Chondroitin 4-O-Sulfotransferase-1 Regulates E Disaccharide Expression of Chondroitin Sulfate Required for Herpes Simplex Virus Infectivity*

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We have demonstrated a defect in expression of chondroitin 4-O-sulfotransferase-1 (CAST-1) in murine sog9 cells, which are poorly sensitive to infection by herpes simplex virus type 1 (HSV-1). Sog9 cells were previously isolated as CS-deficient cells from gro2C cells, which were partially resistant to HSV-1 infection and defective in the expression of heparan sulfate (HS) because of a splice site mutation in the EXT1 gene encoding the HS-synthesizing enzyme. Here we detected a small amount of CS chains in sog9 cells with a drastic decrease in 4-O-sulfation compared with the parental gro2C cells. RT-PCR revealed that sog9 cells had a defect in the expression of CAST-1 in addition to EXT1. Gel filtration analysis showed that the decrease in the amount of CS in sog9 cells was the result of a reduction in the length of CS chains. Transfer of CAST-1 cDNA into sog9 cells (sog9-CAST-1) restored 4-O-sulfation and amount of CS, verifying that sog9 cells had a specific defect in CAST-1. Furthermore, the expression of CAST-1 rendered sog9 cells significantly more susceptible to HSV-1 infection, suggesting that CS modified by CAST-1 is sufficient for the binding and infectivity of HSV-1. Analysis of CS chains of gro2C and sog9-CAST-1 cells revealed a considerable proportion of the E disaccharide unit, consistent with our recent finding that this unit is an essential component of the HSV receptor. These results suggest that CAST-1 regulates the expression of the E disaccharide unit and the length of CS chains, the features that facilitate infection of cells by HSV-1.

Glycosaminoglycans (GAG)4 are ubiquitous molecules distributed on the cell surface and in the extracellular matrices (1–4). GAGs are linear, sulfated polysaccharides composed of repeating disaccharide units consisting of alternating uronic acid and N-acetylatedhexosamine residues, and synthesized as proteoglycans linked to specific Ser residues in the core protein (1, 2, 4). Sulfated GAGs are typically divided into two types, chondroitin sulfate (CS) and heparan sulfate (HS), on the basis of the N-acetylatedhexosamine in the disaccharide units (1, 3, 4). CS and HS contain N-acetylgalactosamine (GalNAc) and N-acetylgalactosamine (GlcNAc) residues, respectively. Compelling evidence has shown that GAGs play crucial roles in a number of physiological phenomena such as cell adhesion, morphogenesis, neural network formation, and cell division (5, 6).

Herpes simplex virus type 1 (HSV-1) is a member of the neurotropic alphaherpesvirus subfamily, part of the Herpesviridae family. Many glycoproteins of HSV-1 decorate the virion envelope, and some play essential roles in viral attachment to and entry into host cells. In particular, gB, gC, and gD utilize cell surface GAGs and some other receptors to effectively bind to and infect host cells (7–9). Among these glycoproteins, the interaction between gC and HS has been extensively studied (8). Furthermore, recent experiments using cell lines deficient in expression of GAGs have suggested that gC also binds to CS characterized by the E disaccharide unit, and that the CS-E unit is a potent inhibitor of HSV infectivity and an essential component of the receptor for HSV (10).

Previously, in the course of the screening to isolate cells non-permissive for lytic infection by HSV-1, gro2C cells were isolated from mouse L cell fibroblasts. Gro2C were subsequently shown to be an HS-deficient mutant cell line due to the dysfunction of EXT1, which encodes a glycosyltransferase essential for the synthesis of HS chains (11–15). Although gro2C cells cannot synthesize HS and survived the lytic infection with

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HSV-1, some susceptibility of these cells to HSV-1 was detected (15). Further selection for HSV-1 resistant gro2C cells led to the isolation of sog9 cells, a cell line >99% resistant to HSV-1 infection (16), and Tufaro and colleagues reported that sog9 cells could synthesize neither HS nor CS. Although exactly why CS could not be synthesized in sog9 cells remained unclear (16), it was speculated that a gene mutated in these cells is critical for the biosynthesis of CS, and that the product of this gene is a key enzyme or factor regulating the biosynthesis. This prompted us to investigate sog9 cells to better understand the biosynthesis of CS.

