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Chondroitin Sulfate Characterized by the E-disaccharide Unit Is a Potent Inhibitor of Herpes Simplex Virus Infectivity and Provides the Virus Binding Sites on gro2C Cells*

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Although cell surface chondroitin sulfate (CS) is regarded as an auxiliary receptor for binding of herpes simplex virus to cells, and purified CS chain types A, B, and C are known to interfere poorly or not at all with the virus infection of cells, we have found that CS type E (CS-E), derived from squid cartilage, exhibited potent antiviral activity. The IC_{50} values ranged from 0.06 to 0.2 μg/ml and substantially exceeded the antiviral potency of heparin, the known inhibitor of virus binding to cells. Furthermore, in mutant gro2C cells that express CS but not heparan sulfate, CS-E showed unusually high anti-herpes virus activity with IC_{50} values of <1 ng/ml. Enzymatic degradation of CS-E with chondroitinase ABC abolished its antiviral activity. CS-E inhibited the binding to cells of the purified virus attachment protein gC. A direct interaction of gC with immobilized CS-E and inhibition of this binding by CS-E oligosaccharide fragments greater than octasaccharide were demonstrated. Likewise, the gro2C-specific CS chains interfered with the binding of viral gC to these cells and were found to contain a considerable proportion (13%) of the E-disaccharide unit, suggesting that this unit is an essential component of the CS receptor for herpes simplex virus on gro2C cells and that the antiviral activity of CS-E was due to interference with the binding of viral gC to a CS-E-like receptor on the cell surface. Knowledge of the determinants of antiviral properties of CS-E will help in the development of inhibitors of herpes simplex virus infections in humans.

Cell surface glycosaminoglycan (GAG)[3] chains such as heparan sulfate (HS) and/or chondroitin sulfate (CS) serve as initial receptors for several human and animal viruses, and such interactions are known targets for antiviral intervention with GAG-like molecules (1, 2). Cell surface CS is regarded as an auxiliary receptor for herpes simplex virus (HSV) that in the absence of HS chains can support initial interaction of HSV with cells (3, 4). A major CS-binding domain of HSV-1 attachment glycoprotein C (gC) has been shown to overlap with the HS binding site of this protein, except for residues Lys-114, Arg-117, Lys-120, and Ile-142, which supported gC binding to CS but not to HS (4). CS chains are composed of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc) at alternating positions. The former residue can be ester *O*-sulfated at C2 or rarely at C3, whereas the latter can carry sulfate groups at C4 and/or C6. Several types of CS chains can be distinguished based on the disaccharide units characterized by sulfate groups at specific position(s). In particular, CS-A comprises GlcAβ1–3GalNAc(4S) as a typical unit, whereas disaccharides L-iduronic acid(2S)β1–3GalNAc(4S), GlcAβ1–3GalNAc(6S), GlcA(2S)β1–3GalNAc(6S), and GlcAβ1–3GalNAc(4S,6S) predominate in CS-B (dermatan sulfate; DS), CS-C, CS-D, and CS-E, respectively, where 2S, 4S, and 6S stand for 2-*O*-, 4-*O*-, and 6-*O*-sulfate. Combination of disaccharide units with different sulfation patterns may form functional domain sequences as observed in HS chains (for review, see Ref. 5).

CS chains that contain di-sulfated disaccharide units are frequently isolated from marine organisms. Of these, CS-E has been purified from squid cartilage; its chains comprising ~61% of the E-disaccharide unit can also be sulfated at C3 of GlcA (6), although it should be emphasized that the GlcA(3S)-containing CS disaccharides have never been reported for mammalian cells or tissues. Such extensively sulfated chains may interact with heparin-binding proteins and/or interfere with the binding of these proteins to cell surface CS or HS. Indeed, recent functional studies have demonstrated that CS-E was capable of binding to a variety of brain-expressed heparin binding growth/differentiation factors with affinities comparable with that of heparin (7). In addition, CS-E inhibited neuronal cell adhesion by binding to neuroregulatory growth factor molecule midkine (8) and prevented interaction of selectins and various chemokines with proteoglycans (9).

From an antiviral point of view it is important to note that the preparations of purified CS-A, CS-B, and CS-C poorly or not at all interfered with HSV infection in HS-expressing cells (3, 10). In light of these data, it was surprising to learn that CS-E blocked HSV invasion of cells at substantially lower concentrations than the standard HS-related compound heparin. This intriguing finding implied that certain structures present in CS-E may fit better than heparin to the attachment/entry domains of viral components. Here, we describe a CS-E-like structure as the gro2C cell surface receptor for HSV and an anti-HSV potential of exogenous CS-E derived from squid cartilage.

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[3] The abbreviations used are: GAG, glycosaminoglycan; HS, heparan sulfate; CS, chondroitin sulfate; CS-A, CS-B, CS-C, CS-D, and CS-E, CS types A, B, C, D, and E, respectively; DS, dermatan sulfate; GMK AH1, green monkey kidney AH1 cells; HSV, herpes simplex virus; gC, glycoprotein C; EMEM, Eagle's minimal essential medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; pfu, plaque-forming unit; GlcA, D-glucuronic acid; GalNAc, *N*-acetyl-D-galactosamine; ΔHexA, 4,5-unsaturated hexuronic acid; 2S, 3S, 4S, and 6S, 2-, 3-, 4-, and 6-*O*-sulfate; ΔDi-0S, ΔHexAα1–3GalNAc; $\Delta Di\text{-}6S_C$, ΔHexAα1–3GalNAc(6S); $\Delta Di\text{-}4S_A$, ΔHexAα1–3GalNAc(4S); $\Delta Di\text{-}diS_D$, ΔHexA(2S) α1–3GalNAc(6S); $\Delta Di\text{-}diS_E$, ΔHexAα1–3GalNAc(4S,6S).

