Using a 3-*O*-Sulfated Heparin Octasaccharide To Inhibit the Entry of Herpes Simplex Virus Type 1[†]

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ABSTRACT: Heparan sulfate (HS) is a highly sulfated polysaccharide and is present in large quantities on the cell surface and in the extracellular matrix. Herpes simplex virus type 1 (HSV-1) utilizes a specialized cell surface HS, known as 3-*O*-sulfated HS, as an entry receptor to establish infection. Here, we exploit an approach to inhibiting HSV-1 infection by using a 3-*O*-sulfated octasaccharide, mimicking the active domain of the entry receptor. The 3-*O*-sulfated octasaccharide was synthesized by incubating a heparin octasaccharide (3-OH octasaccharide) with HS 3-*O*-sulfotransferase isoform 3. The resultant 3-*O*-sulfated octasaccharide has a structure of ΔUA2S-GlcNS6S-IdoUA2S-GlcNS6S-IdoUA2S-GlcNS3S6S-IdoUA2S-GlcNS6S (where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid, GlcN is D-glucosamine, and IdoUA is L-iduronic acid). Results from cell-based assays revealed that the 3-*O*-sulfated octasaccharide has stronger activity in blocking HSV-1 infection than that of the 3-OH octasaccharide, suggesting that the inhibition of HSV-1 infection requires a unique sulfation moiety. Our results suggest the feasibility of inhibiting HSV-1 infection by blocking viral entry with a specific oligosaccharide.

Heparan sulfate (HS)[1] is a highly sulfated linear polysaccharide present ubiquitously on the cell surface and in the extracellular matrix. HS plays a role in regulating embryonic development, inflammatory response, blood coagulation, and assisting viral/bacterial infections (*1*). Heparin, a commonly used anticoagulant drug, is a special form of HS containing glucuronic (GlcUA)/iduronic acid (IdoUA) and glucosamine, each carrying sulfo groups (Figure 1A). The uniquely distributed sulfation pattern of HS polysaccharide is believed to regulate its functional specificity (*2–4*). Thus, understanding the structure−function relationship of HS attracts considerable interest in a bid to improve the anticoagulant efficacy of heparin and to exploit heparin or heparin-like molecules for the development of anticancer and antiviral drugs (*5–7*). The major difficulty in dissecting the structure−function relationship of HS is obtaining HS oligosaccharides or polysaccharides with defined structures. Chemical synthesis has been the major route for preparing structurally defined oligosaccharides to mimic the functions of HS. However, the synthesis of those molecules larger than hexasaccharides is extremely difficult. Using HS biosynthetic enzymes to prepare biologically active polysaccharides and oligosaccharides has recently gained momentum (*8–12*).

The biosynthesis of HS occurs in the Golgi apparatus. HS is initially synthesized as a linear copolymer of GlcUA and GlcNAc, which is then followed by various modifications. These modifications are carried out by specialized sulfotransferases and a glucuronyl C_5-epimerase. The glucosaminyl *N*-deacetylase/*N*-sulfotransferase converts an *N*-acetylated glucosamine (GlcNAc) residue to an *N*-sulfoglucosamine (GlcNS) residue. After *N*-sulfation, C_5-epimerase converts some GlcUA residues to IdoUA residues. The resultant polysaccharide is further modified by 2-*O*-sulfotransferase, 6-*O*-sulfotransferase, and 3-*O*-sulfotransferase to introduce the 2-*O*-sulfo group to IdoUA and GlcUA and 6-*O*-sulfo and 3-*O*-sulfo groups to the glucosamine residue, respectively. Unlike the case for proteins and DNA, biosynthesis of HS is not a template-driven process. The control of the sulfation pattern at the polysaccharide is largely unknown. However, enzymatic synthesis of structurally defined oligosaccharides

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[1] Abbreviations: HS, heparan sulfate; HSV-1, herpes simplex virus type 1; ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; GlcN, D-glucosamine; GlcNAc, *N*-acetylated glucosamine; GlcNS, *N*-sulfoglucosamine; IdoUA, L-iduronic acid; GlcUA, D-glucuronic acid; 3-OST-3, heparan sulfate 3-*O*-sulfotransferase isoform 3; gD, glycoprotein D; PAMN-HPLC, silica-based polyamine high-performance liquid chromatography; 2-AB, 2-aminobenzamide; ESI-MS, electrospray ionization mass spectrometry; CF, cornea fibroblast.

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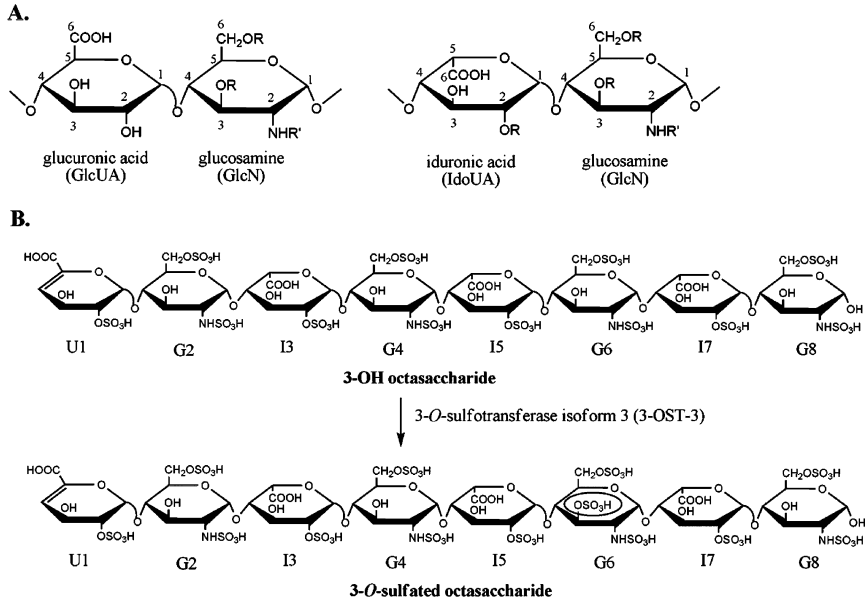

FIGURE 1: HS structure and the preparation of 3-*O*-sulfated octasaccharide by 3-OST-3. Panel A shows the structures of the disaccharide building blocks that make up heparin and heparan sulfate. Numbers denote positions. Panel B illustrates the scheme for preparing 3-*O*-sulfated octasaccharide using the 3-OST-3 enzyme. For clarity, each sugar unit of the octasaccharide is labeled as U1, G2, etc., where U is uronic acid, G is glucosamine, and I is iduronic acid. Also, R can be either H or SO_3H, while R′ can be either H, Ac, or SO_3H, where Ac is an acetyl group.

appears to be possible using a structurally defined oligosaccharide substrate (*11*).

Herpes simplex virus type 1 (HSV-1) belongs to the alphaherpesviruses subfamily of the herpesvirus family. Infections with HSV-1 are highly prevalent in humans and cause localized but recurrent mucocutaneous lesions and encephalitis in rare cases (*13*). Further, infection of HSV-1 in the eye is a leading cause of blindness in the United States (*14*). Cell surface HS plays an important role in assisting attachment of HSV-1 to host cells, as well as in inducing entry of virus into target cells (*15*). The attachment process primarily involves the interaction between HS and the virion envelope glycoproteins gC and/or gB (*16*). Following attachment, HSV-1 enters the target cells by interacting with specific cell surface entry receptors to establish the infection (*17*). Three families of HSV-1 entry receptors are known, and all of the known receptors interact with HSV-1 envelope protein gD. HVEM (herpesvirus entry mediator) and nectin-1 represent two families of those receptors, which belong to the TNFR (tumor necrosis factor receptor) family and the immunoglobulin superfamily, respectively (*18, 19*). The 3-*O*-sulfated HS, which is generated by six 3-OST isoforms, represents the third family of HSV-1 entry receptors (*20–24*). This receptor is unique as it is a polysaccharide and contains a specific saccharide structure. Further structural analysis of a gD-binding oligosaccharide revealed that the binding of HS to gD requires a 3-*O*-sulfated glucosamine unit (*25*).