Here, we report that sog9 cells are defective in the expression of chondroitin 4-O-sulfotransferase-1 (C4ST-1), which transfers a sulfate to the 4-O-position of a GalNAc residue in CS chains (17, 18). The deficiency in the expression of C4ST-1 was found to lead to a drastic decrease in the 4-O-sulfation of a GalNAc residue and notably the E unit, a highly sulfated disaccharide unit consisting of GlcUAβ1–3GalNAc(4S,6S) (19), where 4S and 6S represent 4-O- and 6-O-sulfate. CS containing the E unit was recently reported as one of the cellular receptors for HSV-1 (10). The introduction of C4ST-1 into sog9 cells increased the length of CS chains and the susceptibility of the cells to HSV-1. These results indicate that C4ST-1 regulates the length, 4-O-sulfation of CS chains and the subsequent formation of the E unit thus facilitating infection of cells by HSV-1.

EXPERIMENTAL PROCEDURES

Materials—[3H]Gal (285.2 mCi/mmol) and [3H]GlcNH₂ (10 Ci/mmol) were purchased from NEN Life Science Products. A Superdex™ 200 column, and DEAE-Sephacel and prepacked disposable PD-10 columns containing Sephadex G-25 were obtained from Amersham Biosciences. Chondroitinase ABC (EC 4.2.2.4), heparitinase (EC 4.2.2.8), heparinase (EC 4.2.2.7), and the mouse mononal anti-CS antibody 2H6 were obtained from Seikagaku Corp. (Tokyo, Japan). Actinase E was purchased from Kaken Pharmaceutical Co. (Tokyo, Japan). 2-AB was purchased from Nacalai Tesque (Kyoto, Japan). Benzyl acetoamido-2-deoxy-α-D-galactopyranoside (benzyl-α-D-GalNAc) was supplied by Sigma. Glc-free RPMI medium and 0.1% BSA/PBS) at room temperature for 1 h. To confirm the specificity of the staining with the antibody, sog9 cells were pretreated with chondroitinase ABC protease-free (2 milli international units) to remove CS and then processed for immunostaining as described above. Fluorescent images were obtained using a laser-scanning confocal microscope, FLUOVIEW (Olympus, Tokyo, Japan).

Derivatization of GAGs from gro2C and sog9 Cells Using a Fluorophore, 2AB—Cells were homogenized in acetone, and air-dried. The dried materials were digested with heat-pre-treated (60 °C, 30 min) actinase E in 200 µl of 0.1 m borate-sodium, pH 8.0, containing 10 mM calcium acetate at 60 °C for 24 h. Following incubation, each sample was treated with trichloroacetic acid and the resultant precipitate was removed by centrifugation. The soluble fraction was extracted with ether. The aqueous phase was neutralized with 1.0 M sodium carbonate and adjusted to contain 80% ethanol. The resultant precipitate was dissolved in 50 mM pyridine acetate and subjected to gel filtration on a PD-10 column using 50 mM pyridine acetate as an eluent. The flow through fractions were collected and evaporated dry. The dried sample was dissolved in water. Digestion with chondroitinase ABC (5 milli-international units) was conducted as described previously at 37 °C for 1 h in a total volume of 10 µl (20). Reactions were terminated by boiling for 1 min. Each digest was derivatized with 2-AB, then analyzed by HPLC as reported previously (21).

Metabolic Labeling—Gro2C and sog9 cells were initially starved in a Glc-free medium for 1 h. The cells were then labeled metabolically with d-[3H]Gal (285.2 mCi/mmol) and [3H]GlcNH₂ (285.2 mCi/mmol) in a Glc-free RPMI 1640 medium containing 5% dialyzed fetal bovine serum, 50 mM Glc and 0.5 µM benzyl-α-D-GalNAc at 37 °C for 24 h (22). The cell layer was solubilized with 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl and 1% Triton X-100 with gentle rocking at 4 °C overnight and centrifuged. The supernatant fluids were treated with 0.5 M LiOH at room temperature overnight to release the O-linked sugar from core proteins, and neutralized with acetic acid. Labeled GAGs were isolated by anion exchange chromatography using DEAE-Sephacel and subjected to gel filtration on a PD-10 column using 50 mM pyridine acetate as an eluent. The flow-through fraction was collected and evaporated dry. The dried samples were dissolved in water. Purified GAGs were digested with either chondroitinase ABC, a mixture of heparitinase and heparinase, or both simultaneously, and subjected to gel filtration on a Superdex 200 column.

