel a). These results reinforce our finding that cells expressing ZF 3-*O*ST-3 allow cell fusion to occur, and thus potentially could facilitate spread of HSV-1 in an *in vivo* infection.

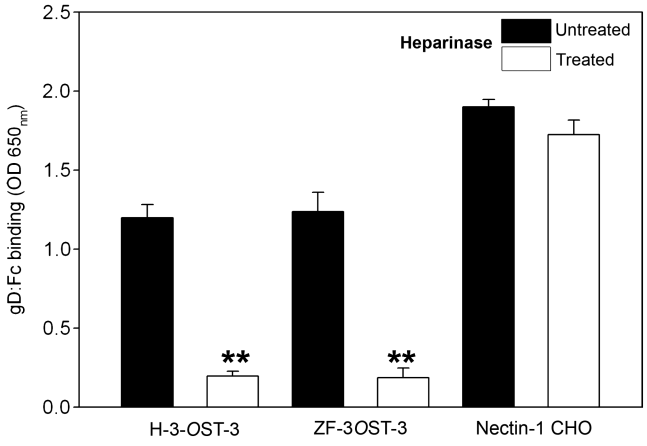

FIG. 2. Enzymatic removal of 3-*O*ST-3-modified heparan sulfate from cells removes glycoprotein D (gD)–binding receptors. A soluble recombinant form of HSV-1 gD (gD:Fc) was allowed to bind cells expressing the 3-*O*ST isoforms (ZF 3-*O*ST-3 and H 3-*O*ST-3) and the cells expressing nectin-1 as indicated. The cells were either treated with a mixture of heparinase-II/-III (treated at 4 U/mL) 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. The values shown represent the amount of reaction product detected spectrophotometrically (OD_{650}). The data shown are the means of triplicate measures and are representative of three independent experiments. Double asterisks indicate significant difference from other treatments ($p < 0.01$, *t*-test); error bars represent SD.

3-OST-3 is required for HSV-1 entry into ZF fibroblast

We next verified the significance of ZF-encoded 3-*O*ST-3 entry receptor for HSV-1 entry in ZF fibroblast (ZF4) cells. The cells were pretreated with heparinase enzyme to remove both HS and 3-*O*ST-3 receptor from the cell surface. Less viral entry was observed for cells when heparinase (Fig. 4A) had been used in both ZF4 and CHO-K1 cells expressing H 3-*O*ST-3 receptor; however, HeLa cells that use nectin-1 as the gD receptor showed no decrease in entry (Fig. 4A), suggesting that ZF-encoded 3-*O*ST-3 receptor plays a significant role in the entry process.

ZF-encoded 3-OST-3 acts a major receptor for HSV-1 entry into ZF fibroblast

To prove that 3-*O*ST-3 receptor is critical for HSV-1 entry in ZF fibroblast; 3-*O*ST-3 receptor was downregulated using siRNA against ZF 3-OST-3 in ZF fibroblast cells. Significantly less viral entry (Fig. 4B) and gD binding to cells (Fig. 4C) was observed when 3-*O*ST-3 was downregulated by siRNA compared to scramble siRNA, suggesting that the 3-*O*ST-3 may be major mediator of HSV-1 entry into ZF fibroblasts.

Discussion

Our previous studies demonstrated that H (human) 3-*O*ST-3-modified HS serves as a receptor for HSV-1 entry in primary cultures of human corneal fibroblasts and viral-glycoprotein-induced cell fusion.[22,24] Because more than 70% sequence similarities were identified between ZF-encoded 3-*O*ST-3 and H 3-*O*ST-3 gene,[19] but not with other isoforms of 3-*O*STs, it appeared logical to test ZF HS modified by 3-*O*ST-3 isoform for their ability to mediate entry and fusion for HSV-1. The