Our research attention has been focused on identifying a specific HS structure for inhibiting herpes infections. Numerous reports have been published on using sulfated compounds, especially sulfated polymers, to block herpes infections (*26, 27*). However, the sulfated polymers that are structurally heterogeneous could bind to a variety of physiologically important proteins, and thus, the risk of toxicity to the host is increased. In this paper, we exploited the possibility of inhibiting HSV-1 infection by targeting the gD-mediated membrane fusion step that is required for virus penetration. The inhibition was achieved by using a 3-*O*-sulfated heparin octasaccharide, which mimics the gD-binding site of the 3-*O*-sulfated HS polysaccharide. This octasaccharide was synthesized by incubating purified 3-*O*-sulfotransferase isoform 3 (3-OST-3) and a heparin-derived octasaccharide, 3-OH octasaccharide (Figure 1B). The structure of the 3-*O*-sulfated octasaccharide was proven to be ΔUA2S-GlcNS6S-IdoUA2S-GlcNS6S-IdoUA2S-GlcNS3S6S-IdoUA2S-GlcNS6S (where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosylurmic acid, S is sulfate, GlcN is D-glucosamine, and IdoUA is L-iduronic acid). The synthetic octasaccharide exhibited much more effective inhibition of HSV-1 infection than 3-OH octasaccharide at ≥40 μM in a cell-based assay. Our results suggest the feasibility of inhibiting HSV-1 infection with a specific oligosaccharide as a therapeutic reagent to treat herpes simplex virus infection.

EXPERIMENTAL PROCEDURES

Preparation of Heparin Tetra-, Hexa-, and Octasaccharides. A solution containing 1.0 g of heparin (150 units/mg from Celsus, Cincinnati, OH) in 15 mL of 50 mM sodium phosphate buffer (pH 7.0) and heparin lyase I (92 munits) was incubated in a polyethylene vial at 37 °C for 10 h. When the reaction was 30% complete, the mixture was heated to 100 °C for 5 min to inactivate the enzyme. The denatured protein was removed by centrifugation at 12000*g* for 10 min. The low-molecular mass heparin oligosaccharides (average molecular mass of <5000 Da) were obtained by pressure filtration. Fractionation was carried out on a Bio Gel P-10 (fine) column (4.8 cm × 1.5 m) eluted with 200 mM sodium chloride at a flow rate of 0.5 mL/min which afforded sized oligosaccharide fractions (detected at 232 nm). The tetrasaccharides, hexasaccharides, and octasaccharides were collected and desalted on a Bio Gel P-2 column (4.8 cm × 70 cm) using water as the mobile phase and detected at 232 nm.

The resulting sized oligosaccharide mixtures were freeze-dried and charge-separated using strong anion-exchange high-pressure liquid chromatography (SAX-HPLC) on a semi-preparative SAX S5 Spherisorb column (Waters) with a 0.1 to 2 M NaCl (pH 3.5) linear gradient. The major peak from each oligosaccharide mixture was collected, desalted, and freeze-dried. The structures isolated were confirmed by ESI-MS (*28*) and ^{1}H NMR spectroscopy (*29*).

Preparation of 3-O-[^{35}S]Sulfated Oligosaccharides. To prepare 3-*O*-sulfated oligosaccharides, individual reaction mixtures consisting of 1−5 μg of the oligosaccharide (either tetra-, hexa-, or octasaccharide) were mixed with approximately 140−240 ng of purified 3-OST-3 enzyme (*30*) and [^{35}S]PAPS (2×10^6 cpm) in a buffer containing 50 mM MES, 1% Triton X-100, 5 mM $MgCl_2$, 10 mM $MnCl_2$, and 100 μg/mL bovine serum albumin (pH 7) in a final volume of 65 μL. The reaction mixture was incubated for 1.5 h at 37 °C. The reaction was stopped by boiling the mixture at 100 °C for 2 min. The resultant solution was centrifuged at 14000 rpm for 2 min to remove any insoluble materials. The supernatant was then subjected to a 200 μL DEAE column, and 3-*O*-[^{35}S]sulfated oligosacccharides were eluted with 1000 mM NaCl.

Expression and Purification of the 3-OST-3 Enzyme. N-Terminally $(His)_6$-tagged 3-OST-3 catalytic domain (Gly^{139}−Gly^{406}) was expressed in *Escherichia coli*. The enzyme was purified with a Ni^{2+}-agarose column as described previously (*30*).

Assessment of the Binding of 3-O-[^{35}S]Sulfated Oligosaccharides to gD. The assay for assessing the binding of various sized 3-*O*-[^{35}S]sulfated oligosaccharides to gD was carried out by an immunoprecipitation procedure using purified gD and DL6, an anti-gD monoclonal antibody (*20*). Either the 3-*O*-[^{35}S]sulfated tetra-, hexa-, or octasaccharide (1−10 pmol) was incubated in 50 μL of buffer containing 50 mM Tris, 0.001% Triton X-100 (pH 7.4) (binding buffer), and 20 μg of gD at room temperature for 30 min. The anti-gD monoclonal antibody DL6 (5 μL) was added, and the reaction mixture was incubated on ice for 1 h, followed by the addition of protein A−agarose beads with agitation at 4 °C for an additional 1 h. The protein A−agarose beads were then washed stepwise with 150 mM NaCl in the binding buffer described above. The bound oligosaccharides were eluted with 1000 mM NaCl.

Affinity Co-Electrophoresis. The binding affinity between the 3-*O*-[^{35}S]sulfated oligosaccharides and gD was determined by an affinity co-electrophoresis approach as previously described (*20, 31*). Briefly, purified gD was casted in 1% low-melting point agarose separation zones in a degassed gel buffer containing 125 mM sodium acetate and 50 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (pH 7) at six final concentrations ranging from 0 to 60 μM gD in each zone. The 3-*O*-[^{35}S]sulfated octasaccharide (≈28000 cpm) or 3-*O*-[^{35}S]sulfated hexasaccharide (≈28000 cpm) was loaded into each separation zone. The gel electrophoresis was performed at 350 mA for 3 h in circulated cold water. The gel was dried and analyzed on a PhosphorImager (Amersham Biosciences, Storm 860). The autoradiography image was obtained by exposing the gel to X-ray film at −80 °C for 1 week.

Digestion with $\Delta^{4,5}$-Glycuronate-2-sulfatase. The 3-*O*-[^{35}S]sulfated octasaccharide was digested with $\Delta^{4,5}$-glycuronate-2-sulfatase (Sigma) as described previously (*30*).

Reducing End Labeling with 2-AB (2-Aminobenzamide). The coupling of the 3-*O*-[^{35}S]sulfated oligosaccharides with 2-aminobenzamide (2-AB) was achieved using a similar procedure as previously described (*32, 33*). The 3-*O*-[^{35}S]sulfated oligosaccharide (1−50 nmol) was dried. An aliquot (5−20 μL) of a freshly prepared coupling reagent containing 0.35 M 2-AB, 1 M $NaBH_3CN$, and 30% (v/v) acetic acid in DMSO was added to the dried sample and incubated for 3 h at 65 °C. The resultant reaction mixture was spotted via paper chromatography. The paper strips were washed with 1 mL of acetonitrile three times, while the labeled oligosaccharide was eluted with 1 mL of distilled water. The labeled oligosaccharides were dried and reconstituted in 50−100 μL of distilled water for further analysis.

Preparation of Larger Quantities of 3-O-Sulfated Octasaccharide. Scale-up preparation of the 3-*O*-sulfated octasaccharide was achieved by coupling 3-OST-3 modification with a PAPS regeneration system (*10*). The reaction mixture containing 50 mM MES (pH 7), 40 μM PAP, 1 mM PNPS, 0.1 mg/mL arylsulfotransferase IV, and 10 μg/mL purified 3-OST-3 was incubated at room temperature for 15 min. To the reaction mixture was added 3-OH octasaccharide (50 μg/mL) substrate, and the reaction mixture was rotated at room temperature overnight. The reaction was terminated by boiling for 2 min. The resultant material was loaded onto a 1 mL DEAE column equilibrated with 150 mM NaCl. The 3-*O*-sulfated octasaccharide was eluted from the DEAE column with 1 M NaCl.