Establishment of an Expression Vector for C4ST-1 and Preparation of Cells That Stably Overexpress C4ST-1—The cDNA fragment encoding mouse C4ST-1 was amplified by reverse transcription-PCR with total RNA derived from a mouse heart Marathon-Ready cDNA library as a template using a 5′-primer (5′-AGAGCCTCGGTGAAGCTA-3′) containing a SacII site and a 3′-primer (5′-ATAACCGCTCTCCATAGAATTC-3′) containing a SalI site. PCR was carried out with KOD-Plus-DNA polymerase (TOYOBO, Tokyo) for 30 cycles at 94 °C for 30 s, 53 °C for 42 s, and 68 °C for 120 s in 5% (v/v) dimethyl sulfoxide. The PCR fragments were subcloned into the SacII-SalI site of the pCMV expression vector (Invitrogen, La Jolla, CA). The nucleotide sequence of the amplified cDNA was determined in a 377 DNA sequencer (PE Applied Biosystems). The expression plasmid (6.7 µg) was transfected into sog9 cells on 100-mm plates using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Transfectants were cultured in the presence of 300 µg/ml of G418. Then, resultant colonies were picked up and propagated for experiments.
Regulation of Chondroitin Sulfate Biosynthesis by C4ST-1

Viruses—The HSV strains used were HSV-1 KOS 321, a plaque-purified isolate of wild-type strain KOS (23), and HSV-1 gC-39, a gC-null derivative of KOS 321 (24). For infectivity assays, stocks of virus were titrated on GMK AH1 cells. For attachment assays, [methyl-3H]thymidine-labeled virions were purified from culture media by centrifugation through a three step discontinuous sucrose gradient as described previously (25). Relative amounts of purified virions in the preparations were estimated based on the quantification of VP5 (26).

Viral Infectivity and Effects of Enzymatic Degradation of CS—Sog9, sog9-C4ST-1-1, sog9-C4ST-1-5, and sog9-C6ST-1 cells were grown to confluence in 6-well plates. For determination of the effects of an enzymatic treatment, chondroitinase ABC at a concentration of 0.1 unit/ml in PBS-A (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4) supplemented with 1 mM CaCl2, 0.5 mM MgCl2, and 0.1% BSA, or buffer alone, was added at a volume of 0.6 ml/well, and the cells were incubated at 37 °C for 40 min then cooled at 4 °C for 20 min. After rinsing with cold PBS-A, the virus was added in 5-fold dilutions to duplicate wells and the infected cells were incubated at 4 °C for 1 h. After further rinsing with culture medium, a fresh medium containing 1% methylcellulose was added and the infected cells were incubated at 37 °C for 4–5 days. Cells were stained with crystal violet solution to count the viral plaques.

Viral Attachment and Effects of Enzymatic Degradation of CS—Sog9 and sog9-C4ST-1-1 cells were grown to confluence in 24-well plates. Cells were either mock-treated or treated with chondroitinase ABC at a concentration of 0.1 unit/ml, as described above. After rinsing, cells were blocked for 30 min at 4 °C with 1% BSA solution in PBS-A supplemented with 1 mM CaCl2 and 0.5 mM MgCl2. [methyl-3H]Thymidine-labeled KOS 321 and gC-39 viruses were prediluted in PBS-A supplemented with 1 mM CaCl2, 0.5 mM MgCl2 and 0.1% BSA to contain the same number of relative VP5 units, and 200 μl of the virus suspension (271 and 113 cpm/μl for KOS 321 and gC-39, respectively) were added per well. Plates were incubated with continuous shaking at 37 °C for 1 h, and the cells were washed three times with PBS-A. Plates were lysed with 5% SDS and transferred to scintillation vials for quantification of radioactivity.

