Enzymes Responsible for Synthesis of Corneal Keratan Sulfate Glycosaminoglycans

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Keratan sulfate glycosaminoglycans are among the most abundant carbohydrate components of the cornea and are suggested to play an important role in maintaining corneal extracellular matrix structure. Keratan sulfate carbohydrate chains consist of repeating N-acetyllactosamine disaccharides with sulfation on the 6-O positions of N-acetylglucosamine and galactose. Despite its importance for corneal function, the biosynthetic pathway of the carbohydrate chain and particularly the elongation steps are poorly understood. Here we analyzed enzymatic activity of two glycosyltransferases, β1,3-N-acetylgalactosaminyltransferase-7 (β3GnT7) and β1,4-galactosyltransferase-4 (β4GalT4), in the production of keratan sulfate carbohydrate in vitro. These glycosyltransferases produced only short, elongated carbohydrates when they were reacted with substrate in the absence of a carbohydrate sulfotransferase; however, they produced extended GlcNAc-sulfated poly-N-acetyllactosaminyltransferase-7 (CGn6ST) followed by keratan sulfate galactose 6-O sulfotransferase treatment. We also observed that production of highly sulfated keratan sulfate in cultured human corneal epithelial cells was dramatically reduced when expression of β3GnT7 or β4GalT4 was suppressed by small interfering RNAs, indicating that these glycosyltransferases are responsible for elongation of the keratan sulfate carbohydrate backbone.

In higher eukaryotes, high numbers of cells interact with each other and form complicated but well organized tissue structures. During the tissue formation in the developmental stage, cells recognize the surrounding environment and interact with neighboring cells and the extracellular matrix. Proteoglycans (PGs) are glycoproteins that carry linearly elongated polysaccharides called glycosaminoglycans (GAGs) on core proteins and that are largely found in the extracellular matrix and play important roles for development, maintenance, and function of the tissues.

Production of the GAG chain takes place mostly in the Golgi apparatus. Several glycosyltransferases and carbohydrate-modifying enzymes such as sulfotransferases act on GAG elongation and modification. So far almost all of the enzymes involved in GAG production are cloned and analyzed for their enzymatic activities; however, the process of GAG production is still unknown in some GAGs because of the presence of multiple enzymes with redundant activities.

Keratan sulfate (KS) PGs are major components of the cornea and also found in cartilage and brain. Because of the high concentration of these molecules in the cornea, their biological function has been extensively studied and found to include maintenance of corneal extracellular matrix structure (1–9).

Corneal KS PGs consist of PG core proteins, such as lumican, keratocan, and mimecan, carrying KS GAGs in an N-linked manner (10–12). KS GAG is a linear carbohydrate chain made of sulfated disaccharide repeats of -3Galβ1–4GlcNAcβ1- with sulfate on the 6-O position of GlcNAc and galactose (10–12) and exhibiting modifications such as fucose and siaic acid (13, 14). Production of the KS GAG chain on PGs is processed by glycosyltransferases and sulfotransferases localized in the Golgi apparatus, and matured KS PGs are secreted into the extracellular matrix. Elongation of the carbohydrate backbone of the KS GAG chain is catalyzed by enzymes of two glycosyltransferase families, β1,3-N-acetylgalactosaminyltransferase (β3GnT) and β1,4-galactosyltransferase (β4GalT), and sulfation of the chain is catalyzed by two carbohydrate sulfotransferases.

Recent studies of carbohydrate sulfotransferases have identified enzymes responsible for sulfation of the corneal KS GAG chain as KS galactose 6-O sulfotransferase (KSG6ST) and corneal N-acetylgalactosamine 6-O sulfotransferase (CGn6ST, also known as GlcNAc6ST-5 and GST4β) (15, 16). Because CGn6ST only transfers sulfate on the nonreducing terminal GlcNAc but not onto internal GlcNAc, sulfation of GlcNAc...
residues of KS GAG is coupled with KS GAG elongation (16). On the other hand, KSG6ST transfers sulfate on galactose located both internally and on the nonreducing terminal of the carbohydrate chain (15, 17). KSG6ST also prefers a sulfated carbohydrate as substrate (15, 17), suggesting that galactose sulfation occurs after production of the GlcNAc-sulfated poly-N-acetyllactosamine chain and that GlcNAc sulfation is necessary for sulfation of galactose residues by KSG6ST.

Patients with macular corneal dystrophy type I, which is caused by deficiency of functional CGn6ST, have no detectable highly sulfated KS in the cornea (18), serum, and cartilage (19, 20), suggesting that GlcNAc sulfation is required to produce highly sulfated KS GAG. Observations that macular corneal dystrophy patients with CGn6ST mutations develop corneal opacities (21) and that mice lacking the orthologous sulfotransferase display corneal thinning with abnormalities in corneal extracellular matrix structure (9) indicate important roles for KS GAG sulfation in the function and maintenance of the cornea. However, unlike KS sulfation, mechanisms underlying KS GAG chain elongation are poorly understood.

In humans, β3GnT and β4GalT enzymes are encoded by eight and seven genes, respectively. Among these, β3GnT7 and β4GalT4 are thought to be responsible for elongation of the KS carbohydrate chain because these enzymes have higher activity for sulfated than nonsulfated substrates (22, 23); however, direct evidence in support of this hypothesis has not been reported.