Expression and Purification of Recombinant gD. A truncated form of gD (Lys^{25}−His^{332}) with a $(His)_6$ tag at the C-terminus was expressed in *E. coli*. The cDNA of gD was isolated from recombinant baculovirus expressing gD306t (a gift from G. Cohen and R. Eisenberg, University of Pennsylvania, Philadelphia, PA) amplified by PCR using two primers, 5′-ATTATTATCATATGAAATATGCCTTGGCGGATGC-3′ (*Nde*I site underlined) and 5′-ATAATATAAAGCTTATGGTAAGGCGTCGCGGCGT-3′ (*Hin*dIII site underlined). The PCR product was cloned into the pET21b vector (Novagen) using *Nde*I and *Hin*dIII sites, and the plasmid was sequenced to confirm the product (University of North Carolina DNA Sequencing Core Facility). Expression of gD was carried out in Origami-B cells (Novagen) carrying the pGro7 plasmid (Takara, Japan) expressing chaperonin proteins GroEL and GroES of *E. coli*. Transformed cells were grown in LB medium supplemented with 12.5 μg/mL tetracycline, 15 μg/mL kanamycin, 20 μg/mL chloramphenicol, and 50 μg/mL carbenicillin at 37 °C. When the OD_{600} reached 0.6−0.8, isopropyl β-thiogalactopyranoside (IPTG) (200 μM) and arabinose (1 mg/mL) were added to induce the expression of gD and chaperone, respectively. The cells were then allowed to shake overnight at 22 °C. The cells were harvested, and recombinant gD was purified with a Ni^{2+}-Sepharose column (Amersham). The protein was further purified with a HiLoad 16/60 Superdex column (Amersham Biosciences) which was eluted with a buffer containing 20 mM Tris and 1 M NaCl (pH 7). The purity of the resulting gD was typically greater than 85% as determined by precasted 12% SDS−PAGE (Bio-Rad). This two-step pu-

rification allowed for the recovery of approximately 5 mg of purified gD from 1 L of bacterial culture.

PAMN-HPLC. The 3-*O*-[^{35}S]sulfated oligosaccharides were analyzed by monitoring their elution profiles when applied to a silica-based polyamine (PAMN) HPLC column (0.46 cm × 25 cm, Waters) (*30*). The column was eluted using a linear gradient of KH_2PO_4 from 300 to 1000 mM over 60 min at a flow rate of 0.5 mL/min.

Determination of the Purity and Concentration of 3-O-Sulfated Octasaccharide. The purity of the 3-*O*-sulfated HP octasaccharide was determined using a nonporous DEAE-NPR column (0.46 cm × 7.5 cm, Tosohass). The DEAE-NPR column was eluted using a linear gradient of NaCl from 100 to 1000 mM in 20 mM Tris (pH 7.0) over 100 min at a flow rate of 0.5 mL/min. The eluted materials were monitored by both an online radioactivity and UV detector. The concentration of 3-*O*-sulfated octasaccharide was determined by comparing the area of the UV (232 nm) peak with that of the unmodified octasaccharide with a known amount.

Preparation of 3-O-[^{35}S]Sulfated Disaccharide and 3-O-[^{35}S]Sulfated Tetrasaccharide as Well as the Corresponding 2-AB-Labeled Standards. The 3-*O*-[^{35}S]sulfated tetrasaccharide standard that has a structure of ΔUA2S-[3-*O*-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6S was prepared by incubating a heparin tetrasaccharide substrate (ΔUA2S-GlcNS6S-IdoUA2S-GlcNS6S) and purified 3-OST-3 enzyme. The 3-*O*-sulfated tetrasaccharide was purified with a DEAE column, and the structural characterization was described in a previous publication (*30*). The 3-*O*-[^{35}S]sulfated disaccharide standard (ΔUA2S-[3-*O*-^{35}S]GlcNS3S6S) was prepared by digesting the 3-*O*-[^{35}S]sulfated tetrasaccharide using a mixture of heparin lyase I, II, and III followed by purification via PAMN-HPLC as described previously (*30*). The 2-AB-labeled tetra- and disaccharide standards were prepared by incubating the 3-*O*-[^{35}S]sulfo tetrasaccharide or the 3-*O*-[^{35}S]sulfated disaccharide with 2-aminobenzamide as described above. The labeled products were analyzed by PAMN-HPLC to confirm the completion of the labeling reaction. The 2-AB-labeled di- or tetrasaccharides carried both ^{35}S radioactivity and absorbance at 310 nm, which is from the 2-AB tag.

Electrospray Ionization Mass Spectrometry. The sample was dialyzed against 25 mM ammonium acetate using a MWCO 1000 membrane (Spectrum). The sample was dissolved in a solution contained in 70% acetonitrile and 10 μM imidazole and was introduced by direct infusion (10 μL/min) into the electrospray ionization mass spectrometer (Agilent 11090 MSD-Trap). Experiments were preformed in negative ionization mode (2000 V at 200 °C, dry gas at 15 psi, nebulizing gas at 5 L/min).

Viral Entry Assays. Viral entry was quantified for the level of β-galactosidase in the target cells infected by a recombinant HSV-1 (KOS gL86), in which β-galactosidase expression can be induced by early viral protein synthesis upon HSV infection. Cells [HeLa and cornea fibroblast (CF) cells] were plated at a density of 2×10^4 cells/well in 96-well plates at least 16 h prior to infection. HSV virions were preincubated with octasaccharides before infection. The activity of β-galactosidase was determined by using *o*-nitrophenyl β-D-galactopyranoside (ONPG) or by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a substrate as previously described (*20*). Microscopy was performed with the 20× objective of an inverted microscope (Axiovert 100 M; Zeiss). All experiments were repeated a minimum of three times unless otherwise noted.

Virus-Free Cell-to-Cell Fusion Assay. The CHO-K1 cells designated effector cells were cotransfected with plasmids expressing four HSV-1(KOS) glycoproteins, pPEP98 (gB), pPEP99 (gD), pPEP100 (gH), and pPEP101 (gL), along with plasmid pT7EMCLuc, which expresses the firefly luciferase gene under the control of the T7 promoter (*22*). Wild-type CHO-K1 cells lack functional entry receptors; therefore, the cells are resistant to both HSV entry and virus-induced cell fusion (*20, 22–24*). The effector cells after a 30 min incubation with octasaccharides were cocultured with CHO-K1 target cells cotransfected with the plasmid expressing 3-OST-3 to synthesize an entry receptor and pCAGT7, which expresses T7 RNA polymerase with the chicken actin promoter and the CMV enhancer (*20, 23*). Effector cells expressing pT7EMCLuc and pcDNA3 (devoid of any viral glycoproteins) and target CHO-K1 transfected with T7 RNA polymerase alone (no expression of 3-OST-3) were used as negative controls. Activation of the reporter luciferase gene, as a measurement of cell fusion, was examined by a reporter lysis assay (Promega) 24 h after mixing as previously described.

RESULTS

Enzymatic Synthesis of 3-O-Sulfated Octasaccharide. The starting material, 3-OH octasaccharide, was isolated from partially depolymerized heparin (*29*). The octasaccharide was eluted as a major component with absorbance at 232 nm when analyzed by DEAE-NPR-HPLC (Figure 2A), suggesting that the purity of the starting octasaccharide is adequate. The 3-*O*-sulfated octasaccharide was prepared by incubating 3-OST-3 enzyme, 3-OH octasaccharide, and ^{35}S-labeled PAPS as illustrated in Figure 1B. The resultant octasaccharide product, carrying a 3-*O*-[^{35}S]sulfo group, afforded a prominent peak at 62 min (Figure 2B) that carried ^{35}S radioactivity (Figure 2B, inset). We estimated that the purity of the 3-*O*-sulfated octasaccharide was between 70 and 80% based on the elution profile. We noted that 20−30% of the ^{35}S-labeled oligosaccharides were eluted at 58 min, raising the question of whether this fraction of oligosaccharides binds to gD. To address this question, we affinity-purified some 3-*O*-sulfated octasaccharides by an immunoprecipitation approach using gD and anti-gD antibody as described in Experimental Procedures. We then compared the elution profile of gD affinity-purified octasaccharides to that of the unpurified ones. Quite noticeably, we did not find any difference between the relative ratio in the intensity of the ^{35}S peak eluted at 58 min versus the one at 62 min (chromatograms not shown), suggesting both components bind to gD. Our efforts in the structural characterization of the octasaccharide were then focused on the component that was eluted at 62 min.