RESULTS

Sog9 Cells Synthesize CS Chains—Previously, sog9 cells, a murine L cell mutant defective in both HS and CS synthesis, were isolated by selection for cells resistant to lytic HSV-1 infection (16). Investigation revealed that this cell line contains a specific defect in the EXT1 gene, which encodes a glycosyltransferase related to the formation of the HS backbone (27). However, the cause of the CS deficiency in sog9 cells has remained elusive. To confirm that CS cannot be synthesized in sog9 cells, immunocytochemistry with sog9 and parental mutant gro2C cells was performed using an anti-CS monoclonal antibody, 2H6, which mainly recognizes CS-A consisting of 4-O-sulfated disaccharide units (Fig. 1B). Unexpectedly, clear signals were detected in sog9 cells, and the signals were eliminated by chondroitinase ABC digestion (Fig. 1, C and D), suggesting that sog9 cells synthesize CS chains.

As the immunohistochemical analysis suggested that sog9 cells may form CS, we next measured the glycosyltransferase activity required for the biosynthesis of CS using a cell lysate as an enzyme source. When GalNAcT-I activity, which initiates the biosynthesis of CS by transferring the first GalNAc residue to the linkage tetrasaccharide, was measured using the authentic synthetic substrate GlcUAβ1–3Galβ1-O-benzylxycarbonyl, analogous to the linkage tetrasaccharide as an acceptor substrate, significant activity comparable to that of gro2C cells was detected (data not shown). In addition, GalNAcT-II and GlcAT-II activities responsible for the formation of the repeating disaccharide units in CS were similar to levels of activity by gro2C cells (data not shown). Hence, the enzymes involved in the biosynthesis of the chondroitin backbone appeared to be present in sog9 cells as in gro2C cells. These results indicated that glycosyltransferases participating in the biosynthesis of CS are not affected in sog9 cells.

Sog9 Cells Express CS with a Low Degree of 4-O-Sulfation—To examine whether sog9 cells in fact synthesize CS, GAGs isolated from this cell line were chemically analyzed. The acetone powder of sog9 or gro2C cells was digested with actinase E, and the resultant GAG-peptides were purified as described under “Experimental Procedures.” The purified GAG peptides were subjected to digestion with chondroitinase ABC or a mixture of heparitinase and heparinase. The resultant disaccharides were derivatized with 2-aminobenzamide (2-AB) followed by anion-exchange HPLC on a PA-03 column. As shown in Fig. 2A, gro2C cells contained CS disaccharides dominated by ΔDI-4S, which occupied 69% of the total. As expected, sog9 cells, which have been regarded as a CS-deficient cell line (16), also contained a detectable amount of CS, although the total amount of disaccharide units was approximately one-third in
that of gro2C cells (Fig. 2B and Table 1). Notably, the proportions of ΔDi-4S and ΔDi-diS₄ᵦ units in sog9 cells were dramatically reduced (Table 1). Whereas the proportion of ΔDi-4S (27%) in sog9 cells was less than half that (69%) in gro2C cells, the proportion of ΔDi-diS₄ᵦ in sog9 cells exhibited a drastic reduction, being about one-tenth (1%) that (10%) in gro2C cells (Table 1). As the 4-O-sulfation of GalNAc residues is catalyzed by the chondroitin/dermatan 4-O-sulfotransferase (C4ST/ D4ST) family, one member of this family may be defective in sog9 cells and this sulfotransferase may play a critical role in regulating CS content and the formation of the ΔDi-diS₄ᵦ unit (28).

Sog9 Cells Are Defective in the Expression of C4ST-1—To date, four sulfotransferases, C4ST-1, C4ST-2, C4ST-3, and D4ST-1, which are involved in the 4-O-sulfation of GalNAc residues in CS/DS, have been identified. As C4ST-1, C4ST-2, and D4ST-1 are the major contributors to the 4-O-sulfation of CS/DS in terms of level of enzymatic activity and tissue distribution (28), the expression of the mRNA for these genes was evaluated by RT-PCR using cDNA libraries prepared from gro2C and sog9 cells. It was revealed that C4ST-1 mRNA was not expressed in sog9 cells, whereas the levels of C4ST-2 and D4ST-1 mRNA in sog9 cells were indistinguishable from those in gro2C cells (Fig. 3). These results indicated that the C4ST-1 locus might be mutated in sog9 cells, and the residual 4-O-sulfated GalNAc in sog9 cells is most likely formed by the actions of C4ST-2 and/or D4ST-1.