Here, using soluble forms of recombinant enzymes and glycosidase-assisted column chromatography, we demonstrate that β3GnT7 and β4GalT4 can produce KS GAG carbohydrate in vitro. We also observed that suppressing expression of either β3GnT7 or β4GalT4 reduced highly sulfated KS GAG production in cultured human corneal cells, indicating that these glycosyltransferases are responsible for KS GAG elongation.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors Encoding Soluble Forms of Enzymes**—cDNAs encoding the catalytic domains of β4GalT4 and β3GnT7 were PCR-amplified using human placental Marathon-Ready cDNA (Clontech, Mountain View, CA) as template and the following primers: 5′-ATGGAATCTTTGCACTAGTGGTTCCAAAGCAGG1-3′ and 5′-TAACCTCGACTCTGACCACTACAAACC1-3′; and β3GnT7, 5′-GGTTATATGATATTATTTACCTCGGCTGTCGAGCCAGG1-3′ and 5′-TTCCTGTGCAGAACGAGGTCCTGGCGCTGTCAGTA1-3′. Amplified DNAs were digested with EcoRV and XhoI and cloned into the corresponding sites of pcDNA3.1-HSH (24). To construct an expression vector for soluble KSG6ST, we amplified cDNA encoding the catalytic domain of KSG6ST using the primer pair, 5′-ATGGAATCTTTGCACTAGTGGTTCCAAAGCAGG1-3′ and 5′-TTATGTGACCACTACAAACC1-3′ and 5′-CCCAGGTCCTGGCGCTGTCAGTA1-3′. Amplified DNAs were digested with EcoRV and XhoI and cloned into the corresponding sites of pcDNA3.1-HSH. Construction of expression vectors for intact and soluble CGn6ST was described previously (25).

**Preparation of Soluble Enzymes**—Expression vectors or an empty pcDNA3.1-HSH were transfected individually into Lec20 cells (26), kindly provided by Dr. Pamela Stanley, using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. The cells were then cultured for 24 h with a-minimum essential medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal bovine serum, and the medium was replaced with Opti-MEM medium (Invitrogen) supplemented with 40 μg/ml of l-proline and cultured cells for 24 h at 37 °C. We then concentrated the culture medium up to 200-fold using a Microcon YM-30 (Millipore Corp., Bedford, MA) and mixed that medium (1:1) with glycerol for storage at −20 °C. Protein production was confirmed by Western analysis using alkaline phosphatase-conjugated anti-T7 tag antibody (Novagen, Madison, WI) and the LumiPhos WB chemiluminescence solution (Pierce).

**KS Synthesis in Vitro**—As a starting substrate, we prepared the monosulfated trisaccharide carbohydrate 35SO4−6GlcNAcβ1–6Manα1–6Manα-octyl by treatment of the chemically synthesized trisaccharide GlcNAcβ1–6Manα1–6Manα-octyl with soluble CGn6ST enzyme as described (16), except the amount of the trisaccharide (2.5 nmol) and 35S-PAPS (10 μCi; PerkinElmer Life Sciences) was changed to maximize labeling efficiency (5–10 × 106 cpm/2.5 nmol of substrate). For enzymatic KS synthesis in vitro, we incubated a 50-μl reaction mixture containing 50 mM HEPES-NaOH, pH 7.2, 5 mM MnCl2, 1 mM 5′-AMP, 0.25 mM UDP-galactose (Sigma), 0.3 mM UDP-GlcNAc (Sigma), 100 μg/ml proline chloride, 1 mM PAPS (Sigma), 3 × 106 cpm of the starting substrate, and a mixture of concentrated medium of 0.5 μl of β4GalT4, 5 μl of β3GnT7, and 5 μl of CGn6ST with or without 4 μl of KSG6ST at 37 °C for 16 h. We then stopped the reaction by incubation in boiling water and removed insoluble debris by centrifugation before column chromatography. For additional KSG6ST treatment, the reaction mixture of β3GnT7/β4GalT4/CGn6ST was incubated in boiling water and incubated for 21 h at 37 °C after the addition of KSG6ST and PAPS.

**Column Chromatography**—To determine the size of reaction products, we performed gel filtration chromatography with a column (1.0-cm diameter × 120-cm length) of Bio-Gel P-4 (Bio-Rad) equilibrated with 100 mM ammonium acetate buffer, pH 6.8. The reaction mixture samples were applied to the column, and the eluate was collected in 1-ml fractions. The elution pattern of carbohydrate products was monitored by counting 35S radioactivity using a liquid scintillation counter. For further analyses, we pooled radioactive fractions, desalted them by Sephadex G25 gel filtration (Sigma) equilibrated with 7% (v/v) 1-propanol/water medium, and lyophilized the samples again.