Electrospray ionization mass spectrometric analyses were performed for the 3-OH and 3-*O*-sulfated octasaccharides. The MS spectrum of 3-OH octasaccharide is shown in Figure 2C. The sample exhibited two prominent molecular ion peaks. These were $[M - 7H + 2Na]^{5-}$ at *m*/*z* 469.3 (M_r = 2307.5 Da) and $[M - 5H + Na]^{4-}$ at *m*/*z* 580.3 (M_r = 2303.2

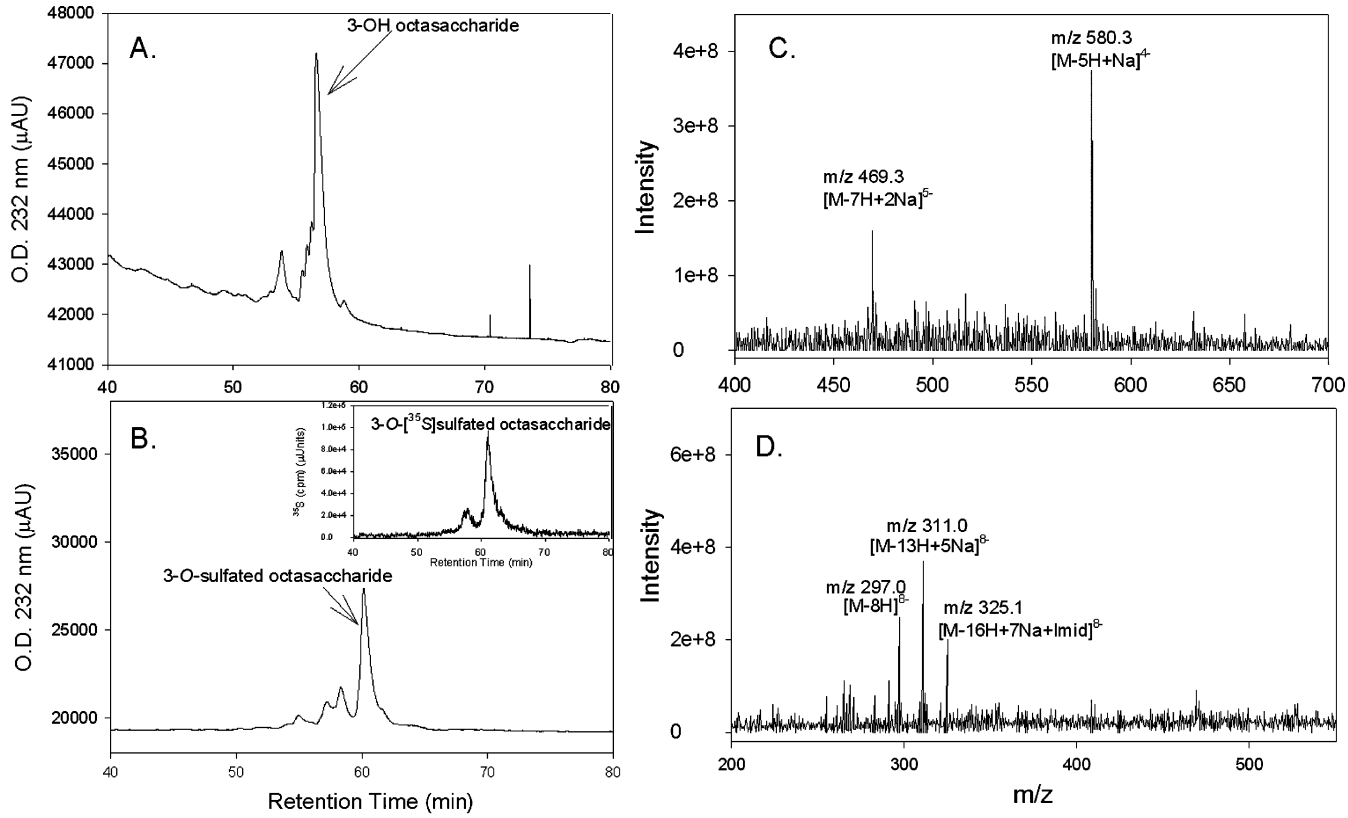

FIGURE 2: HPLC chromatograms and MS spectra of the 3-OH octasaccharide and 3-*O*-sulfated octasaccharide. The 3-OH octasaccharide (A) and the 3-*O*-sulfated octasaccharide (B) were subjected to DEAE-NPR-HPLC. The inset of panel B shows the ^{35}S elution profile of 3-*O*-[^{35}S]sulfated octasaccharide. Panel C shows the mass spectrum of 3-OH octasaccharide. Panel D shows the mass spectrum of 3-*O*-sulfated octasaccharide. The expected ions are indicated, where Na and Imid represent sodium and imidazole adducts, respectively.

Da). From these data, the molecular mass of the 3-OH octasaccharide was determined to be 2305.4 ± 2.2 Da which is close to the anticipated molecular mass (M_r = 2306.9 Da). The 3-*O*-sulfated octasaccharide was purified by DEAE-NPR-HPLC, and it gave three molecular ions, which were $[M - 8H]^{8-}$ at *m/z* 297 (M_r = 2384.0 Da), $[M - 13H + 5Na]^{8-}$ at *m/z* 311 (M_r = 2386.1 Da), and $[M - 16H + 7Na + Imid]^{8-}$ at *m/z* 325.1 (M_r = 2387.9 Da) (Figure 2D). From these data, the molecular mass of the 3-*O*-sulfated octasaccharide was determined to be 2386.0 ± 1.6 Da. This determination is consistent with the molecular mass of 2386.8 Da calculated for an octasaccharide carrying one 3-*O*-sulfo group. These results confirm that the product is the octasaccharide carrying one extra sulfo group.

Determination of the Binding Affinity of the 3-O-Sulfated Octasaccharide for gD. The binding constant (K_d) of gD and 3-*O*-[^{35}S]sulfated octasaccharide was determined by using an affinity co-electrophoresis method (*31*). The 3-*O*-[^{35}S]sulfated octasaccharide was separated under electrophoresis conditions through agarose gel zones containing gD concentrations ranging from 0 to 60 μM. The electrophoretic migration profile of the 3-*O*-[^{35}S]sulfated octasaccharide was visualized using a PhosphorImager and by autoradiography (Figure 3A). The migration of the 3-*O*-[^{35}S]sulfated octasaccharide was clearly retarded by gD in a concentration-dependent manner (Figure 3A), suggesting that this octasaccharide interacts with gD. The K_d for the 3-*O*-[^{35}S]sulfated octasaccharide binding to gD was determined to be 19 μM (Figure 3B), which is very similar to the K_d of a previously characterized gD-binding 3-*O*-sulfated octasaccharide isolated from HS (18 μM) (*25*).

We also assessed the binding of 3-*O*-sulfated tetrasaccharide and 3-*O*-sulfated hexasaccharide to gD. In these experiments, the 3-*O*-sulfated tetrasaccharide and 3-*O*-sulfated hexasaccharide were prepared by incubating a tetrasaccharide (ΔUA2S-GlcNS6S-IdoUA2S-GlcNS6S) or a hexsaccharide substrate (ΔUA2S-GlcNS-IdoUA2S-GlcNS6S-IdoUA2S-GlcNS6S) with 3-OST-3 enzyme and [^{35}S]PAPS. The resultant 3-*O*-sulfated hexasaccharide and tetrasaccharide did not show any significant binding to gD (data not shown), suggesting that octasaccharide represents the minimum size of a 3-*O*-sulfated HS that can exhibit detectable binding affinity for gD. We did not determine the binding affinity between gD and 3-OH octasaccharide because it is known that full-length heparin does not bind to gD (*20*).

Determination of the Site of 3-O-Sulfation in the Octasaccharide. The 3-OH octasaccharide has four glucosamine residues, G2, G4, G6, and G8 (Figure 1B), that might accept a 3-*O*-sulfo group (also see the Supporting Information). We then determined which saccharide unit carried the 3-*O*-[^{35}S]sulfo group using a combination of chemical and enzymatic degradations from both nonreducing and reducing ends.