Rescue of sog9 Cells by Introducing C4ST-1—To verify that C4ST-1 can restore levels of CS and the E disaccharide unit, a pCMV-Script expression vector, which possesses the cytomegalovirus promoter and the neomycin-resistance gene, and harbors the open reading frame of mouse C4ST-1, was transfected into sog9 cells, followed by positive selection in the presence of a neomycin analog, G418. Each of the resultant colonies was picked up and propagated for experiments. As shown in Fig. 2, C and D, the disaccharide composition and the amount of CS isolated from the two stable clones with the different expression levels of C4ST-1 introduced (sog9-C4ST-1-1 (high expression) and sog9-C4ST-1-5 (low expression) cells) were analyzed by HPLC as above. The results showed that the disaccharide composition and the amount of CS in sog9-C4ST-1-1 cells were similar to those in gro2C cells, verifying that sog9 cells are deficient in C4ST-1 expression. In addition, differences in disaccharide composition and amount of CS were observed between the two stable clones, corresponding to the difference in the expression level of the mouse CAST-1 introduced (Table 1). Notably, the proportion of ΔDi-diS₄ᵦ unit in sog9-C4ST-1-1 cells was closer to that in gro2C cells, suggesting that C4ST-1 is critical to the formation of this unit. In addition, the increase in the percentage of the disaccharide units ΔDi-4S and ΔDi-diS₄ᵦ, which contain 4-O-sulfate, among all disaccharide units was concomitant with the increase in the total amount of CS (Table 1). These results suggest that 4-O-sulfation of CS formed by C4ST-1 or the expression of C4ST-1 protein itself may be one of the determinants of the total amount of CS. To examine whether the regulation of the amount of CS is specific to C4ST-1, C4ST-2, D4ST-1, or chondroitin 6-O-sulfotransferase-1 (C6ST-1) required for sulfation of the 6-O-position of GalNAc in CS (28–30), was introduced into sog9 cells and the CS extracted from these cells was analyzed. Although higher expression of C4ST-2 and D4ST-1 mRNA was observed in sog9-C4ST-2 and sog9-D4ST-1 cells, respectively, than sog9 cells (data not shown), the disaccharide composition and the amount of CS in sog9-C4ST-2 and sog9-D4ST-1 cells were sim-

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**FIGURE 2. Disaccharide composition of CS chains from gro2C, sog9, sog9-C4ST-1-1, and sog9-C4ST-1-5 cells.** The GAG fractions prepared from gro2C, sog9, sog9-C4ST-1-1, and sog9-C4ST-1-5 cells were digested with chondroitinase ABC. Each digest was labeled with 2-AB and analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column. The elution positions of authentic 2-AB-labeled disaccharide standards derived from CS are indicated by numbered arrows: 1, ∆HexA-GalNAc; 2, ∆HexA-GalNAc(6S); 3, ∆HexA-GalNAc(4S); 4, ∆HexA(2S)-GalNAc(6S); 5, ∆HexA-GalNAc(4S,6S). A, CS disaccharides from gro2C cells; B, CS disaccharides from sog9 cells; C, CS disaccharides from sog9-C4ST-1-1; D, CS disaccharides from sog9-C4ST-1-5.

**TABLE 1**

Disaccharide composition of CS from gro2C, sog9, sog9-C4ST-1-1, sog9-C4ST-1-5, sog9-C4ST-2, sog9-D4ST-1, and sog9-C6ST-1 cells