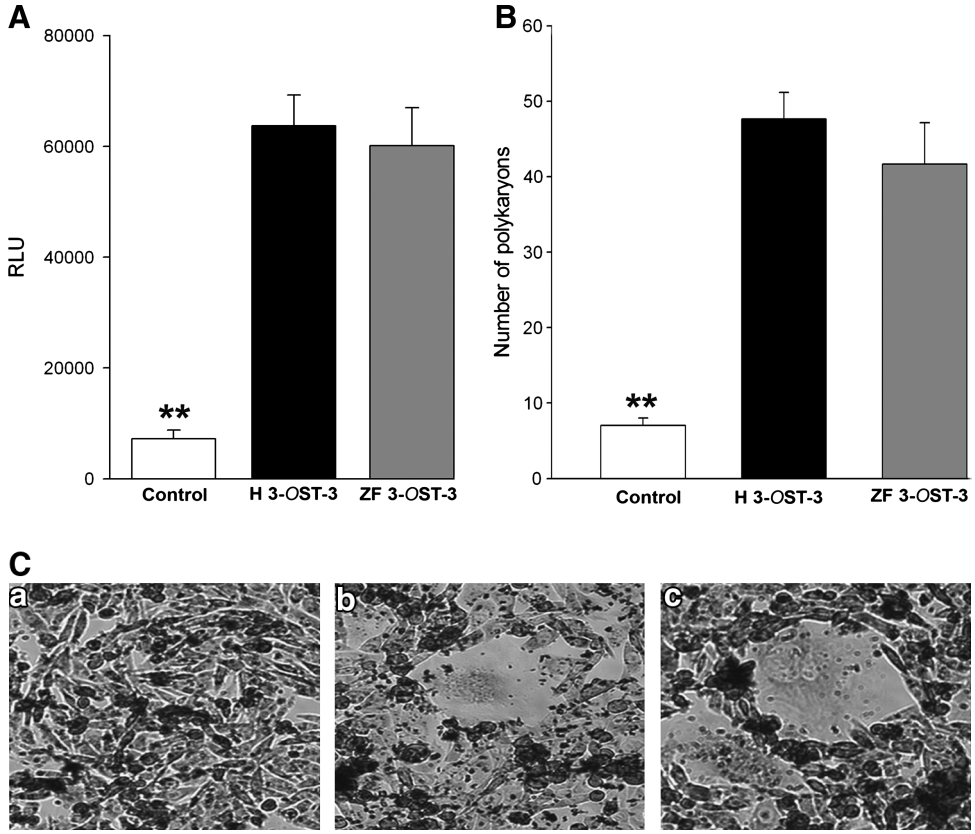

FIG. 3. (**A**) ZF 3-*O*ST-3-expressing target CHO-K1 cells gain the ability to fuse with effector cells coexpressing HSV-1 glycoproteins gB, gD, gH, and gL. The target cells were transfected with plasmids expressing H 3-*O*ST-3 and ZF 3-*O*ST-3 (as indicated), and luciferase reporter gene. The effector 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) using a Sirius luminometer (Berthold Detection System). (**A**) Expression of ZF 3-*O*ST-3 isoform results in the fusion of CHO-K1 cells with HSV-1-glycoprotein-expressing cells as measured by a luciferase assay system with reporter lysis buffer (Promega). The data shown are the means of triplicate measures and are representative of three independent experiments. Double asterisks indicate significant difference from other treatments ($p < 0.01$, *t*-test); error bars represent SD. (**B**) Quantitative determination of the number of fused polykaryocytes formed after coculture of target cells expressing either ZF-encoded 3-*O*ST-3 or human-encoded 3-*O*ST-3 receptors with the effector CHO-K1 cells expressing HSV-1 glycoproteins. Target CHO-K1 cells expressing empty vector (pDream2.1) cultured with effector CHO-K1 cells were used as negative controls. The data shown are the means of triplicate measures with three independent experiments. Double asterisks indicate significant difference from other treatments ($p < 0.01$, *t*-test); error bars represent SD. (**C**) Multinucleated cells or polykaryocytes were microscopically observed with cells expressing H 3-*O*ST-3 (panel **b**) and/or ZF 3-*O*ST-3 (panel **c**), but not with cells expressing control vector pDream2.1 (panel **a**). CHO-K1 target cells transfected with plasmids expressing H 3-*O*ST-3 or ZF 3-*O*ST-3 were mixed with the HSV-1 glycoprotein expressing effector cells, and stained with Giemsa at 24 h postmixing. The effects of H 3-*O*ST-3- or ZF 3-*O*ST-3-expressing CHO-K1 cells on multinucleated polykaryocytes formation were observed. Shown are photographs of representative cells (Nikon) after 24 h.

novel finding presented herein is that 3-*O*S HS generated by ZF-encoded 3-*O*ST-3 isoform can also mediate HSV-1 entry and cell-to-cell fusion by polykaryocyte formation in CHO-K1 cells that lack endogenous 3-*O*-sulfation.[6] The significance of ZF-encoded 3-*O*ST-3 receptor was further demonstrated in ZF fibroblast cells, where downregulation of 3-*O*ST-3 receptors significantly reduced the HSV-1 entry (Fig. 4B). These results suggest the possible involvement of 3-*O*ST-3 receptor in the embryo or adult fish model of HSV-1 infection.

The knowledge that ZF 3-*O*S HS modified by 3-*O*ST-3 is the mediator for polykaryocyte formation in cell culture can be useful for following reasons: (1) it can lead the way for the development of anti-3-*O*S HS compounds that can potentially block 3-*O*S HS in the spread of infection in ZF models, (2) 3-*O*ST-3 and other 3-*O*ST isoforms knockouts in ZF can be tested for HSV-1 infection, (3) because the 3-*O*ST-3 gene family is differentially regulated in ZF,[19] it is possible that we might generate a HSV-1 tropism model in animals and understand viral pathogenesis in more detail in terms of spread or the establishment of latency, and (4) a model animal for herpes stromal keratitis can be also established in ZF cornea, which in turn is similar to human cornea by structure and development.[25] An important question remains to be demonstrated is whether ZF-encoded 3-*O*ST-3 alone or other isoforms of ZF-encoded 3-*O*S HS can also mediate the HSV-1 spread or polykaryocyte formation in the infected model of ZF. There is