EXPERIMENTAL PROCEDURES

Materials—Chondroitinase ABC (EC 4.2.2.4 from *Proteus vulgaris*) and chondroitinase AC-I (EC 4.2.2.5 from *Flavobacterium heparinum*) were obtained from Sigma and from Seikagaku Corp. (Tokyo, Japan). Chondroitinase B (EC 4.2.2 from *F. heparinum*), chondroitinase C (EC 4.2.2 from *F. heparinum*), and hyaluronidase (EC 3.2.1.35 from sheep testis) were purchased from Sigma. CS-A (from sturgeon notochord), CS-C and CS-D (from shark cartilage), and CS-E (from squid cartilage) were obtained from Seikagaku Corp. [*methyl*-^{3}H]Thymidine (25 Ci/mmol) was from Amersham Biosciences, and $Na_2[^{35}S]O_4$ (specific activity 1,325 Ci/mmol) was from PerkinElmer Life Sciences. Pooled human γ-globulin was from Aventis Behring (Marburg, Germany).

Cells and Viruses—African green monkey kidney epithelial-like (GMK AH1) cells (11) were cultivated in Eagle's minimal essential medium (EMEM) supplemented with 2% newborn calf serum and 0.05% Primaton substance (Kraft Inc., Norwich, CT). Mutant HS-deficient gro2C cells derivative of murine L fibroblasts (12) were propagated in Dulbecco's modified EMEM supplemented with 10% fetal calf serum. These cells were kindly provided by Dr. Frank Tufaro, University of British Columbia (Vancouver). For cytotoxicity assay, GMK AH1 cells that were seeded in 96-well cluster plates and had reached ~80–90% confluence at day 2 of culture were incubated for 24 h at 37 °C with 0.1 ml of serial 3-fold dilutions of CS-E in EMEM. The cytotoxicity of CS-E for cells was measured by using the tetrazolium-based CellTiter96 assay according to the manufacturer's protocol (Promega, Madison, WI). HSV-2 333 strain (13), HSV-1 KOS 321 strain (14), and its gC-negative derivative designated gC$^-$39 (15) were used.

Preparation of GAGs from gro2C Cells—CS chains were prepared from gro2C cells by the method of Lyon *et al.* (16) with some modifications. Briefly, to facilitate identification of GAG fractions the cells were cultured in the presence of trace amounts of $Na_2[^{35}S]O_4$ for 3 days at 37 °C. The culture medium was aspirated and soluble proteoglycans concentrated by adsorption-elution on DEAE-Sephacel beads while the cells were washed with phosphate-buffered saline (PBS) and harvested by scraping. Approximately 10^9 cells or the soluble proteoglycan fraction were digested with 25 mg of Pronase (Sigma) for 4 h at 37 °C and then for a further 20 h with an additional 25 mg of the enzyme. After centrifugation at 1,000 × *g* for 15 min, the supernatant medium was boiled for 15 min to inactivate the enzyme and centrifuged again at 5,000 × *g* for 20 min. The supernatant was supplemented with 0.1% Triton X-100 and applied onto a column containing 5 ml of DEAE-Sephacel beads. The beads were washed with 0.02 M phosphate buffer (pH 7.0) supplemented with 0.3 M NaCl and 0.1% Triton, then with 0.05 M sodium acetate buffer (pH 4.0) containing 0.15 M NaCl and 0.1% Triton, and finally with the detergent-free phosphate buffer/0.3 M NaCl. The adsorbed material was eluted with 1.5 M NaCl and precipitated with ethanol. The amount of isolated GAGs was determined by quantification of hexuronic acid using the *meta*-hydroxydiphenyl method (17) with commercial preparation of CS-C as a standard.

Preparation of 2-Aminobenzamide-derivatized GAGs from gro2C Cells—Gro2C cells were homogenized in acetone and air-dried. The dried materials were digested with heat-activated (60 °C, 30 min) actinase E in 200 μl of 0.1 M boric acid-NaOH, pH 8.0, containing 10 mM calcium acetate. The incubation was carried out at 60 °C for 24 h. Following incubation, trichloroacetic acid was added to the sample to give 5%, and the resultant precipitate was removed by centrifugation. The soluble fraction was extracted with ether to remove trichloroacetic acid. The aqueous phase was neutralized with 1 M sodium carbonate and adjusted to 80% ethanol. The resultant precipitate was dissolved in 50 mM pyridine acetate, pH 5.0, and subjected to gel filtration on a PD-10 column (Amersham Biosciences) using 50 mM pyridine acetate, pH 5.0, as an eluent. The flow-through fractions were collected and evaporated to dryness. The dried materials were dissolved in water and then digested with chondroitinase ABC as described previously, using 5 mIU of chondroitinase ABC for 1 h at 37 °C in a total volume of 10 μl (18). Reactions were terminated by boiling for 1 min. The digests were derivatized with a fluorophore 2-aminobenzamide and then analyzed by high performance liquid chromatography as reported previously (19).

Preparation of Size-defined CS-E Oligosaccharides—Even-numbered CS-E oligosaccharides were prepared by enzymatic fragmentation of commercial squid cartilage CS-E with sheep testicular hyaluronidase, followed by fractionation using gel filtration column chromatography on Bio-Gel P-10 as described previously (6).

Preparation and Binding Assays of Purified Virions and Viral gC to Cells—[*methyl*-^{3}H]Thymidine-labeled virions of HSV were purified from infectious culture medium of GMK AH1 cells by centrifugation through a three-step discontinuous sucrose gradient as described (20, 21). The viral gC was purified from lysates of extracellular virus particles and virus-infected cells by immunoaffinity chromatography as previously described (21). The effect of CS-E on the binding of purified virions to cells was tested by the HSV attachment-inhibition assay described previously (22). The effect of CS-E on the binding of purified gC to cells was tested as follows. Purified gC (0.2 μg) was preincubated for 15 min at 4 °C with serial dilutions of CS-E prior to the addition to GMK AH1 cells growing in 96-well cluster plates. Following an adsorption period of 1 h at 4 °C, the bound gC was detected by immunoassay with the use of an anti-gC monoclonal antibody C4H11B6, alkaline phosphatase-conjugated AffiniPure F(ab′)$_2$ fragment of goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) as the secondary antibody, and *p*-nitrophenyl phosphate as a substrate (10).