To identify the degree of sulfation of products, we separated them by anion exchange HPLC using a Whatman Partisil SAX-10 column (4.6-mm diameter × 25-cm length). The column was first equilibrated with 60% acetonitrile/water, and then after loading a sample, the products were eluted under the following conditions: 60% acetonitrile/water for 5 min; a linear gradient from 60% acetonitrile/water to either 45 mM (up to tetrasulfated materials); or 70 mM (up to heptasulfated materials) KH2PO4 containing 60% acetonitrile/water for 75 min. The flow rate was 1 ml/min, 1-ml fractions were collected, and 35S radioactivity was monitored as described. To evaluate sulfation status, we prepared multi-sulfated carbohydrate standards by enzymatic reactions as described previously (16).
Enzymes Responsible for Corneal KS GAG Synthesis

Glycosidase-associated Carbohydrate Structure Determination—To determine nonreducing terminal structure of materials in P-4 gel filtration fractions, the purified materials were treated with glycosidases and analyzed as described above. For β-galactosidase and hexosaminidase A treatments, lyophilized samples were dissolved in water and incubated with either 25 milliunits of Jack bean β-galactosidase (Seikagaku Co., Tokyo, Japan) or 5 milliunits of human placental hexosaminidase A (Sigma) in a 30-μl mixture containing 50 mM sodium citrate buffer pH 3.5, at 37 °C for 5 min (β-galactosidase) or for 16 h (hexosaminidase A). The samples were then boiled for 5 min, and the digests were separated by column chromatography. For keratanase treatment, we incubated purified samples with 75 milliunits of keratanase from Pseudomonas sp. (Seikagaku Co.) in a 20-μl mixture containing 100 mM Tris-HCl, pH 7.5, at 37 °C for an hour, stopped the enzymatic reaction by boiling, and subjected samples to column chromatography.

KS Synthesis in Cultured Human Corneal Epithelial Cells—SV40-immortalized human corneal epithelial cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 50/50 mix medium (Mediatech, Inc., Herndon, VA) supplemented with 15% fetal bovine serum, 4.2 μg/ml of bovine insulin, 0.8 μg/ml of cholera toxin (Invitrogen), 8.3 μg/ml of mouse epidermal growth factor (Invitrogen) and 33 μg/ml gentamycin (Sigma). To produce sulfated KS, we co-transfected CGn6ST and KSG6ST expression vectors into 1 × 10^6 cells using Nucleofector electroporator (Axam Inc. Gaithersburg, MD) according to the manufacturer’s instruction and cultured cells in the above medium at 37 °C for 48 h. To analyze effects of glycosyltransferase expression on KS production, we co-transfected CGn6ST and KSG6ST expression vectors together with specific glycosyltransferase siRNAs (sequence information is listed in supplemental Table S1), which were obtained from Ambion (Austin, TX). As a negative control, we used a commercially available negative control siRNA (NC#2; Ambion).

Western Blot Analysis of Highly Sulfated KS—Transfected cells were washed with cold PBS five times, and lysates were prepared using 120 μl of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, a 1× proteinase inhibitor mixture (Sigma), and 1 mM phenylmethylsulfonyl fluoride. After SDS-PAGE, the proteins were electroblotted to an Immobilon-P transfer membrane (Millipore). The membranes were treated with blocking buffer (10% nonfat milk in PBS) at room temperature for 1 h and then probed with 5D4 anti-KS antibody (1:4000) (Seikagaku Co.) in blocking buffer for 1 h. The membranes were washed in 0.05% Tween 20 in PBS three times and reacted with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:4000) in blocking buffer for 1 h. After washing membranes in 0.05% Tween 20 in PBS three times, signals were detected using the ECL Plus reagent (GE Healthcare, Piscataway, NJ). Signal intensity was calculated using NIH Image 1.62 software.

Quantitative Reverse Transcription-PCR Analysis—Total RNA was isolated from transfected cells by RNeasy Mini kit (Qiagen) according to the manufacturer’s instruction. After DNase I treatment, we subjected samples to reverse transcription using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR analysis was carried out using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) with a specific primer set for each glycosyltransferase gene (see supplemental Table S2). Amplification of DNA products was monitored by the Mx 3000 QPCR system (Stratagene, La Jolla, CA) using the following reaction conditions: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 30 s.

RESULTS

GlcNAc-sulfated Poly-N-acetyllactosamine Production by β3GnT7, β4GalT4, and CGn6ST in Vitro—To analyze enzymatic activity of β3GnT7 and β4GalT4 for KS carbohydrate synthesis in vitro, we constructed expression vectors encoding soluble glycosyltransferases and the sulfotransferases, KSG6ST and CGn6ST, and transfected Lec20 cells, a cell line derived from Chinese hamster ovary cells, with vectors individually to secrete enzymes into the medium (Fig. 1). Because Lec20 cells do not produce endogenous β4GalT1 (26), most glycosyltransferase activity in the medium originates from the transfected expression vector (Fig. 1B). Enzymes prepared from the culture medium of mock-transfected Chinese hamster ovary cells but not in that of mock-transfected Lec20 cells.
medium were mixed in several combinations and incubated with a monosulfated trisaccharide substrate, \( ^{35} \text{SO}_3 \)-6GlcNAcβ1–6Man\( \alpha \)-1–6Man\( \alpha \)-octyl, and carbohydrate donor substrates, UDP-GlcNAc, UDP-Gal, and PAPS. After 16 h of incubation at 37 °C, the reaction products were analyzed by P-4 gel filtration column chromatography (Fig. 2). When the substrate was reacted with a mixture of \( \beta \)-3GnT7 and \( \beta \)-4GalT4 glycosyltransferases, two major products were detected as molecules larger than the substrate (Fig. 2A), indicating that the two enzymes can add carbohydrate onto the carbohydrate sub-
Enzymes Responsible for Corneal KS GAG Synthesis

the product was found to be a monosulfated carbohydrate (Fig. 3A). From these results, we conclude that the product was monosulfated tetrasaccharide Galβ1-4\(^{32}\)SO\(_4\)\(^{-}\)-GalNAcβ1-6Manα1-6Manα1-octyl.