| Composition  | gro2C cells | sog9 cells | sog9-C4ST-1-1 cells | sog9-C4ST-1-5 cells | sog9-C4ST-2 cells | sog9-D4ST-1 cells | sog9-C6ST-1 cells |
|--------------|-------------|------------|---------------------|---------------------|------------------|------------------|------------------|
| ΔDi-0S       | 51.2 (15)   | 70.3 (61)  | 49.8 (16)           | 78.0 (48)           | 74.9 (68)        | 53.1 (64)        | 13.2 (12)        |
| ΔDi-6S       | 21.9 (6)    | 13.2 (11)  | 11.8 (4)            | 13.6 (8)            | 7.1 (6)          | 6.9 (8)          | 80.6 (74)        |
| ΔDi-4S       | 234.8 (69)  | 31.5 (27)  | 222.9 (72)          | 65.7 (40)           | 28.8 (26)        | 23.2 (28)        | 13.0 (12)        |
| ΔDi-diS₄ᵦ    | ND          | ND         | ND                  | ND                  | ND               | ND               | ND               |
| Total (pmol/mg) | 340.6      | 115.8      | 310.4               | 163.6               | 110.8            | 83.2             | 108.8            |
| Molar ratio of sulfate to disaccharide | 0.95 | 0.40 | 0.92 | 0.56 | 0.32 | 0.36 | 0.90 |

* ND, not detected.
similar to those in sog9 cells (Table 1). In addition, HPLC analysis for sog9-C6ST-1 cells showed no increase in the amount of CS despite a drastic increase in the proportion of ΔDi-6S (Table 1). These results indicate that the amount of CS is specifically regulated by C4ST-1.

**Deficiency of C4ST-1 Expression Leads to a Reduced Chain Length of CS**—To evaluate whether the decrease in the amount of CS in sog9 cells was caused by a reduction in the length or number of CS chains, the length of CS chains obtained from gro2C and sog9 cells was compared. GAGs of each cell line were radiolabeled with \[^{3}H\]GlcNH\_2 and \[^{3}H\]Gal in the presence of gro2C and sog9 cells. GAGs of each cell line were subjected to gel filtration chromatography on a Superdex 200 column. Inset shows the calibration curve, showing a linear relation between the log \(M_r\) and the elution volume, which was generated using the data obtained with size-defined commercial polysaccharides; dextran (average \(M_r\): 200,000, 65,500, 37,500, and 18,100; all from Sigma). Arrowheads indicate the size of molecular standards. Closed square, gro2C cells; closed triangle, sog9 cells; open square, sog9-C4ST-1-1 cells; open circle, sog9-C4ST-1-5 cells.

**Infectivity and Attachment of HSV to sog9 Cells before and after Introduction of C4ST-1**—Because earlier studies (16, 31) had indicated that CS may function as an initial receptor for HSV-1, we investigated whether transfection of sog9 cells with C4ST-1 cDNA can rescue their susceptibility to HSV-1 infection. Compared with sog9 cells, a 7- or 5-fold increase in the number of plaques of an HSV-1 wild-type strain, KOS 321, was observed on the sog9-C4ST-1-1 or sog9-C4ST-1-5 cells, respectively (Fig. 5), suggesting that the expression level of C4ST-1 correlates with the susceptibility to HSV-1 infection. In contrast, the susceptibility of the sog9-C6ST-1 cells to HSV-1 KOS 321 was comparable to that of sog9 cells, confirming the importance of 4-O-sulfation of CS formed by C4ST-1 (Fig. 5).

Enzymatic digestion of CS with chondroitinase ABC abrogated most of this increment in the sensitivity of the sog9-C4ST-1-1 cells to HSV-1, but had no significant effect on the number of plaques on sog9 cells (data not shown). These findings, in conjunction with our recent finding that CS with the E disaccharide unit provides HSV binding sites on gro2C cells, indicate that the rescue of the expression of the E disaccharide unit of CS chains on the surfaces of sog9 cells resulted in a rise in viral infectivity (10). Furthermore, the gC-null virus (gC\(^{-}\)39) showed a less than 2-fold increase in the number of plaques on the C4ST-1-transfected cells as compared with the over 5-fold increase with the gC-positive virus, strongly suggesting that gC/CS interactions contributed to the enhanced viral replication seen in the sog9-C4ST-1-1 and sog9-C4ST-1-5 cells. We next conducted an attachment assay, in which an enhancement
The dysfunction of C4ST-1 in sog9 cells resulted in a remarkable decrease in the proportion of the E disaccharide unit accompanied by less 4-O-sulfation. Whereas the proportion of Di-4S unit obtained from sog9 cells was about half that from gro2C cells, the proportion of Di-diS_E unit revealed a drastic decrease to approximately one-tenth of that in gro2C cells. Notably, despite the fact that sog9 cells expressed mRNAs of other 4-O-sulfotransferases such as C4ST-2 and D4ST-1 (Fig. 3), few E disaccharide units were formed. Moreover, overexpression of C4ST-2 or D4ST-1 in sog9 cells led to no increase in the proportion of the E disaccharide unit or in the amount of CS (Table 1). These results indicated that C4ST-1 expression is a prerequisite for the formation of the E disaccharide unit, and C4ST-2 and D4ST-1 expression could not compensate for this role of C4ST-1. Recently, Wrana et al. (32) generated C4ST-1-deficient mice by inserting lacZ into the C4ST-1 locus via gene trap mutation and showed that this mouse develops severe chondrodysplasia characterized by a disorganized cartilage growth plate as well as specific alterations in the orientation of chondrocyte columns. Consistent with our results shown here, they observed that loss of C4ST-1 led to a drastic decrease in both the proportion of 4-O-sulfated CS and the amount of CS (32). Thus, C4ST-1 regulates the biosynthesis and function of CS in vivo.