An Enzyme-linked Immunosorbent-based Assay of Binding of Viral gC to Immobilized CS-E—Commercial squid cartilage CS-E was biotinylated as previously reported (7). Biotinylated CS-E (4 μg each) was immobilized on 96-well streptavidin-coated microtiter plates (BD Biocoat Assay Environments; BD Biosciences) at 4 °C overnight. The wells were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20, HSV-1 gC (0.1 μg each) was added, and the plate was incubated for 2 h at room temperature. Bound gC protein was detected using mouse anti-gC monoclonal antibody B1C1B4, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG/IgM. Alkaline phosphatase activity was detected using *p*-nitrophenyl phosphate as a substrate, and the absorbance was measured at 415 nm. Reactivity of the commercial CS-E with the gC protein was evaluated by inhibitory enzyme-linked immunosorbent assay where the gC component was preincubated with test CS-E oligo- or polysaccharides for 30 min at room temperature, and then the mixture was added to the CS-E-immobilized microtiter plates.

Viral Plaque Assays—The effect of CS-E on HSV infectivity was tested by the plaque number reduction assay as previously described (22). The effect of CS-E on cell-to-cell spread of HSV was tested by the plaque size reduction assay (22) and by the infectious center assay (23). Briefly, for plaque size reduction assay, CS-E was diluted in an overlay medium composed of EMEM and 1% methylcellulose or 0.5% pooled human γ-globulin. The experiments were carried out in monolayer cultures of densely growing GMK AH1 cells. The overlay medium was added to cells after their infection with HSV and left on the cell monolayer throughout the entire period of the development of viral plaques. Images of 20 neighboring plaques were captured and subjected to plaque area determination by using a Leica DC300 digital camera and IM500 image software. For the infectious center assay, infected cells, prepared by their dissociation with trypsin-versene solution at 4–5 h

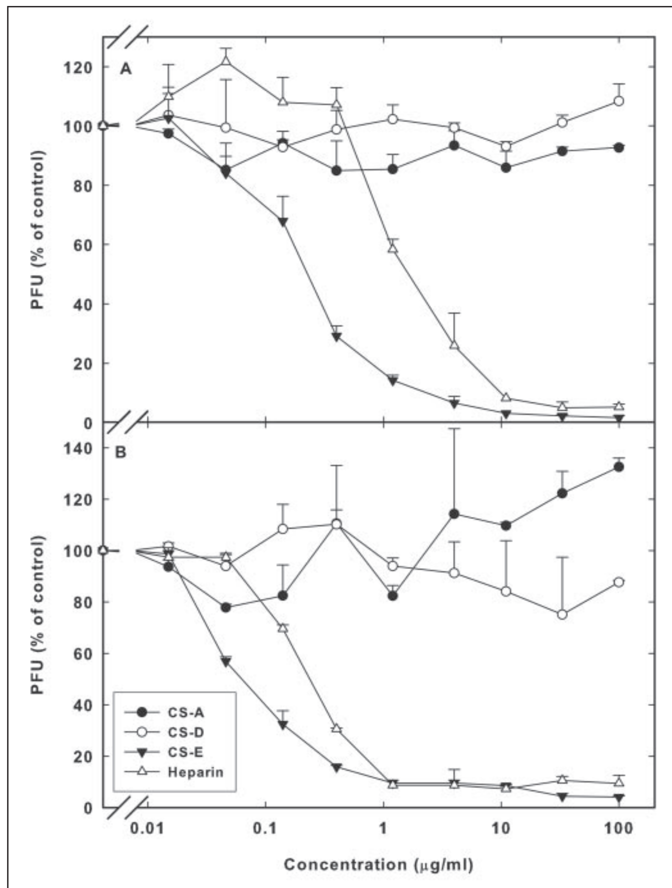

FIGURE 1. **Interference of CS-E with HSV infectivity.** CS-A, CS-D, CS-E, or heparin at different concentrations was incubated with ~200 pfu of HSV-1 KOS321 (*A*) or HSV-2 333 (*B*) for 15 min prior to and during a 1-h period of virus infection of GMK AH1 cells. The results are expressed as a percentage of the number of viral plaques (*pfu*) found with compound-treated virions relative to mock-treated controls. At least two separate experiments were carried out in duplicate for each compound.