We next analyzed the carbohydrate structure found in fraction II (Fig. 2B) using the same strategy. A chromatogram of fraction II on a P-4 gel filtration column showed a broader peak (Fig. 2D), but the pattern was converted to a sharper peak by β-galactosidase treatment (fraction II’ in Fig. 2D). Meanwhile, hexosaminidase A treatment, which hydrolyzes both nonsulfated GlcNAc and 6-O-sulfated GlcNAc located on nonreducing terminal of a carbohydrate substrate (27), converted constituents of fraction II into two components (II-1 and II-2 in Fig. 2D). Because the retention positions of fractions II’ and II-1 were very close but not identical and because both peaks overlapped with the original broad peak of fraction II (Fig. 2D), we conclude that fraction II contains a mixture of fractions II’ and II-1 and that the II-1 component is hydrolyzed by β-galactosidase and converted to component II’ (Fig. 2D). We also performed sequential digestion of fraction II components with β-galactosidase followed by hexosaminidase A and found that all products were converted to a single fraction having the same elution position as fraction II-2 and also

strate. When we incubated the substrate with a mixture of \(\beta3\)Gnt7, \(\beta4\)GalT4, and CGn6ST, we detected several products of much larger size (Fig. 2B) than products from a mixture lacking CGn6ST (Fig. 2A).

To identify the carbohydrate structure of the products, we collected products separately and analyzed them by glycosidase treatment followed by column chromatography. Fraction I, containing the most abundant product, was slightly larger than the starting substrate by P-4 gel filtration column chromatography, and its size was not altered by hexosaminidase A treatment (Fig. 2C). However, a component of fraction I was converted to a digested fraction with the same retention position as the starting substrate by β-galactosidase treatment (Fig. 2C). This result indicates that the product in fraction I was a tetrasaccharide carbohydrate resulting from addition of one galactose on the nonreducing terminal of the starting trisaccharide substrate by \(\beta4\)GalT4. This tetrasaccharide product was next analyzed by SAX-10 anion exchange chromatography, and fraction I (Fig. 2D). These results indicate that the carbohydrate backbone structure of fraction II is a mixture of a pentasaccharide, GlcNAcβ1-3Galβ1-4GlcNAcβ1-6Manα1-6Manα1-octyl (fraction II’), and a hexasaccharide, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6Manα1-6Manα1-octyl (fraction II-1), and that hexosaminidase A treatment converted the pentasaccharide product (fraction II’) into a tetrasaccharide carbohydrate, Galβ1-4GlcNAcβ1-6Manα1-6Manα1-Octyl (fraction II-2).

The sulfation status of components of II, II-1, and II-2 was analyzed by a SAX-10 column (Fig. 3, B–D). Fraction II was separated into two components, monosulfated material and disulfated material (Fig. 3B). Fraction II-1 was also separated into two components, both of which eluted at the same positions as fraction II, but the proportion of mono- to disulfated materials in fraction II-1 was nearly 1:1 (Fig. 3C). Because increased sulfation was apparently due to the presence of CGn6ST, disulfated components found in fractions II and II-1
were sulfated on GlcNAc in the carbohydrate products. Fraction II-2, which was originally a pentasaccharide component in fraction II and was converted to a tetrasaccharide by hexosaminidase A, was identified as a single component of mono- and disulfated carbohydrate (Fig. 3D). However, because hexosaminidase A hydrolyzes the nonreducing terminal GlcNAc both with or without sulfate modification, the degree of sulfation of fraction II-2 does not represent the sulfation status of pentasaccharide components originally found in fraction II. Thus, we estimated the presence of disulfated pentasaccharide components and calculated the amount of mono- and disulfated pentasaccharide components in fraction II (see supplemental Tables S3–5). From these results, we conclude that fraction II consists of four molecules: mono- and disulfated hexasaccharide, Galβ1–4(±SO3–6)GlcNAcβ1–3Galβ1–4(±SO3–6)GlcNAcβ1–6Manα1–6Manα1-octyl, and mono- and disulfated pentasaccharide, ±SO3–6GlcNAcβ1–3Galβ1–4(±SO3–6)GlcNAcβ1–6Manα1–6Manα1-octyl.

Similar to fraction II, components of fractions III and IV were separated into two fractions by hexosaminidase A treatment (Fig. 2E and F), indicating that each fraction contains carbohydrates of two different lengths, with or without exposed GlcNAc at the nonreducing terminal. Similar to what was seen in fraction II, sequential treatment of the carbohydrate components in fraction III with β-galactosidase and hexosaminidase A converted the two carbohydrates into a single component exhibiting a sharp peak with a retention position identical to fraction II-1 on the P-4 chromatogram (Fig. 2E). This result indicates that carbohydrate components of fraction III are hepta- and octasaccharides, both of which are elongated products of fraction II-1 carbohydrates, to which either Galβ1–4GlcNAcβ1- or GlcNAcβ1- structures have been added onto the fraction II-1 carbohydrate backbone. These results suggest that all observed carbohydrate products were made of an incomplete poly-N-acetyllactosamine backbone consisting of repeating N-acetyllactosamine units, with or without galactose on the nonreducing terminal on the starting substrate. We therefore estimated that fraction IV consisted of nona- and decasaccharide carbohydrates made of poly-N-acetyllactosamine repeats attached to the starting substrate.