The strategy from the reducing end analysis of 3-*O*-sulfated octasaccharide for identifying the position of the 3-*O*-[^{35}S]sulfo group is illustrated in Figure 4. First, 2-aminobenzamide (2-AB) was coupled to the reducing end of the octasaccharide by reductive amination. Then, the 2-AB-labeled 3-*O*-sulfated octasaccharide was subjected to partial digestion with heparin lyases to yield a ^{35}S-labeled tetrasaccharide and a ^{35}S-labeled disaccharide that could be resolved by PAMN-HPLC. As expected, heparin lyases treated 2-AB-

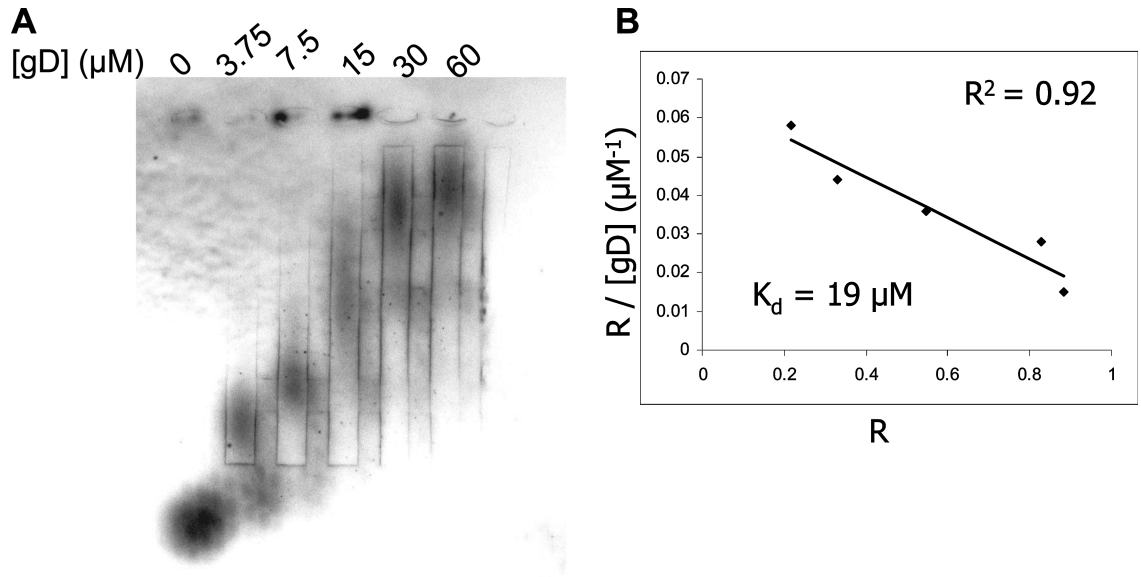

FIGURE 3: Determining the binding constant (K_d) between gD and 3-*O*-[^{35}S]sulfated HP octasaccharide. Panel A presents the autoradiography of the agarose gel in which purified 3-*O*-[^{35}S]sulfated octasaccharide was subjected to electrophoresis through separation zones containing gD at the indicated concentrations. Approximately 28000 cpm (4×10^{-12} mol) per lane of the 3-*O*-[^{35}S]sulfated octasaccharide was loaded into each zone. Panel B represents the plot of $R/[gD]_{total}$ vs R, where $R = (M_0 - M)/M_0$, where M_0 is the migration of free 3-*O*-[^{35}S]sulfated octasaccharide and M is the observed migration of 3-*O*-[^{35}S]sulfated octasaccharide in the presence of gD at various concentrations. The plot yields a straight line with a slope of $-1/K_d$. The linear coefficient is presented as an R^2 value and is shown in panel B.

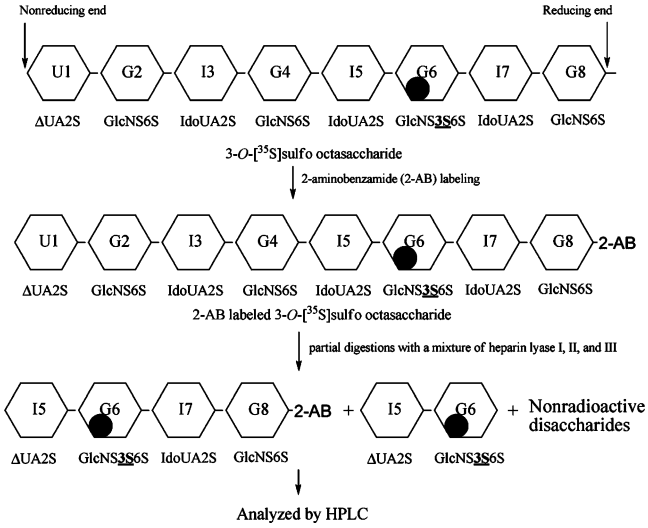

FIGURE 4: Schematic diagram for the structural characterization of the 3-*O*-sulfo octasaccharide. The 3-*O*-[3-^{35}S]sulfo octasaccharide is subjected to reducing end labeling with 2-AB followed by partial digestion with a mixture of heparin lyases (I, II, and III). The resultant ^{35}S-labeled species can be resolved by HPLC. Black circles represent locations of the 3-*O*-[3-^{35}S]sulfo group.

labeled 3-*O*-sulfated octasaccharide exhibited two ^{35}S-labeled components as analyzed by PAMN-HPLC (Figure 5A). The ^{35}S-labeled component that was eluted at 55 min was confirmed to be ΔUA2S-[3-*O*-^{35}S]GlcNS3S6S as it coeluted with the standard (Figure 2 of the Supporting Information). The other ^{35}S-labeled component that was eluted at 94 min co-eluted with a tetrasaccharide standard with a structure of ΔUA2S-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6S-(2-AB) (Figure 5C), but not with ΔUA2S-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6S (Figure 5B), clearly indicating that the 3-*O*-[^{35}S]sulfo group is located on the G6 glucosamine residue. Additional experiments were carried out to further confirm that the 3-*O*-[^{35}S]sulfo group was located at the G6 residue but not at the G2 residue (Supporting Information).

3-O-Sulfated Octasaccharide-Inhibited Herpes Simplex Virus 1 Infection and Cell−Cell Fusion. Because the binding of cellular entry receptor and gD is needed for the entry of HSV-1 into the target cells (*20, 21, 34*), we examined whether the 3-*O*-sulfated octasaccharide could serve as an inhibitor for this interaction by binding to gD in a cell-based assay. First, we monitored the entry of a recombinant HSV-1 expressing β-galactosidase (*18*) into primary cultures of human corneal fibroblasts (CFs) (Figure 6A). We have previously demonstrated that entry of HSV-1 into CFs is mediated by 3-*O*-sulfated HS (*35*). As shown in Figure 6A, the 3-OH octasaccharide showed no obvious inhibition of the viral infection as demonstrated by the fact that the number of blue cells observed with the 3-OH octasaccharide treatment was comparable to the number without treatment. However, at the concentration that was used, 3-*O*-sulfated octasaccharide completely blocked viral entry, suggesting that the 3-*O*-sulfated octasaccharide has a stronger ability to inhibit HSV-1 infection.

Further, we discovered that 3-*O*-sulfated octasaccharide also exhibited stronger efficacy in inhibiting the infection with HSV-1 of HeLa cells in a dose−response manner (Figure 6B). In this experiment, the recombinant HSV-1 was incubated with different concentrations of octasaccharides before it was exposed to the cells. The inhibition activity of 3-*O*-sulfated octasaccharide becomes significantly greater at >40 μM. At 60 μM, 3-*O*-sulfated octasaccharide nearly completely blocked the entry of HSV-1 into HeLa cells, while 3-OH octasaccharide inhibited only 50% of the viral entry.