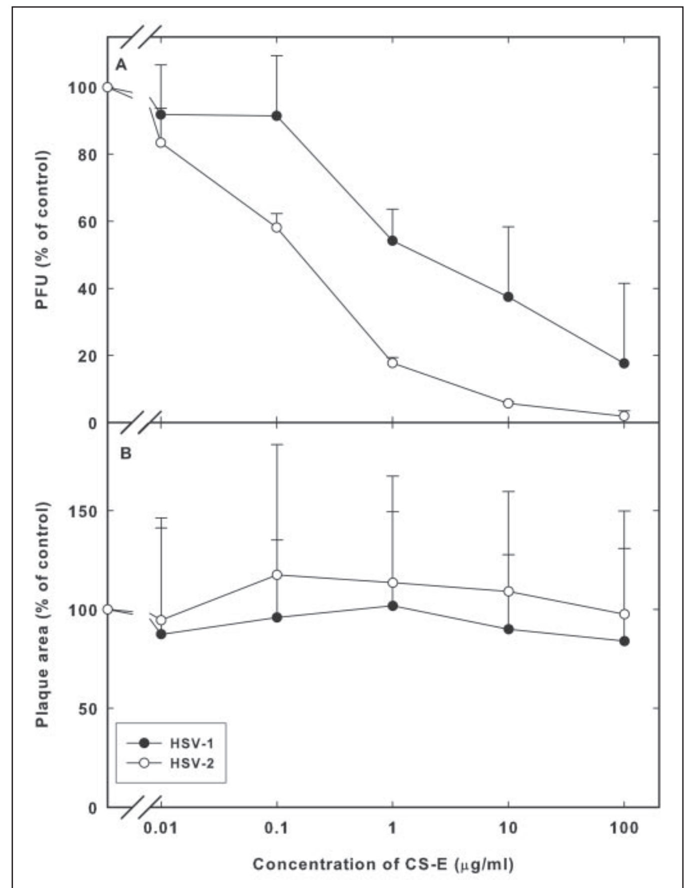

FIGURE 2. **Interference of CS-E with the cell-to-cell spread of HSV.** *A*, HSV-infected cells (~200), prepared as described under "Experimental Procedures," were added to and co-incubated with a confluent monolayer of GMK AH1 cells for 2–3 days at 37 °C in the presence of pooled human γ-globulin and different concentrations of CS-E. The results are expressed as a percentage of the number of viral plaques (*pfu*) found with compound-treated cells relative to mock-treated controls. Two separate experiments were carried out in duplicate for each virus. *B*, GMK AH1 cells were infected with ~200 pfu of either HSV-1 KOS321 or HSV-2 333 and then overlaid with EMEM supplemented with 1% methylcellulose and different concentrations of CS-E. The results are expressed as a percentage of the average area of viral plaques developed in drug-treated cells relative to mock-treated controls. Images of 20 viral plaques per each dilution of the compounds were captured and subjected to area determinations using the IM500 software. The average area of HSV-1 plaques in control wells was 0.14 mm^2 ± 0.09 and of HSV-2 plaques was 0.30 mm^2 ± 0.17.

after infection with HSV, were added to monolayer cultures of GMK AH1 cells in the medium comprising 0.5% human γ-globulin and specific concentrations of CS-E. The viral plaques were visualized by staining the cells with 1% solution of crystal violet after 2 (HSV-2) or 3 (HSV-1) days of incubation at 37 °C.

Chondroitinase Digestion Assays—The anti-HSV activity of chondroitinase-treated CS-E was assayed as follows. CS-E (100 μg) in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$) supplemented with 0.1% BSA (PBS-BSA) was digested for 10 min at 37 °C with different numbers of chondroitinase ABC units. Immediately afterward, the mixture was boiled for 1 min to inactivate the enzyme and then tested in the viral plaque assays for residual CS-E activity. The susceptibility of chondroitinase-treated gro2C cells to HSV was tested as follows. Gro2C cells seeded in 6-well cluster plates were washed with warm Dulbecco's-modified EMEM, and 1-ml volumes of PBS-BSA supplemented with different numbers of chondroitinase ABC, AC-I, B, or C units were added. Following incubation for 30 min at 37 °C, the cells were left at 4 °C for 30 min, washed twice with cold PBS-BSA, and then incubated for 1 h at 4 °C with 1 ml of PBS-BSA containing ~200 plaque-forming units (pfu) of HSV-1. Subsequently, the cells were washed twice with PBS-BSA and overlaid with 3 ml of 1% methylcellulose solution in Dulbecco's medium supplemented with 2% fetal calf serum. Following incubation for 4 days at 37 °C, the viral plaques were visualized by staining with 1% solution of crystal violet.

RESULTS

Antiviral Potency of CS-E—The squid cartilage-derived CS-E was examined for its effect upon HSV infection of GMK AH1 cells. For comparative purposes, two other types of CS chains, CS-A and CS-D, and the standard HS-related polysaccharide heparin were included. In CS-E chains the number of sulfate groups/disaccharide unit averages 1.7, which is almost twice that of CS-A but only approximately two-thirds that of heparin. According to the manufacturer's data CS-D contains 23% of di-sulfated disaccharides and its degree of sulfation is 1.2. The effect of CS-E on HSV-1 or HSV-2 infectivity was assayed by incubating the CS-E with the virus for 15 min prior to addition to cells and during the 1-h period of virus infection of cells (Fig. 1, *A* and *B*). The concentrations of CS-E that reduced the number of viral plaques by 50% (IC_{50}) were 0.2 μg/ml for HSV-1 and 0.06 μg/ml for HSV-2, thus substantially exceeding the antiviral potency of heparin. In contrast, within the concentration range tested, CS-A and CS-D showed no anti-HSV activity. CS-E exerted no cytotoxic effect when incubated with GMK AH1 cells for 24 h at concentrations of up to 500 μg/ml (data not shown).

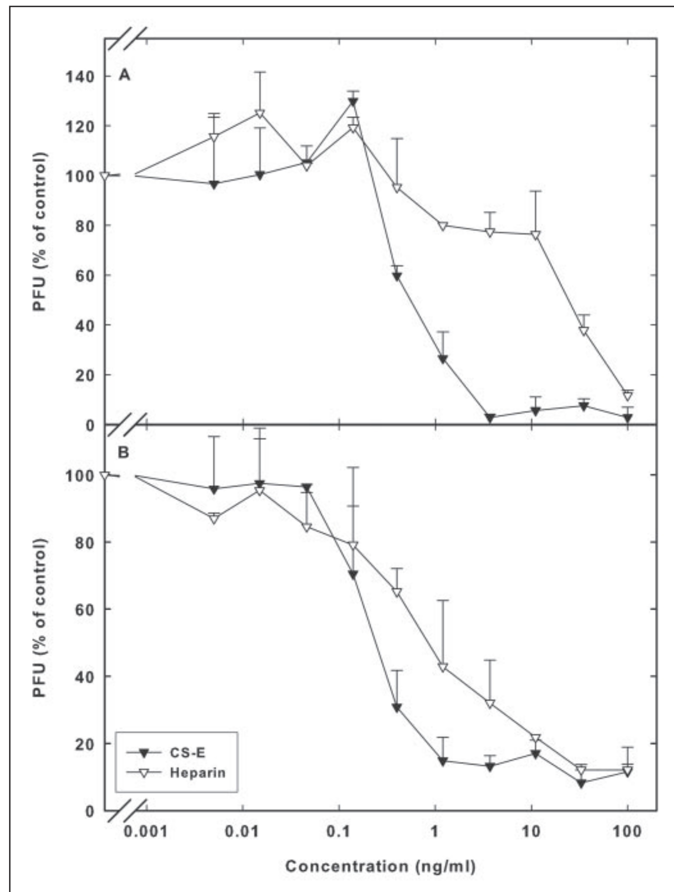

FIGURE 3. **Interference of CS-E with HSV infectivity in mutant gro2C cells expressing CS but not HS.** CS-E or heparin at specific concentrations was incubated with ~200 pfu of HSV-1 KOS321 (*A*) or HSV-2 333 (*B*) for 15 min prior to and during a 1-h period of virus infection of gro2C cells. For further explanations, see the legend to Fig. 1.