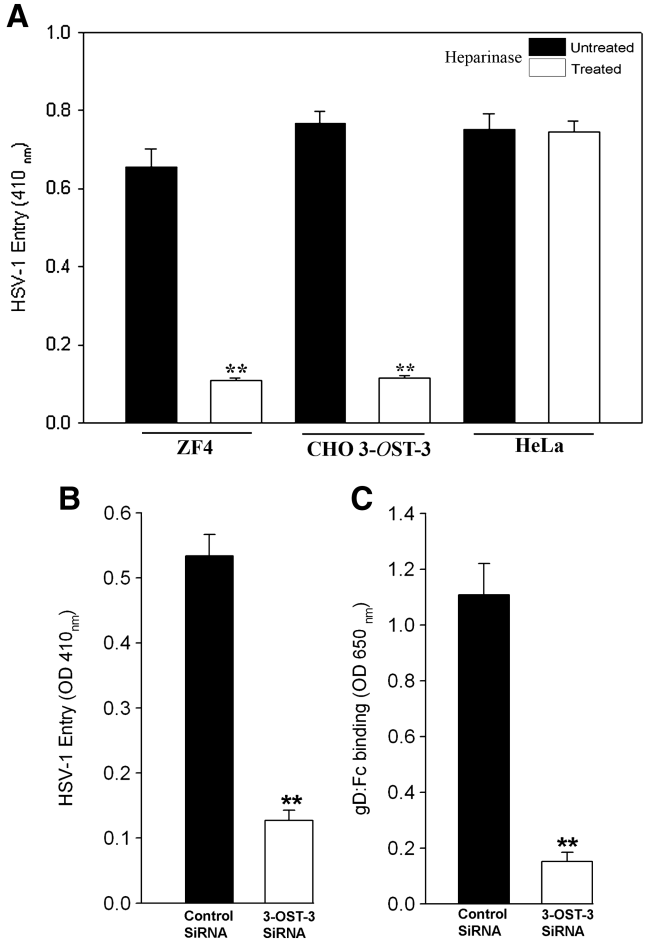

FIG. 4. (**A**) Effect of heparinase treatment on HSV-1 entry into ZF fibroblast (ZF4) cells. Cultured ZF4, control CHO-K1 cells expressing H 3-*O*ST-3 receptor and HeLa cells were treated with heparinase-II and -III (4 U/mL) in 96-well tissue culture dishes. A separate set of cultured cells were mock treated with phosphate-buffered saline alone. The cells were then exposed to HSV-1 (KOS) gL86 at 25 pfu/cell, and viral entry was quantitated 6 h later by *o*-nitrophenyl-*β*-D-galactopyranoside assay. Data shown are the means of triplicate measures. (**B**). siRNA downregulation of 3-*O*ST-3 receptor significantly affects HSV-1 entry in ZF4 cells. HSV-1 entry was determined in ZF4 cells transfected with ZF-encoded 3-*O*ST-3-specific siRNA. Cells transfected with an equal amount of scrambled siRNA were used as control. The cells were then exposed to HSV-1 (KOS) gL86 at 25 pfu/cell, and viral entry was quantitated 6 h later by *o*-nitrophenyl-*β*-D-galactopyranoside assay. Data shown are the means of triplicate measures. (**C**) HSV-1 glycoprotein D (gD) binding to ZF4 cells is significantly affected by siRNA down-regulation of 3-*O*ST-3 receptor. A soluble recombinant form of HSV-1 gD (gD:Fc) was allowed to bind ZF4 cells transfected with control siRNA (black bar) and 3-*O*ST-3-specific siRNA (white bar). Binding of gD:Fc was detected by use of a secondary antibody against rabbit IgG:Fc and a horseradish peroxidase detection system. The values shown represent the amount of reaction product detected spectrophotometrically (OD_{650}). The data shown are the means of triplicate measures and are representative of three independent experiments. Double asterisks indicate significant difference from other treatments ($p < 0.01$, *t*-test); error bars represent SD.

growing literature on the use of ZF to model viral disease including HSV-1.[20,26–30] While the study of disease processes in the ZF is a powerful tool in its own right, the development of ZF models of viral entry and spread is also a first step for their use in antiviral drug discovery, especially for anti-3*O*S HS compounds targeted against HSV. ZF's competitive advantage over other model systems is optical clarity in a vertebrate embryo amenable to large-scale screening, including genetic and small molecule drug screens.

In summary, our work attempts to appreciate the potential contribution of ZF-encoded 3-*O*ST-3 as a receptor for HSV-1. One recent study indicated that ZF is an excellent experimental organism to study the biological roles of glycosaminoglycans because of the difference in the disaccharide and sulfation pattern that exists among ZF of different stages of embryonic development.[31] In this context the differential pattern of ZF 3-*O*ST-3 expression may become even more interesting to test if the 3*O*S HS variability reflects in any change in HSV-1 pathogenesis or cell tropism. Our study is likely to advance ZF as an alternate model system to study unique interactions between HSV-1 gD and modified forms of HS.

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Disclosure Statement

No competing financial interests exist.

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