We next examined whether 3-*O*-sulfated octasaccharide could inhibit the fusion of cells mediated by HSV-1 glycoproteins. Fusion of cells mimics the fusion of virion and target cells that occurs during viral entry (*36*). In this experiment, Chinese hamster ovary (CHO-K1) cells, designated "effector" cells, were generated. The effector cells express all viral glycoproteins essential for the entry via

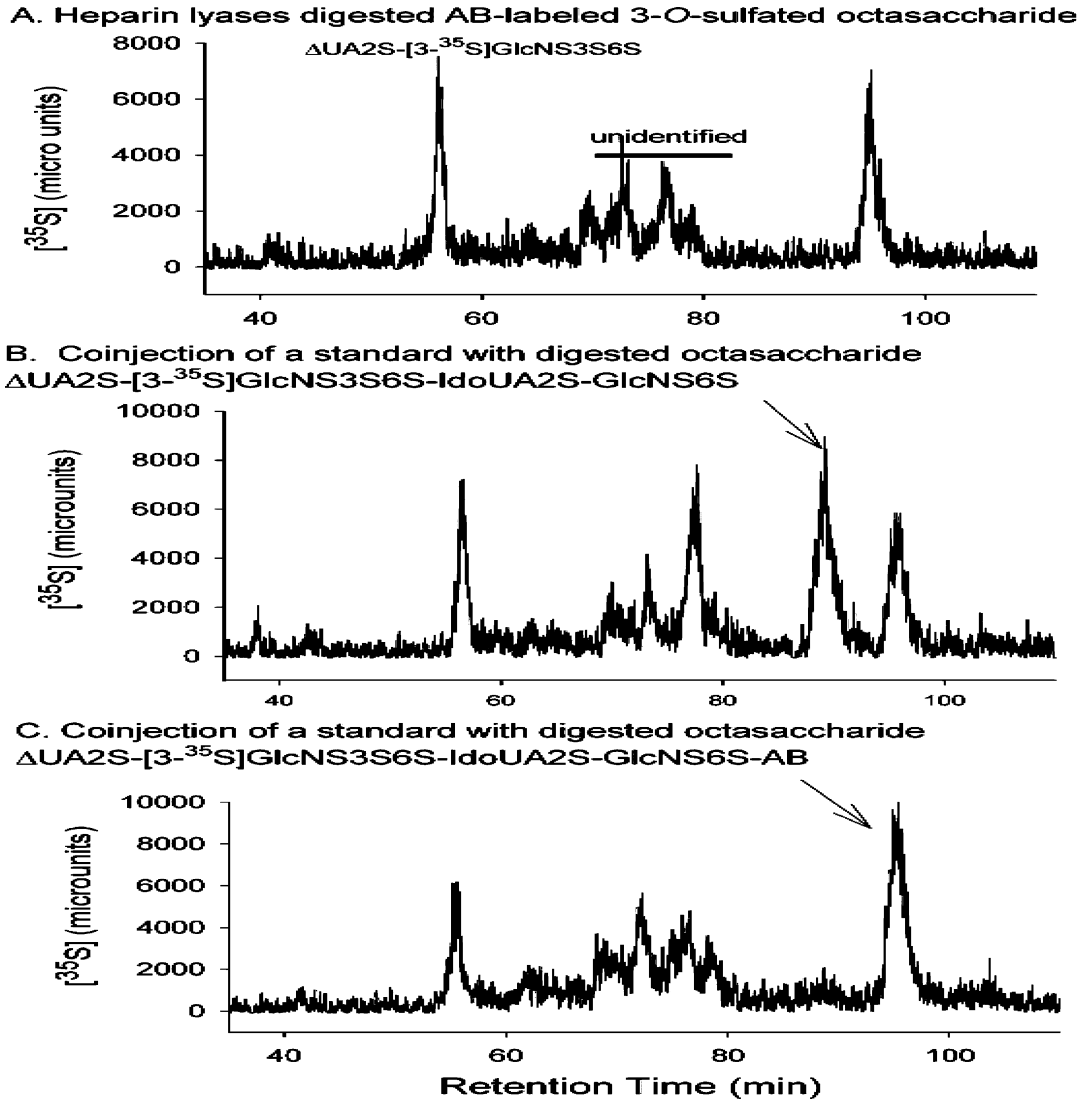

FIGURE 5: Reducing end sequence analysis of 3-*O*-sulfated octasaccharide. Purified 3-*O*-sulfated octasaccharide was digested with heparin lyases, including lyases I, II, and III. The products were analyzed by PAMN-HPLC. The identities of the digested products were confirmed by co-injection with appropriate tetrasaccharide standards: (A) digestion of the 2-AB-labeled 3-*O*-sulfated octasaccharide alone, where both di- and tetrasaccharides are eluted, (B) profile of the digested octasaccharide co-injected with ΔUA2S-[3-^{35}S]GlcNS6S3S-IdoUA2S-GlcNS6S, and (C) profile of the digested octasaccharide co-injected with ΔUA2S-[3-^{35}S]GlcNS6S3S-IdoUA2S-GlcNS6S-AB. The arrow in panel B indicates the elution position of ΔUA2S-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6S and the arrow in panel C the elution position of ΔUA2S-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6S-AB.

transfection of plasmids expressing gB, gD, gH, and gL, along with the pT7EMCLuc plasmid. Another group of CHO-K1 cells, designated "target" cells, that expressed viral entry receptor 3-*O*-sulfated HS and T7 RNA polymerase was generated, which induces the expression of luciferase in effector cells when the target cells fuse with the effector cells (*36*). Both effector and target cells were mixed for 18 h before the luciferase activity was measured to determine the extent of cell−cell fusion and the inhibitory effects of the octasaccharides. The 3-*O*-sulfated octasaccharide significantly decreased the level of luciferase expression compared to the 3-OH octasaccharide (Figure 6C), suggesting that 3-*O*-sulfated octasaccharide but not the 3-OH octasaccharide has the ability to block the fusion process. Since fusion is important not only for entry of the exogenous virions but also for the spread of the endogenous viruses, our data also suggest that 3-*O*-sulfated octasaccharide might be capable of stopping viral spread among neighboring cells.

DISCUSSION

HS assists HSV infection at both viral attachment and viral entry steps by interacting with different viral envelope proteins. The critical role of HS has attracted considerable interest in developing HS-based compounds as anti-herpes agents. Soluble HS and heparin inhibit HSV infections effectively (*16, 37*). Furthermore, synthetic polymers carrying sulfate or sulfonate groups also display a strong activity for blocking HSV infections (*26, 27*). However, because the HS polysaccharide and synthetic polymers carry a large number of sulfate or sulfonate groups, these molecules interact with a variety of proteins, raising concerns about potential toxicity issues. Although the toxic effects can generally be reduced by narrowing the structural complexity of the polymers, it is still extremely difficult to prepare homogeneous sulfated or sulfonated polymers with existing techniques. Thus, it has been quite challenging to develop any structurally homogeneous preparations of sulfated polymers for use as antiviral