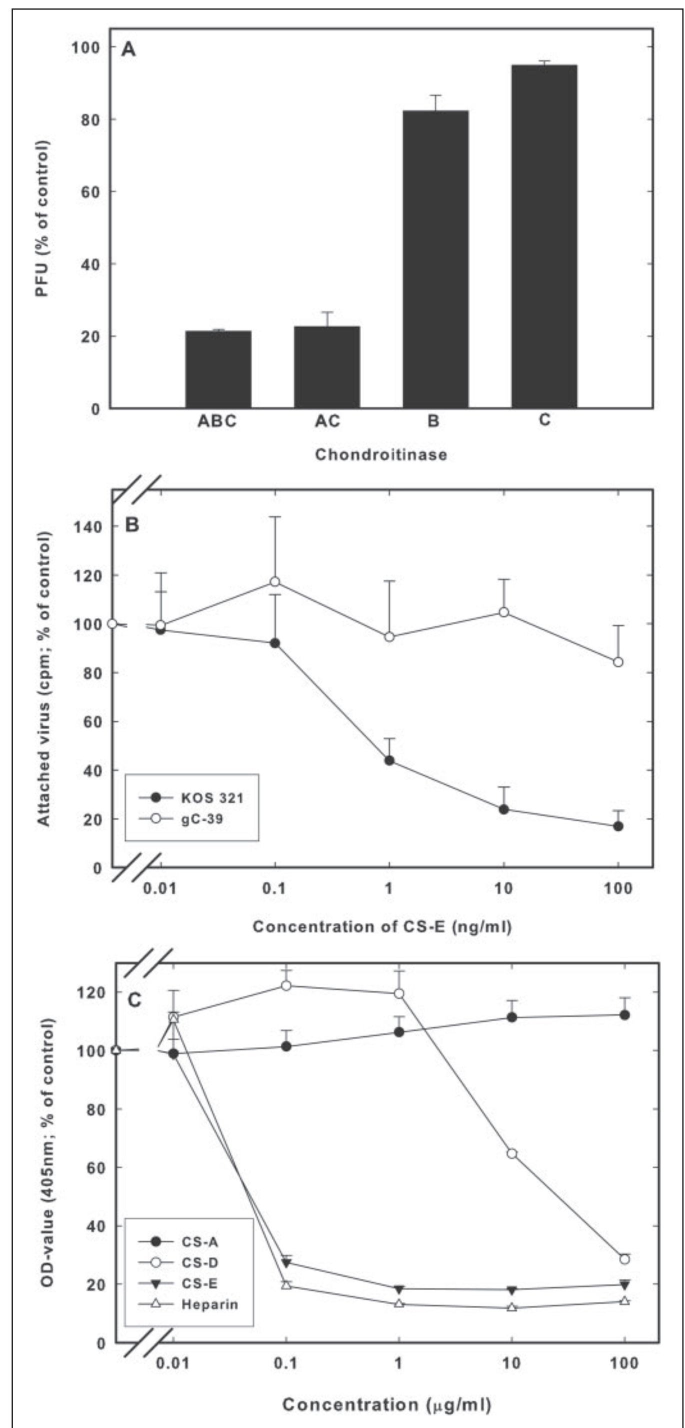

FIGURE 4. **Interference of CS-E- or CS-degrading enzymes with the binding of HSV-1 or isolated HSV-1 gC to cells.** *A*, gro2C cells were treated for 30 min at 37 °C with an excess of chondroitinase ABC (2 units), AC-I (1 unit), B (15 units), or C (17 units) prior to their infection with HSV-1 KOS321 strain. Activities of all enzymes are shown in Sigma units. One IU corresponds to ~600 Sigma units. The results are expressed as a percentage of the number of viral plaques (*pfu*) found in enzyme-treated cells relative to mock-treated controls. *B*, CS-E at different concentrations was incubated with [*methyl*-^{3}H]thymidine-labeled particles of HSV-1 KOS321 or its gC-null derivative gC$^-$39 for 15 min prior to and during a 1-h period of virus binding to gro2C cells. The results are expressed as a percentage of the attached viral cpm found with compound-treated virions relative to mock-treated controls. The mean number of cell-associated cpm in control wells was 6,858 for KOS321 and 5,723 for the gC$^-$39 strain. *C*, CS-A, CS-D, CS-E, or heparin at different concentrations was incubated with purified gC of HSV-1 for 15 min prior to and during a 1-h period of gC adsorption to gro2C cells. The results are expressed as a percentage of the absorbance of attached viral gC found with CS-E-treated protein relative to mock-treated controls. Two separate experiments were carried out in duplicate or in triplicate (*C*) for each agent tested.

Two different assays were used to investigate the effect of CS-E on cell-to-cell spread of HSV. An infectious center assay evaluates virus transmission from an exogenously added infected cell to a non-infected cell of the cell monolayer and may reflect the host-to-host transmission of HSV via infected cells. The plaque size reduction assay assesses the virus spread between the densely growing cells of the monolayer culture and may indicate the capability of the compound to penetrate into the narrow intercellular spaces. Both assays were carried out in the presence of either pooled human γ-globulin or methylcellulose to exclude virus transmission by routes other than the direct cell-to-cell spread. CS-E inhibited HSV-2, and to some extent HSV-1, transmission from exogenously added infected cells to non-infected cells (Fig. 2*A*), although it had no effect on the size of HSV-1 and HSV-2 plaques developed (Fig. 2*B*).