The sulfation status of carbohydrates in fractions III and IV was again analyzed by SAX-10 HPLC before and after hexosaminidase A treatment (Fig. 3, E–F). By this analysis, we observed multi-sulfated carbohydrate products in the fractions up to tetrasulfated status (Fig. 3, H and I). Because we conclude that fraction III contains hepta- and octasaccharides, both of which carry 3 GlcNAc in their carbohydrate backbones, and because we included CgMan6ST in the reaction mixture, the product should be trisulfated carbohydrate if the molecules were fully sulfated. By SAX-10 HPLC analysis, however, we found that more than 50% of the products were mono- and disulfated products in fraction III (Fig. 3E), indicating that the products were not fully sulfated on their GlcNAc residues. To understand the sulfation pattern of undersulfated carbohydrate products, we treated components of fraction III with keratanase, which recognizes GlcNAc-sulfated disaccharide SO3–6GlcNAcβ1–3Gal linked to GlcNAc (with or without sulfation) via a β1–4 linkage and hydrolyzes that linkage (Fig. 4A). Thus, when a nonradioactive sulfate is attached to GlcNAc located close to ±SO3–6GlcNAc, the molecule will be hydrolyzed and converted to radiolabeled trisaccharide. If nonradioactive sulfate is attached to GlcNAc located close to nonreducing terminal, the molecule will be converted to pentasaccharide (Fig. 4A). Following keratanase treatment, almost all fraction III components were converted to a trisaccharide component (Fig. 4B). The sulfation status of components of fraction III, which was

![Figure 4](image-url)
TABLE 1
Determined carbohydrate structures and their molar ratio in GlcNAc-sulfated poly-N-acetyllactosamine product synthesized by a mixture of β3GnT7/β4GalT4/CGn6ST in vitro

- Galactose; ■ GlcNAc; ○ Mannose; *, actual components found in these fractions are the illustrated structures without nonreducing terminal GlcNAc, because of hexosaminidase A treatment. **, these structures are estimated from the results of keratanase treated disulfated hepta/octasaccharide structures.

| Fraction in Fig. 2 | Determined structures | Molar ratio (%) |
|--------------------|-----------------------|-----------------|
| before hexosaminidase A | after hexosaminidase A |                  |
| I                  | -                     | 82.2            |
| II                 | (II-2)*               | 6.05            |
|                    |                       | 0.440           |
| II-1               |                       | 2.73            |
| III                | (III-2)*              | 0.297           |
|                    |                       | 1.08            |
|                    |                       | 0               |
| III-1              |                       | 0.411           |
| IV                 | (IV-2)*               | 1.25            |
|                    |                       | 0.077           |
|                    |                       | 0.512           |
|                    |                       | 0.080           |
| IV-1               |                       | 0.239           |
|                    |                       | 0.443           |
Enzymes Responsible for Corneal KS GAG Synthesis

FIGURE 5. Average efficiency of GlcNAc-sulfated poly-N-acetyllactosamine synthesis in vitro. Percentages in boxes indicate averages of the reaction efficiencies at each step of the enzymatic reaction starting from monosulfated pentasaccharide product to tetrasulfated decasaccharide product (see supplemental Fig. S1).

originally identified as a mixture of mono-, di-, and trisulfated carbohydrates, was also identified as a monosulfated product following keratanase treatment, indicating that carbohydrates in fraction III were sulfated on the internal GlcNAc located closest to $^{35}$SO$_3^-$-GlcNAc. This result suggests that GlcNAc sulfation of carbohydrate products by a mixture of $\beta$3GnT7/$\beta$4GalT4/CGn6ST was consecutively but not randomly occurring during elongation of carbohydrate backbone. Thus, we determined the carbohydrate structure of the products synthesized by the three enzymes $\beta$3GnT7, $\beta$4GalT4, and CGn6ST in Table 1. Based on the result, we illustrated synthetic flow of carbohydrate products generated by the three enzymes and calculated efficiencies of each enzymatic reaction step (supplemental Fig. S1). We also calculated average reaction efficiency for each enzymatic reaction that repeatedly takes place by the same enzyme for production of the same nonreducing terminal structure during GlcNAc-sulfated poly-N-acetyllactosamine chain production (Fig. 5).