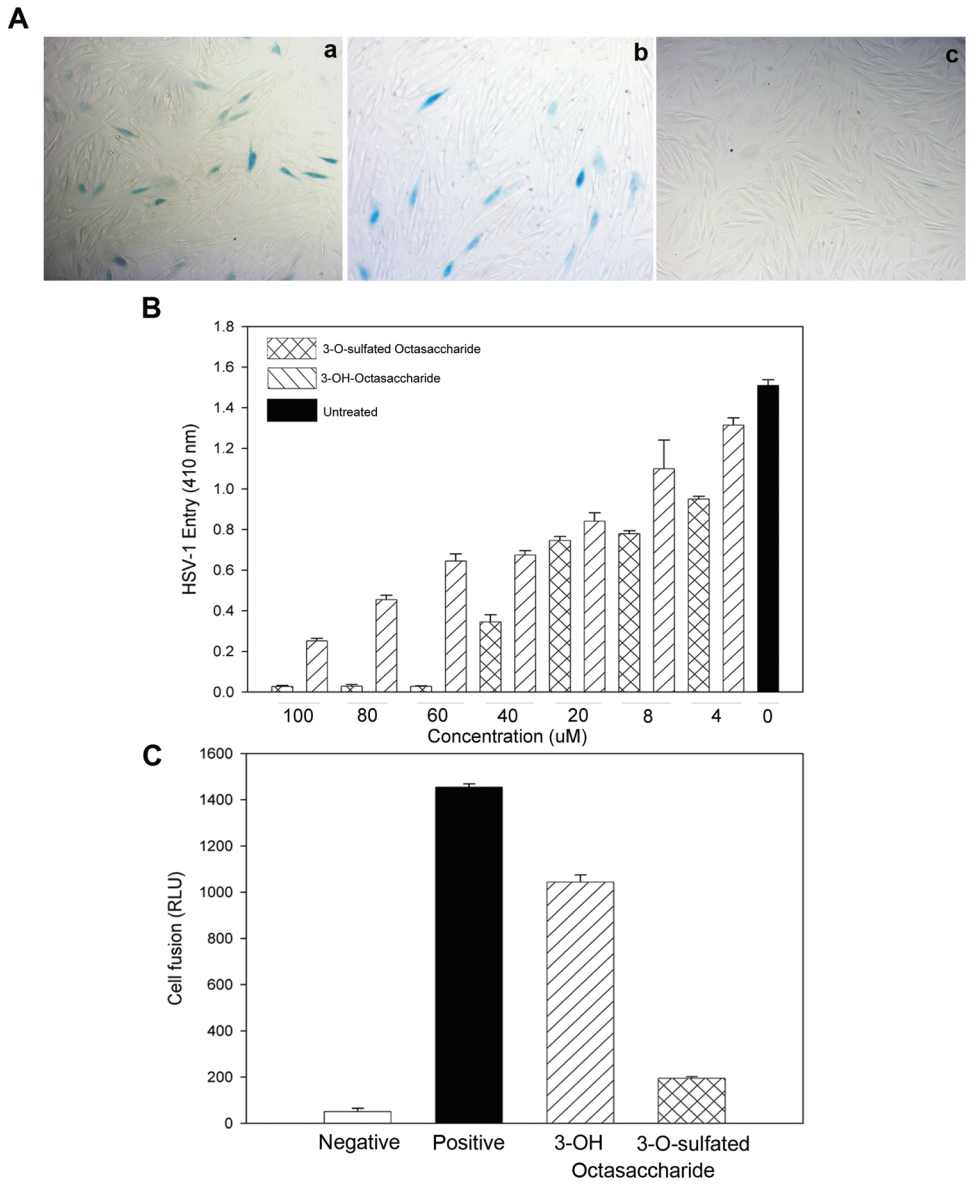

FIGURE 6: 3-*O*-Sulfated octasaccharide inhibits HSV-1 entry. Panel A shows the inhibition of entry of HSV-1 into cultured corneal fibroblasts (CFs). CFs were infected with 1 PFU (plaque-forming units)/well of HSV-1(KOS-gL86) preincubated with buffer alone (a), 100 μM 3-OH octasaccharide (b), or 100 μM 3-*O*-sulfated octasaccharide (c). Six hours later, the cells were washed, fixed, and incubated with X-gal to identify infected cells (dark cells). Panel B shows the dose-dependent inhibition of entry of HSV-1 into HeLa cells. HeLa cells were infected with 1 PFU (plaque-forming units)/well of HSV-1(KOS-gL86) preincubated with indicated concentration of 3-OH octasaccharide or 3-*O*-sulfated octasaccharide as shown. Untreated cells were used as a control. Approximately 6 h after infection, the cells were lysed for the quantification of β-galactosidase activity as a measure of viral entry. Absorbance at 410 nm (OD_{410}) of ONPG reaction products was plotted against the concentration of the octasaccharides used. Panel C shows the specific inhibition of HSV-1 glycoprotein-induced membrane fusion. CHO-K1 cells were used as effector and target cells. Effector cells were transfected with plasmid expressing HSV-1 glycoproteins and luciferase reporter plasmids. Target cells were transfected with T7 RNA polymerase and the plasmid-expressing 3-OST-3. Luciferase activity was measured 24 h after mixing and cocultivating the effector and target cells. The luciferase activity is from one experiment performed in triplicate. The concentration of 3-OH or 3-*O*-sulfated octasaccharides used in this experiment was 100 μM.

drugs. Further, the structural selectivity of HS in HSV attachment has not been clearly established, which calls into question the viability of polymers with specific sulfation patterns in inhibiting HSV attachment. Because a specific 3-*O*-sulfated HS is involved in serving as an entry receptor for HSV-1, a structurally defined mimetic such as 3-*O*-sulfated oligosaccharide should provide the selectivity in inhibiting the infection. However, the feasibility of this approach has not been tested previously because such an oligosaccharide has not been available in sufficient amount to carry out the test.

In this work, we synthesized a structurally defined HS octasaccharide using an enzymatic approach. The structure of the 3-*O*-sulfated octasaccharide is ΔUA2S-GlcNS6S-IdoUA2S-GlcNS6S-IdoUA2S-GlcNS3S6S-IdoUA2S-GlcNS6S as determined by mass spectrometry and sequencing analysis. This octasscharide is structurally distinct from a previous HS-derived octasaccharide, ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S (where Ac is acetyl and UA is either glucuronic or iduronic acid), which was synthesized through a completely different route (*25*). The previous structure was prepared in extremely small

quantities from a 3-OST-3 enzyme-modified HS hexasaccharide library. This method did not afford sufficient quantities for antiviral testing. The 3-*O*-sulfated octasaccharide used in this study was prepared from a one-step modification of a heparin octasaccharide substrate, permitting us to prepare submilligram quantities and, thus, allowing us to test its antiviral efficacy. This 3-*O*-sulfated octasaccharide binds to HSV-1 gD with an affinity of 19 μM, which is very similar to that of the previously reported HS-derived octasaccharide, suggesting that gD can bind to octasaccharides with somewhat different structures.

HSV-1 utilizes three types of cell surface molecules as entry receptors for infection, HVEM (herpesvirus entry mediator), nectin-1, and 3-*O*-sulfated HS (*15*). All three receptors bind to gD at submicromolar affinities. HeLa cells express HVEM and nectin-1 and probably 3-OST-3 (*18, 38*), while CF represents a unique cell type in which HSV-1 entry is primarily mediated by 3-*O*-sulfated HS (*35*). We observed that the 3-*O*-sulfated octasaccharide inhibited the entry of HSV-1 into both HeLa and CF, suggesting that saturating gD with the octasaccharide can serve as a generic viral entry inhibitor independent of which entry receptor is present on the cell surface. Our finding is also important given the origin of CF. These primary cells were cultured from the stroma of the human cornea (*35*). Infection of the stroma is the leading cause of infectious blindness in developed countries (*14*).

Our data support the possibility that the 3-*O*-sulfated octasaccharide inhibits the infection of HSV-1 at both the attachment and entry steps. We note that the unmodified octasaccharide shows modest inhibition of HSV-1 infection, while the 3-*O*-sulfated octasaccharide has a significantly stronger inhibitory effect. Perhaps the difference between the mechanisms of action of the unmodified and 3-*O*-sulfated octasaccharide lies in the ability of 3-*O*-sulfated octasaccharide to inhibit the membrane fusion. It is noteworthy that while 3-OH octasaccharide could block only 50% of entry to HeLa cells at 60 μM, an identical concentration of the 3-*O*-sulfated octasaccharide nearly completely inhibited the entry (Figure 6B). This effect raises the possibility that the 3-*O*-sulfated octasaccharide has acquired an additional ability to specifically block the membrane fusion event by saturating gD. Since both are expected to block the attachment process equally, it is likely that the enhanced effectiveness of the 3-*O*-sulfated octasaccharide is derived from its ability to effectively block membrane fusion. In any case, ours is the first report describing an octasaccharide that can specifically block a membrane fusion event. Previous reports using full-length heparin or HS have found only inhibition of viral attachment to cell surfaces (*39*).

In summary, we describe a novel approach to inhibiting HSV-1 infection by targeting the entry mechanism. This inhibition is achieved by using a unique 3-*O*-sulfated octasaccharide, which was prepared from heparin using an enzymatic approach. Our results demonstrate, for the first time, the infection of HSV-1 can be blocked by saturating the viral envelope glycoprotein gD using a small molecule of defined structure. Although the potency of this inhibitory effect is not very high, the compound exhibits structural selectivity, suggesting that we could design oligosaccharides with specific sulfation patterns to inhibit the infection. The binding affinity of these oligosaccharides for gD will be improved once extensive structure and activity relationship studies are undertaken. For example, the binding affinity of oligosaccharides for gD might be increased by increasing the oligosaccharide size to 10 or 12 saccharide units. Continued efforts to synthesize oligosaccharides having different sulfation patterns could also lead to additional novel molecules that inhibit HSV infections with high potency.