Mechanism of Antiviral Activity of CS-E—A potent inhibition of HSV by CS-E raised questions about the nature of both the cellular GAG receptor, with which the compound competed, and the viral component targeted by CS-E. To clarify these issues, CS-E was tested for an anti-HSV activity in mutant gro2C cells that express CS but not HS (12). CS-E exhibited unusually high anti-HSV potency in these cells with IC_{50} values of 0.5 ng/ml for HSV-1 and 0.2 ng/ml for HSV-2 (Fig. 3, *A* and *B*). Note that the IC_{50} values for CS-E in GMK AH1 cells, which express both HS and CS, were more than 100-fold higher than the corresponding values in gro2C cells (compare Figs. 1 and 3; note differences in scale). In addition to CS-E, heparin exhibited a high antiviral activity in gro2C cells that significantly exceeded that observed in GMK-AH1 cells. These results suggest that in the absence of HS receptor, sulfated

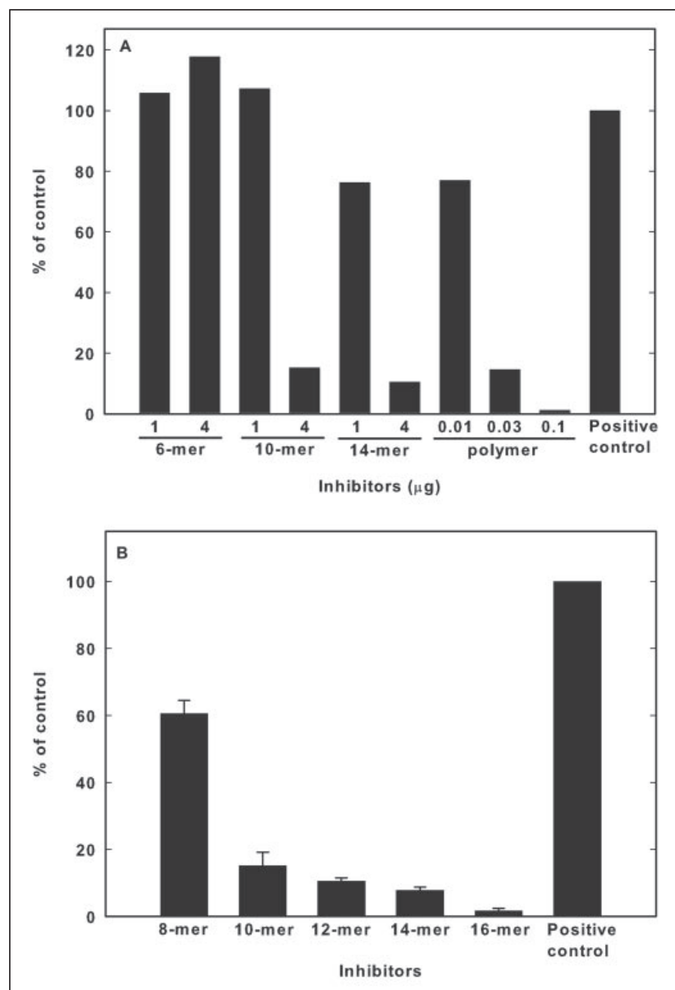

FIGURE 5. **Inhibition of the binding of viral gC to immobilized CS-E by soluble CS-E oligosaccharides.** *A*, the gC protein (0.1 μg, purified from HSV-1 virions) was added with or without indicated amounts of CS-E hexa-, deca-, tetradeca-, or polysaccharides to the CS-E-coated microtiter plates. Bound gC protein was quantified using mouse anti-gC monoclonal antibody B1C1B4 as described under "Experimental Procedures." The mean absorbance (at 415 nm) for binding of viral gC to CS-E in the absence of inhibitor was 1.258, and this value was accepted as 100% (*Positive control*). All other values are expressed as percentages of this control quantity. *B*, inhibitory enzyme-linked immunosorbent assay was carried out as described in *panel A* except that a single dose (4 μg each) of various CS-E oligosaccharides of different sizes was used as inhibitor. Values and the S.E. were obtained from the average of two separate experiments.

polysaccharides such as CS-E or heparin very effectively competed out the available cellular receptor in forms of CS. To identify the type of receptor molecule, gro2C cell surface GAGs were digested with chondroitinases ABC, AC-I, B, and C, which predominantly cleave the corresponding types of CS; some of them may also digest heterologous types, *e.g.* chondroitinase ABC can cleave most CS types, including CS-E and DS (the isomeric form of CS). Cells treated with chondroitinases ABC and AC-I, but not with B or C, were less susceptible to HSV-1 infection (Fig. 4*A*), suggesting that a certain specific structure of CS or DS is the cell surface receptor for HSV on gro2C cells.

To identify the viral component targeted by CS-E, the effect of this compound on binding to cells of radiolabeled HSV-1 wild-type strain (Fig. 4*B*), HSV-1 gC-deficient mutant designated $gC^{-}39$ (Fig. 4*B*), and an isolated gC component of HSV-1 (Fig. 4*C*) was tested. CS-E reduced the binding to gro2C cells of wild-type HSV-1 and its attachment protein gC, whereas HSV-1 gC-null mutant was only marginally affected. Altogether the results shown in Fig. 4 indicate that CS-E inhibited HSV infection of gro2C cells by competing with cell surface CS or DS for binding to viral gC.

TABLE ONE

Disaccharide composition of CS from gro2C, L, and GMK AH1 cells and CS-E from squid cartilage

A glycosaminoglycan fraction was prepared from cells after extensive protease digestion followed by ethanol precipitation and subsequent gel filtration. This fraction was digested with chondroitinase ABC, followed by labeling with a fluorophore 2-aminobenzamide and subsequent anion exchange high performance liquid chromatography to determine disaccharide composition as described under "Experimental Procedures." Note that no disaccharide was generated by digestion with chondroitinase B (not shown).