Highly Sulfated KS Production by $\beta$3GnT7, $\beta$4GalT4, CGn6ST, and KSG6ST in Vitro—Corneal KS GAG is highly sulfated on both GlcNAc and galactose residues (10–12). Sulfation of galactose residues is catalyzed by KSG6ST (15). From its substrate specificity, sulfation of galactose by KSG6ST likely occurs after production of GlcNAc-sulfated poly-N-acetyllactosamine GAG by CGn6ST and glycosyltransferases (11, 16, 23). Using soluble enzymes including KSG6ST, we tested production of highly sulfated KS GAG in vitro. The reaction products of a mixture of $\beta$3GnT7, $\beta$4GalT4, and CGn6ST included elongated carbohydrates of several lengths (Fig. 6A), and one fraction containing a mixture of nona/decasaccharides was identified to have mostly tri- and tetrasulfated structures (Fig. 6E). When a mixture of $\beta$3GnT7/$\beta$4GalT4/CGn6ST products was further treated by KSG6ST, the degree of sulfation of the nona/decasaccharide fraction was increased up to heptasulfated products (Fig. 6F), indicating that GlcNAc-sulfated poly-N-acetyllactosamine carbohydrates were utilized and converted to highly sulfated KS carbohydrate following KSG6ST treatment. When a trisaccharide carbohydrate substrate was treated with all four enzymes ($\beta$3GnT7, $\beta$4GalT4, CGn6ST, and KSG6ST), elongation efficiency was equivalent to that found in a reaction of three enzymes (Fig. 6, A and C). The products were also sulfated up to hexasulfated components (Fig. 6G), which was a higher degree of sulfation than that seen in the $\beta$3GnT7/$\beta$4GalT4/CGn6ST mixture (Fig. 6F) but lower than that seen in a two-step reaction with $\beta$3GnT7/$\beta$4GalT4/CGn6ST followed by KSG6ST (Fig. 6F). Interestingly, treatment of a trisaccharide substrate with a mixture of KSG6ST and $\beta$3GnT7 and $\beta$4GalT4 glycosyltransferases elongated the products up to nona/decasaccharides, and these products were also sulfated up to tetrasulfated components (Fig. 6H). Because treatment of the substrate with two glycosyltransferases without any sulfotransferase only produced up to penta/hexasaccharide carbohydrates (Fig. 2A), this result indicates that KSG6ST enhanced elongation of the poly-N-acetyllactosamine backbone by the glycosyltransferases. From these results, we conclude that four enzymes: $\beta$3GnT7, $\beta$4GalT4, CGn6ST, and KSG6ST, are sufficient to synthesize highly sulfated KS carbohydrate in vitro.

$\beta$3GnT7, $\beta$4GalT4, CGn6ST, and KSG6ST Are Required for Sulfated KS Production in Cultured Corneal Cells—We next analyzed production of KS GAG in cultured corneal cells. Under normal culture conditions, SV40-transformed human corneal epithelial cells do not produce detectable levels of highly sulfated KS GAG, which is recognized by the 5D4 monoclonal antibody (28, 29). However, the corneal cells began producing 5D4-positive, highly sulfated KS GAG, which is recognized by the 5D4 monoclonal antibody (28, 29). However, the corneal cells began producing 5D4-positive, highly sulfated KS carbohydrate when CGn6ST and KSG6ST were overexpressed (Fig. 7, A and D), and this was a consistent result with our previous observation of KS production in HeLa cells (25). These findings indicate that both sulfotransferases are required to produce highly sulfated KS carbohydrate. Human genes $\beta$3GnT7 and $\beta$4GalT4 encode $\beta$3GnT7 and $\beta$4GalT4, respectively, and corneal epithelial cells endogenously express both genes (Fig. 7, G and H).$^4$

When we suppressed $\beta$3GnT7 expression in the cultured corneal epithelial cells by transfection of specific siRNAs, 5D4-positive KS production was dramatically reduced to less than 10% of levels seen in cells transfected with negative control siRNA; even both CGn6ST and KSG6ST were overexpressed (Fig. 7, D and G). Similarly, we observed a 50% reduction of 5D4-positive product in the cells using $\beta$4GalT4 siRNAs (Fig. 7, E and H). Because siRNAs for other glycosyltransferases,

$^4$ K. Kitayama and T. O. Akama, unpublished data.
Enzymes Responsible for Corneal KS GAG Synthesis

such as β1,3-N-acetylgalactosaminyltransferase-2 (β3GnT2) and β1,4-galactosyltransferase-1 (β4GalT1), both of which are likely major contributors to poly-N-acetyllactosamine elongation, did not have a significant effect on production of highly sulfated KS production in cells (Fig. 7, F and I), we conclude that both β3GnT7 and β4GalT4 are required for elongation of the KS carbohydrate backbone in human corneal cells.

DISCUSSION

In this study, we demonstrate that β3GnT7 and β4GalT4 are required for KS GAG production in vitro and in cultured human corneal cells. β3GnT7 was originally identified as a gene involved in tumor invasiveness and later as a member of the β3GnT family (30). β4GalT4 was identified as a member of β4GalT family by expressed sequence tag data base searches (31) and suggested to function in neolacto-series glycosphingolipid biosynthesis (32). Seko et al. (22) reported β4GalT activity that acts preferentially on sulfated substrates in human colorectal mucosa and later found that the enzyme responsible for that activity was β4GalT4. Using in vitro analysis, they suggested that among seven enzymes of the β4GalT family, β4GalT4 functions in biosynthesis of sulfo siaul Lewis X structure as well as in production of KS GAG (22). They also analyzed enzymatic activity of a COS-7 cell membrane fraction containing overexpressed β3GnTs using several carbohydrate substrates and found that β3GnT7 has higher enzymatic activity for sulfated N-acetyllactosamine substrates than for nonsulfated substrates, suggesting that β3GnT7 functions in KS biosynthesis (23). Their studies suggested that four enzymes, β3GnT7, β4GalT4, CGn6ST, and KSG6ST, could produce sulfated KS GAG in vitro (23), but such an observation has not been reported until this study.