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SUPPORTING INFORMATION AVAILABLE

Additional results of the structural analysis of 3-*O*-sulfated octasaccharide. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Esko, J. D., and Selleck, S. B. (2002) Order out of chaos: Assembly of ligand binding sites in heparan sulfate. *Annu. Rev. Biochem. 71*, 435–471.
2. Liu, J., and Pedersen, L. C. (2007) Anticoagulant heparan sulfate: Structural specificity and biosynthesis. *Appl. Microbiol. Biotechnol. 74*, 263–272.
3. Kreuger, J., Spillmann, D., Li, J.-p., and Lindahl, U. (2006) Interactions between heparan sulfate and proteins: The concept of specificity. *J. Cell Biol. 174*, 323–327.
4. Gama, C., Tully, S. E., Sotogaku, N., Clark, P. M., Rawat, M., Vaidehi, N., Goddard, W. A., Nishi, A., and Hsieh-Wilson, L. C. (2006) Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. *Nat. Chem. Biol. 2*, 467–473.
5. Fuster, M. M., and Esko, J. D. (2005) The sweet and sour of cancer: Glycans as novel therapeutic targets. *Nat. Rev. Cancer 5*, 526–542.
6. Shriver, Z., Raguram, S., and Sasisekharan, R. (2004) Glycomics: A pathway to a class of new and improved therapeutics. *Nat. Rev. Drug Discovery* 863–873.
7. Liu, J., and Thorp, S. C. (2002) Heparan sulfate and the roles in assisting viral infections. *Med. Res. Rev. 22*, 1–25.
8. Chen, J., Jones, C. L., and Liu, J. (2007) Using an enzymatic combinatorial approach to identify anticoagulant heparan sulfate structures. *Chem. Biol. 14*, 986–993.
9. Muñoz, E., Xu, D., Avci, F., Kemp, M., Liu, J., and Linhardt, R. J. (2006) Enzymatic synthesis of heparin related polysaccharides on sensor chips: Rapids screening of heparin-protein interactions. *Biochem. Biophys. Res. Commun. 339*, 597–602.
10. Chen, J., Avci, F. Y., Muñoz, E. M., McDowell, L. M., Chen, M., Pedersen, L. C., Zhang, L., Linhardt, R. J., and Liu, J. (2005) Enzymatically redesigning of biologically active heparan sulfate. *J. Biol. Chem. 280*, 42817–42825.
11. Balagurunathan, K., Lech, M. Z., Beeler, D. L., Wu, Z. L., and Rosenberg, R. D. (2003) Enzymatic synthesis of antithrombin III-binding heparan sulfate pentasaccharide. *Nat. Biotechnol. 21*, 1343–1346.
12. Xu, D., Moon, A., Song, D., Pedersen, L. C., and Liu, J. (2008) Engineering sulfotransferases to modify heparan sulfate. *Nat. Chem. Biol. 4*, 200–202.
13. Corey, L., and Spear, P. G. (1986) Infections with herpes simplex viruses. *N. Engl. J. Med. 314*, 686–691, 749−757.
14. Brandt, C. R. (2005) The role of viral and host genes in corneal infection with herpes simplex virus type 1. *Exp. Eye Res. 80*, 607–621.
15. Shukla, D., and Spear, P. G. (2001) Herpesviruses and heparan sulfate: An intimate relationship in aid of viral entry. *J. Clin. Invest. 108*, 503–510.
16. Shieh, M.-T., WuDunn, D., Montgomery, R. I., Esko, J. D., and Spear, P. G. (1992) Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biol. 116*, 1273–1281.
17. Spear, P. G., Eisenberg, R. J., and Cohen, G. H. (2000) Three classes of cell surface receptors for alphaherpesvirus entry. *Virology 275*, 1–8.

18. Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996) Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell 87*, 427–436.
19. Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Spear, P. G. (1998) Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science 280*, 1618–1620.
20. Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999) A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell 99*, 13–22.
21. Xia, G., Chen, J., Tiwari, V., Ju, W., Li, J.-P., Malmström, A., Shukla, D., and Liu, J. (2002) Heparan sulfate 3-O-sulfotransferase isoform 5 generates both an antithrombin-binding site and an entry receptor for herpes simplex virus, type 1. *J. Biol. Chem. 277*, 37912–37919.
22. Xu, D., Tiwari, V., Xia, G., Clement, C., Shukla, D., and Liu, J. (2005) Characterization of Heparan Sulfate 3-O-Sulfotransferase Isoform 6 and Its Role in Assisting the Entry of Herpes Simplex Virus, Type 1. *Biochem. J. 385*, 451–459.
23. O'Donnell, C. D., Tiwari, V., Oh, M. J., and Shukla, D. (2006) A role for heparan sulfate 3-O-sulfotransferase isoform 2 in herpes simplex virus type 1 entry and spread. *Virology 346*, 452–459.
24. Tiwari, V., O'Donnell, C. D., Oh, M. J., Valyi-Nagy, T., and Shukla, D. (2006) A role for 3-O-sulfotransferase isoform-4 in assisting HSV-1 entry and spread. *Biochem. Biophys. Res. Commun. 338*, 930–937.
25. Liu, J., Shriver, Z., Pope, R. M., Thorp, S. C., Duncan, M. B., Copeland, R. J., Raska, C. S., Yoshida, K., Eisenberg, R. J., Cohen, G., Linhardt, R. J., and Sasisekharan, R. (2002) Characterization of a heparan sulfate octasaccharide that binds to herpes simplex viral type 1 glycoprotein D. *J. Biol. Chem. 277*, 33456–33467.
26. Anderson, R. A., Feathergill, K., Diao, X., Cooper, M., Kirkpatrick, R., Spear, P., Waller, D. P., Chany, C., Doncel, G. F., Herold, B., and Zaneveld, L. J. (2000) Evaluation of poly(styrene-4-sulfonate) as a preventive agent for conception and sexually transmitted diseases. *J. Androl. 21*, 862–875.
27. Raghuraman, A., Tiwari, V., Zhao, Q., Shukla, D., Debnath, A. K., and Desai, U. R. (2007) Viral inhibition studies on sulfated lignin, a chemically modified biopolymer and a potential mimic of heparan sulfate. *Biomacromolecules 8*, 1759–1763.
28. Thanawiroon, C., Rice, K. G., Toida, T., and Linhardt, R. J. (2004) LC/MS sequencing approach for highly sulfated heparin-derived oligosaccharides. *J. Biol. Chem. 279*, 2608–2615.
29. Pervin, A., Gallo, C., Jandik, K. A., Han, X.-J., and Linhardt, R. J. (1995) Preparation and Structural Characterization of Large Heparin-Derived Oligosaccharides. *Glycobiology 5*, 83–95.
30. Moon, A., Edavettal, S. C., Krahn, J. X., Munoz, E. M., Negishi, M., Linhardt, R. J., Liu, J., and Pedersen, L. C. (2004) Structural analysis of the sulfotransferase (3-OST-3) involved in the biosynthesis of an entry receptor of herpes simplex virus 1. *J. Biol. Chem. 279*, 45185–45193.
31. Lee, M. K., and Lander, A. D. (1991) Analysis affinity and strutural selectivity in the binding of proteins to glycosamineglycans: Development of a sensitive electrophoretc approach. *Proc. Natl. Acad. Sci. U.S.A. 88*, 2768–2772.
32. Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M., and Parekh, R. B. (1995) Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Anal. Biochem. 230*, 229–238.
33. Kinoshita, A., and Sugahara, K. (1999) Microanalysis of glycosaminoglycan-derived oligosaccharides labeled with a fluorophore 2-aminobenzamide by high-performance liquid chromatography: Application to disaccharide composition analysis and exosequencing of oligosaccharides. *Anal. Biochem. 269*, 367–378.
34. Spear, P. G. (2004) Herpes simplex virus: Receptors and ligands for cell entry. *Cell. Microbiol. 6*, 401–410.
35. Tiwari, V., Clement, C., Xu, D., Valyi-Nagy, T., Yue, B. Y. J. T., Liu, J., and Shukla, D. (2006) A role for 3-*O* sulfated heparan sulfate as the receptor for herpes simplex virus type-1 entry into primary human corneal fibroblasts. *J. Virol. 80*, 8970–8980.
36. Pertel, P. E., Fridberg, A., Parish, M. L., and Spear, P. G. (2001) Cell fusion induced by herpes simplex virus glycoproteins gB, gD, and gH-gL requires a gD receptor but not necessarily heparan sulfate. *Virology 279*, 313–324.
37. WuDunn, D., and Spear, P. G. (1989) Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol. 63*, 52–58.
38. Cocchi, F., Menotti, L., Mirandola, P., Lopez, M., and Campadelli-Fiume, G. (1998) The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. *J. Virol. 72*, 9992–10002.
39. Herold, B., Gerber, S. I., Belval, B. J., Siston, A. M., and Shulman, N. (1996) Differences in the susceptibility of herpes simplex virus types 1 and 2 to modified heparin compounds suggest serotype differences in viral entry. *J. Virol. 70*, 3461–3469.

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