Composition	gro2C[a]	L[a]	GMK AH1[a]	Squid cartilage[b] CS-E
	mol %			
ΔDi-0S	15	10	21	9
ΔDi-6S_C	8	5	13	9
ΔDi-4S_A	64	68	63	21
ΔDi-diS_D	ND[c]	ND[c]	ND[c]	ND[c]
ΔDi-diS_E	13	17	3	61
Total	100	100	100	100

[a] Disaccharide analysis was carried out twice for each sample, and the results were reproducible. Chondroitinase AC-I digestion of these samples showed no larger resistant oligosaccharides, implying no GlcA(3S)-containing structures. However, the possibility that GlcA(3S) may be present in the CS chains cannot be completely excluded.
[b] Data from Kinoshita *et al.* (6) derived from chondroitinase ABC degraded CS-E. It should be noted that the overall GlcA(3S) content in CS-E chains is ~10% (w/w), with significant variations among different preparations (30) as calculated based on the amounts of the isolated oligosaccharides, which were resistant to digestion with hyaluronidase and structurally elucidated by proton NMR spectroscopy (6, 29). However, because GlcA(3S)-containing disaccharide units are destroyed by the action of chondroitinase ABC, unsaturated disaccharides derived from such units cannot be isolated or quantified (35) and are not included here.
[c] ND, not detected.

Knowing that CS-E interfered with interaction between the viral gC and the cell surface CS chains, our further studies focused on these two components. First, we investigated whether viral gC was capable of binding to CS-E chains. To this end the gC component, derived from HSV-1 particles, was added to CS-E chains immobilized at the plastic surface. The viral gC bound to CS-E, and this interaction was substantially inhibited by soluble CS-E polymers at concentrations of ≥0.03 μg and by CS-E deca- or tetradecasaccharide at ≥4 μg (Fig. 5*A*). To identify a minimum size of CS-E chain fragment required for interference with gC binding to immobilized CS-E, a panel of even-numbered size-defined CS-E oligosaccharides was used (Fig. 5*B*). The CS-E oligosaccharides (4 μg of each) composed of 8 monosaccharide units reduced the binding of gC to CS-E by ~40%, whereas decasaccharide or greater CS-E fragments decreased this binding by >50%.

Efficient inhibition of HSV infectivity by CS-E, but not by other CS types, suggested that certain structural features, specific for the CS-E chain, may determine its capability of binding gC and thus the antiviral activity. Treatment of CS-E (100 μg) for 10 min at 37 °C with 0.5 unit (Sigma units) of chondroitinase ABC drastically decreased (>80%) its anti-HSV-1 and anti-HSV-2 activity. Interestingly, CS-E digested with 0.05 unit of the enzyme retained >80% of its original activity against HSV-2 but only ~15% of its anti-HSV-1 activity (data not shown). Because chondroitinase ABC has endolytic eliminase activity, this finding suggests that shorter oligosaccharide fragments of CS-E chains are required for inhibition of HSV-2 than for blocking of HSV-1. However, these results also emphasize the importance of the E-unit structure for interaction with viral gC and raise the possibility that a CS-E-like structure may constitute the actual cell surface receptor for this protein. To investigate this possibility, a GAG fraction was prepared from gro2C cells after extensive protease digestion followed by ethanol precipitation and subsequent gel filtration. This fraction was digested with chondroitinase ABC, followed by labeling with a fluorophore 2-aminobenzamide and subsequent anion exchange high performance liquid chro-

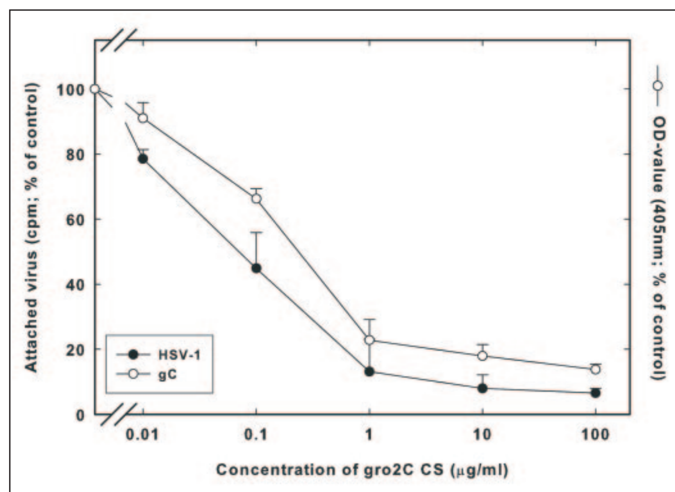

FIGURE 6. **Interference of gro2C CS with the binding of HSV-1 particles or isolated HSV-1 gC to cells.** Gro2C-specific CS was incubated with radiolabeled HSV-1 virions or isolated gC protein for 15 min prior to and during a 1-h period of virus/gC adsorption to gro2C cells. The results are expressed as a percentage of attached viral cpm or the absorbance of attached viral gC found with CS-E-treated ligands relative to mock-treated controls. Two separate experiments were carried out in duplicate for purified virions or in triplicate for viral gC.

matography to determine disaccharide composition. As mentioned above, chondroitinase ABC cleaves most variants of CS chains, including CS-E as well as DS (6, 18). The results revealed the existence of a considerable proportion (13%) of the E-disaccharide unit (ΔDi-diS$_E$) in addition to a major unit ΔDi-4S$_A$ and two minor units, including ΔDi-6S$_C$ and ΔDi-0S (TABLE ONE). No disaccharide was generated by digestion with chondroitinase B specific for DS (data not shown). In addition to mutant gro2C cells, CS chains from murine L fibroblasts, which are parental cells for gro2C, and CS from GMK AH1 cells were analyzed for disaccharide composition. Both L and GMK AH1 cells are known to be susceptible to HSV, and their CS chains contained E-units at proportions of 17 and 3%, respectively (TABLE ONE). The effect of gro2C-specific CS on the binding to cells of purified radiolabeled HSV-1 particles or isolated viral gC is shown in Fig. 6. This form of CS potently inhibited the binding to gro2C cells of HSV virions and viral gC with IC$_{50}$ values of 0.07 and 0.2 μg/ml, respectively. Note that as regards the viral gC, this effect was only ~5-fold weaker than that observed for CS-E but exceeded the anti-gC activity of CS-D by ~100-fold (compare Figs. 4*C* and 6). Furthermore, CS-A that possessed like gro2C CS ~1 sulfate group/disaccharide, but according to the supplier data (Seikagaku) comprised no E-unit structures, did not interfere with the binding of gC to cells (Fig. 4*C*). These results, together with data shown in Fig. 4*A*, strongly suggest that the major cell surface receptor for HSV on gro2C cells is indeed CS chains containing the E-disaccharide units. TABLE ONE also includes data on disaccharide composition of squid cartilage CS-E. Compared with CS from gro2C cells, the squid cartilage CS contained more ΔDi-diS$_E$ units and some extra unique sulfate groups at C3 of GlcA. These structural differences between CS chains expressed in gro2C cells and in squid cartilage seem to be important for the explanation of a potent antiviral activity of CS-E in gro2C cells (Fig. 3).