KS GAG biosynthesis apparently occurs in two stages: the production of GlcNAc-sulfated poly-N-acetyllactosamine chain by β3GnT7, β4GalT4, and CnGn6ST and the production of highly sulfated KS by sulfation of galactose by KSG6ST (11, 16, 22, 23). CnGn6ST transfers sulfate only on the nonreducing terminal GlcNAc (16); therefore, sulfation of GlcNAc residues must be coupled to elongation of the carbohydrate backbone processed by glycosyltransferases. On the other hand, KSG6ST has much higher sulfation activity on substrates containing N-acetyllactosamine disaccharide with sulfate on GlcNAc (15, 17), suggesting that sulfation of galactose occurs after production of the GlcNAc-sulfated poly-N-acetyllactosamine chain. Furthermore, because β3GnT7 has very weak glycosyltransferase activity on a sulfated substrate when the nonreducing terminal galactose is sulfated (23), sulfation of the nonreducing terminal galactose by KSG6ST may inhibit elongation of the KS GAG backbone. However, when we reacted a sulfated carbohydrate substrate with all four enzymes including KSG6ST, elongation of carbohydrate products was not significantly inhibited (Fig. 6C), although the degree of product sulfation was not as high as that seen with β3GnT7/β4GalT4/CnGn6ST treatment followed by KSG6ST reaction (Fig. 6, F and G). This result indicates that production of a GlcNAc-sulfated poly-N-acetyllactosamine chain is not inhibited by the presence of KSG6ST. Surprisingly, when we reacted the substrate with three enzymes (β3GnT7, β4GalT4, and KSG6ST) without CnGn6ST, we observed elongated carbohydrates, which were not seen in a reaction of two glycosyltransferases with no sulfotransferase (Figs. 2A and 6D). This unanticipated result can be explained as follows. Firstly, elongation of the carbohydrate chain by β3GnT7 and β4GalT4 is not coupled to sulfation of galactose by KSG6ST because the nonreducing terminal galactose is a less favored substrate for KSG6ST (17). Second, nonsulfated carbohydrate products made of repeating disaccharides of Galβ1–4GlcNAc are less hydrophilic and therefore less efficient substrates for continuous chain elongation by glycosyltransferases because of poor solubility. Indeed, production of nonsulfated or less sulfated carbohydrate may be a primary cause of corneal deposit formation of macular corneal dystrophy patients (33). Thus, when KSG6ST is present during carbohydrate synthesis by β3GnT7 and β4GalT4, elongated carbohydrates are occasionally but not cooperatively sulfated on galactose residues by sulfotransferase, such that sulfated products become more hydrophilic and are utilized for further elongation by glycosyltransferases. Taken together, we conclude that highly sulfated KS is mainly produced by β3GnT7, β4GalT4, CnGn6ST, and KSG6ST and that biosynthesis of carbohydrate occurs in two steps: production of GlcNAc-sulfated poly-N-acetyllactosamine structure followed by sulfation of galactose residues. Because natural corneal KS GAG chains are efficiently elongated more than 10 sulfated N-acetyllactosamine repeats (11, 12, 34), we hypothesize that in KS GAG-producing cells, β3GnT7, β4GalT4, and CnGn6ST are tightly associated or closely co-localized with each other to achieve efficient production of GlcNAc-sulfated poly-N-acetyllactosamine chains in the Golgi, whereas KSG6ST may be localized elsewhere or not tightly associated with the other enzymes. Further analysis of cellular localization or biochemical examination such as immunoprecipitation is required to test this hypothesis.

A mixture of β3GnT7, β4GalT4, and CnGn6ST produced GlcNAc-sulfated poly-N-acetyllactosamine carbohydrate in vitro. We confirmed production of up to a tetrasulfated decasaccharide (Table 1); however, the products clearly contained longer and more sulfated carbohydrates, because P-4 column chromatography detected peaks that likely contain larger molecules (Fig. 2B). Although long carbohydrate products were made by a simple reaction with a three-enzyme mixture, the length of products was much less than that of corneal KS GAG (11, 12), indicating that reaction conditions of this study were not as high as that in vivo. Further optimization, such as altering the ratio of the three enzymes or the concentration of donor molecules may improve the efficiency of KS carbohydrate production in vitro. In our reaction conditions, although galactosylation of the nonreducing terminal GlcNAc by β4GalT4 was more than 90%, the efficiencies of the other two reactions were about 50% (Fig. 5), indicating that reaction conditions of these steps must be improved to generate longer carbohydrate products. However, the most inefficient step of KS GAG production in vitro was the first step of β3GnT7 reaction, which transfers GlcNAc to the nonreducing terminal galactose of monosulfated tetrasaccharide carbohydrate. Because 82.2% of products were remained as Galβ1–4(SO3-)6GlcNAcβ1–6Manα1–6Manα1-octyl (Table 1), the effi-
Enzymes Responsible for Corneal KS GAG Synthesis

A

\[ \text{Fraction number} \]

\[ 20 \ 25 \ 30 \ 35 \ 40 \ 45 \ 50 \ 55 \ 60 \ 65 \ 70 \ 75 \ 80 \]

\[ \text{\( ^{35} \text{S-radioactivity (cpm)} \)} \]