The decrease in 4-O-sulfation in sog9 cells led to a shortening of CS chains, thereby resulting in a decrease in the amount of CS. Considering that the only difference between gro2C and sog9 cells is in the expression of C4ST-1, it is reasonable to assume that 4-O-sulfated CS or C4ST-1 itself regulates the chain length of CS in these cells. Indeed, the transfection of sog9 cells with C4ST-1 resulted in a recovery of the amount of CS accompanied by an increase in the length of CS chains, and the expression level of C4ST-1 correlated well with the recovery of the amount of 4-O-sulfated CS. In Caenorhabditis elegans and Drosophila melanogaster, CS is synthesized as non-sulfated...
chondroitin and 4-O-sulfated CS, respectively (33, 34). Whereas the chain length of chondroitin in C. elegans is short (~40 kDa), that of CS in D. melanogaster is ~70 kDa (33, 34). In addition, our group previously showed that a 4-O-sulfated tetrasaccharide such as GlcUAβ1-3GalNAc(4S)β1-4GalUAβ1-3GalNAc(4S) isolated from whale cartilage CS-A is a good acceptor substrate for GalNAc transferase-II, which transfers the GalNAc residue to growing CS chains, and enhances GalNAc transferase-II activity up to 3–4 fold compared with a non-sulfated tetrasaccharide (35). Thus, the 4-O-sulfation of CS chains by C4ST-1 may facilitate the elongation of CS chains by enzymes such as chondroitin synthase synthesizing the CS backbone. Hence, it is worth analyzing whether CS polymerase consisting of chondroitin synthase-1 and chondroitin polymerizing factor, which form the disaccharide backbone of CS, interacts with C4ST-1 to regulate the length of CS chains (36, 37).

HSV-1 utilizes cell surface GAG chains and other receptors to effectively bind and infect host cells (8). This virus rigidly binds to HS and CS through a positively charged domain of gC (31) expressed on the envelope of HSV and fuses with host cells after interaction of viral components with several cellular receptors including binding of gD to HS pentasaccharide with unusual sulfation, GlcNH2(3S) (7). In a further characterization of the binding of gC to CS, our group showed that the squid cartilage-derived CS-E, rich in E disaccharide units, exhibits potent anti-HSV-1 activity greater than heparin, which is an analog of HS with more highly sulfated disaccharide units (10). This inhibitory activity of CS-E is directed against gC of HSV-1 and depends on the dose and length of CS-E (10). Furthermore, chondroitinase digestion of CS expressed on the surface of gro2C cells revealed that CS chains containing the E disaccharide unit function as the receptor for HSV-1 binding (10). Supporting this result, in the present study, overexpression of C4ST-1 in sog9 cells not only led to synthesis of the E disaccharide unit on a level close to that in gro2C cells but also rendered the cells sensitive to infection with HSV-1, confirming our findings that the E disaccharide unit is involved in the binding and infectivity of HSV-1. Thus, C4ST-1 regulates the cell susceptibility to HSV-1 through the formation of the E disaccharide units responsible for the binding of the virus. Therefore, the discovery of a specific inhibitor for C4ST-1 activity and characterization of the disaccharide sequence of the CS chains bound to HSV will provide insight into the development of therapeutics for HSV-1 infection.

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