DISCUSSION

In light of known observations that different types of CS exhibited either little or no anti-HSV activity (3, 10, 24), the potent inhibition of HSV infectivity by CS-E and detection of the E-unit structure as an important component of the CS receptor for HSV on gro2C cells were surprising findings. Because functions of cell surface CS in promoting HSV attachment to cells are considered auxiliary or substitutive to those of HS chains (3, 4, 12, 25), the antiviral activities of HS-related inhibitors such as heparin were found to be superior to these of CS and related molecules (10). CS-E seems to be an exception to this rule as its antiviral activity substantially exceeded that of heparin. By and large, an antiviral activity of sulfated polysaccharides positively correlates with the molecular mass of the chain and the degree of its sulfation (for review, see Ref. 26). Although CS-E chains (~70 kDa) are larger than those of heparin (~12.5 kDa), the latter polysaccharide is more extensively sulfated (~2.7 sulfates/disaccharide) than CS-E, which contains ~1.7 sulfates/disaccharide. Interestingly, two other types of CS, *i.e.* CS-B (DS) and CS-D, like CS-E contain the di-sulfated disaccharide units yet demonstrated poor antiviral activity (3, this report). Although this difference could be attributed to the lower content of di-sulfated units in CS-B (~27%) and CS-D (~23%) than in CS-E (~61%), the gro2C-specific CS that contained only 13% of such units (TABLE ONE) appeared to be a more potent inhibitor than CS-D of the binding of viral gC to cells. Hence, one cannot exclude that the specific positioning of sulfates in the predominant CS-E disaccharide unit, *i.e.* GlcAβ1–3GalNAc(4S,6S), is an important determinant of the HSV-1 gC binding activity and thereby antiviral potency of this polysaccharide. Certain sulfated galactans and xylogalactans have been reported to interfere with HSV infection of cells (27, 28), raising the possibility that specific sulfation of galactose residue might be important for its antiviral activity. In addition, it is worth noting that the squid cartilage CS-E chains were found to contain an extra sulfate group at C3 of GlcA (6), a residue that has never been reported for mammalian cell CS. Because up to 10% of GlcA residues can be 3-*O*-sulfated, the CS-E chains may possess a domain-like structure with specific positioning of tri-sulfated disaccharide units relative to di-sulfated units (6, 29, 30). It is not known whether these tri-sulfated units are the key determinants of the antiviral potency of squid CS-E. In this context it is important to note that chemical oversulfation of CS and DS chains, which initially possessed ~1 sulfate group/disaccharide unit and were devoid of antiviral properties, drastically boosted their activity against several viruses, including HSV (31).

According to the most accepted opinion, sulfated polysaccharide inhibitors act by competing with cell surface GAG chains for binding to the viral attachment protein(s). The same seems to be true for CS-E, as this compound interfered with the binding to cells of both purified HSV virions and an isolated viral attachment component gC. In addition, we have observed that viral gC was capable of binding to immobilized CS-E chains and that CS-E-derived oligosaccharide fragments as small as octasaccharide interfered with this interaction. This finding raised the possibility that the E-unit structure might exist in the cell surface CS chains, thus providing receptor sites for the virus binding to cells. Indeed, disaccharide analysis of CS expressed on the surface of gro2C cells revealed the presence of a substantial proportion (13%) of the E-unit. Furthermore, this form of CS interfered with the binding to gro2C cells of HSV particles and viral gC. This observation together with the findings that CS-E exhibited unusually high antiviral activity in gro2C cells and that chondroitinases ABC and AC-I, but not B or C, prevented HSV infection of these cells by degrading the CS chains strongly suggests that the E-disaccharide unit is an essential structural element of CS receptor for HSV-1 at the surface of gro2C cells. Although the importance of E-unit for infection of normal HS/CS-expressing cells remains obscure, our data revealed the presence of this structure in CS chains derived from the HSV-susceptible cells. In addition to marine organisms, the E-disaccharide units typical for CS-E are widely expressed in mammalian cells and tissues, including mast cells, macrophages, cartilage, and brain (for review, see Refs. 5, 6). Recent studies also showed the E-units in the CS chains of appican, a CS proteoglycan form of amyloid precursor protein (32), and syndecan-1 and -4, which are expressed on the surface of mammalian cells such as

murine mammary gland epithelial cells (33, 34). It would be of interest to determine whether the presence of the E-units in neural tissue could facilitate HSV invasion of neurons, a feature that is central to the pathogenesis of HSV infections in humans.

In addition to the effects of CS-E on virus infectivity, the compound was capable of reducing the virus spread from infected cells of exogenous origin during their co-cultivation with non-infected cells. These data suggest that CS-E has a potential to limit the host-to-host transmission of HSV via infected cells. However, because of the relatively high molecular mass CS-E did not reduce the cell-to-cell spread within the monolayer of densely growing cells. Certain sulfated oligosaccharides of low molecular mass such as mannose-containing PI-88 agent or pentosan polysulfate were capable of reducing the cell-to-cell spread of HSV, very likely because of their capability to access the narrow intercellular space (22).

In conclusion, CS chains characterized by the presence of the E-disaccharide unit appeared to be an important ligand for HSV gC. CS chains with a high content of the E-unit potently inhibited HSV infectivity, whereas those with a moderate quantity of these units, such as the gro2C cell-specific CS, were found to serve as receptor sites for binding of the virus to cells. Identification of the specific oligosaccharide sequences, which can promote both virus attachment to cells and inhibition of this activity, will form a structural basis for the development of highly efficient CS-E-like inhibitors of HSV infections in humans.

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