B

\[ \text{Fraction number} \]

\[ 20 \ 25 \ 30 \ 35 \ 40 \ 45 \ 50 \ 55 \ 60 \ 65 \ 70 \ 75 \ 80 \]

\[ \text{\( ^{35} \text{S-radioactivity (cpm)} \)} \]

C

\[ \text{Fraction number} \]

\[ 20 \ 25 \ 30 \ 35 \ 40 \ 45 \ 50 \ 55 \ 60 \ 65 \ 70 \ 75 \ 80 \]

\[ \text{\( ^{35} \text{S-radioactivity (cpm)} \)} \]

D

\[ \text{Fraction number} \]

\[ 20 \ 25 \ 30 \ 35 \ 40 \ 45 \ 50 \ 55 \ 60 \ 65 \ 70 \ 75 \ 80 \]

\[ \text{\( ^{35} \text{S-radioactivity (cpm)} \)} \]

E

\[ \text{Fraction number} \]

\[ 0 \ 10 \ 20 \ 30 \ 40 \ 50 \ 60 \ 70 \ 80 \]

\[ \text{\( ^{35} \text{S-radioactivity (cpm)} \)} \]

F

\[ \text{Fraction number} \]

\[ 0 \ 10 \ 20 \ 30 \ 40 \ 50 \ 60 \ 70 \ 80 \]

\[ \text{\( ^{35} \text{S-radioactivity (cpm)} \)} \]

G

\[ \text{Fraction number} \]

\[ 0 \ 10 \ 20 \ 30 \ 40 \ 50 \ 60 \ 70 \ 80 \]

\[ \text{\( ^{35} \text{S-radioactivity (cpm)} \)} \]

H

\[ \text{Fraction number} \]

\[ 0 \ 10 \ 20 \ 30 \ 40 \ 50 \ 60 \ 70 \ 80 \]

\[ \text{\( ^{35} \text{S-radioactivity (cpm)} \)} \]
ciency of GlcNAc transfer at this step was only 17.8%, which was one-third the average efficiency of β3GnT7 enzyme observed throughout sulfation-coupled poly-
N-acetyllactosamine elongation (Fig. 5), suggesting that the tetrasaccha-
dride, Galβ1–4(SO3-6)GlcNAcβ1–6Manα1–6Manα1-octyl, may not be a suitable β3GnT7 substrate. A commonly recognized carbohydrate structure of N-linked corneal KS consists of an extended linear KS GAG chain attached to a complex-type N-glycan core structure via one or two repeats of nonsulfated N-acetyllactosamine disaccharides (11, 12, 34). β3GnT7 may have less activity on galactose located close to the N-glycan core structure, and formation of N-acetyllactosamine repeats immediately adjacent to the N-glycan core may be cata-
yzed by other glycosyltrans-
ferases. Indeed, among β3GnT family proteins, β3GnT7 shows the lowest activity on a nonsulfated N-acetyllactosamine connected to a complex-type N-glycan core (23), supporting this idea. During continu-
ous sulfation-coupled carbohy-
derate elongation, we observed that both β3GnT7 and β4GalT4 showed at least 4-fold higher activity toward an N-acetyllactosamine structure having sulfated GlcNAc at the position closest to the nonreduc-
ting terminal than toward a termi-
nal structure without sulfate (Fig. 5). This finding is consistent with substrate specificity of these enzymes observed in vitro (22, 23) and strongly suggests that β3GnT7 and β4GalT4 are responsible for KS GAG production. We also observed that there was no carbohydrate product exhibiting more than a tet-
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without sulfotransferase (Fig. 2A), suggesting that a carbohydrate molecule with a nonsulfated tetrasaccharide at the non-reducing terminal is not utilized as a glycosyltransferase substrate. Presently, however, it is unclear whether this is due to lower hydrophilicity of the carbohydrate molecule or less preferable substrate structure.

Decreasing β3GnT7 mRNA levels dramatically reduced sulfated KS production to less than one-tenth of controls in cultured human corneal epithelial cells (Fig. 7D). Reduced β4GalT4 mRNA also inhibited sulfated KS production but less effectively than suppression of β3GnT7 mRNA, although in both cases siRNAs suppressed mRNA expression more than 70% (Fig. 7, G and H). siRNAs specific for β3GnT2 and for β4GalT1 did not significantly suppress sulfated KS production, indicating that reduction of sulfated KS production in cultured corneal cells was due to specific suppression of β3GnT7 or β4GalT4 by corresponding siRNAs. The weaker effect of β4GalT4 siRNAs may be because β4GalT4 has much higher enzymatic activity for elongation of KS GAG chains than β3GnT7; thus, the remaining β4GalT4 mRNA may account for β4GalT4 activity in producing sulfated KS. Further investigation is required to eliminate the possibility that other β4GalT enzymes compensate for β4GalT4 in sulfated KS production.

In summary, we have detected production of sulfated KS by four enzymes, β3GnT7, β4GalT4, C6Gn6ST, and KSG6ST, in vitro and in cultured cells. Because specific suppression of either β3GnT7 or β4GalT4 reduced sulfated KS production in cultured corneal cells, we conclude that β3GnT7 and β4GalT4 play major roles in elongating sulfated KS in the cornea. Further characterization of the glycosyltransferases and the sulfotransferrases in producing sulfated KS may establish roles for the carbohydrate chain for corneal extracellular matrix organization.

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