Enzymatic Synthesis in Vitro of the Disulfated Disaccharide Unit of Corneal Keratan Sulfate

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Among the enzymes of the carbohydrate sulfotransferase family, human corneal GlcNAc 6-O-sulfotransferase (hCGn6ST, also known as human GlcNAc6ST-5/GST4β) and human intestinal GlcNAc 6-O-sulfotransferase (hIGn6ST or human GlcNAc6ST-3/GST4α) are highly homologous. In the mouse, intestinal GlcNAc 6-O-sulfotransferase (mIGn6ST or mouse GlcNAc6ST-3/GST4) is the only orthologue of hCGn6ST and hIGn6ST. In the previous study, we found that hCGn6ST and mIGn6ST, but not hIGn6ST, have sulfotransferase activity to produce keratan sulfate (Akama, T. O., Nakayama, J., Nishida, K., Hiraoka, N., Suzuki, M., McAuliffe, J., Hindsgaul, O., Fukuda, M., and Fukuda, M. N. (2001) J. Biol. Chem. 276, 16271–16278). In this study, we analyzed the substrate specificities of these sulfotransferases in vitro using synthetic carbohydrate substrates. We found that all three sulfotransferases can transfer sulfate to the nonreducing terminal GlcNAc of short carbohydrate substrates. Both hCGn6ST and mIGn6ST, but not hIGn6ST, transfer sulfate to longer carbohydrate substrates that have poly-N-acetyllactosamine structures, suggesting the involvement of hCGn6ST and mIGn6ST in production of keratan sulfate. To clarify further the involvement of hCGn6ST in biosynthesis of keratan sulfate, we reconstituted the biosynthetic pathway in vitro by sequential enzymatic treatment of a synthetic carbohydrate substrate. Using four enzymes, β1,4-galactosyltransferase-I, β1,3-N-acetylgalactosaminyltransferase-2, hCGn6ST, and keratan sulfate Gal 6-O-sulfotransferase, we were able to synthesize in vitro a product that conformed to the basic structural unit of keratan sulfate. Based on these results, we propose a biosynthetic pathway for N-linked keratan sulfate on corneal proteoglycans.

Keratan sulfate proteoglycan is a major component of the corneal stroma. Keratan sulfate proteoglycan is thought to play an important role in maintaining corneal transparency by organizing and providing proper hydration of the extracellular matrix of the corneal stroma. Lumican, keratocan, and minecan have been identified as carriers of keratan sulfate glycosaminoglycans (GAGs) in an N-linked manner (1–5). The expression of these core proteins is regulated during developmental stages and wound healing of the cornea (4–7), suggesting an involvement of keratan sulfate proteoglycans in the reconstruction of the cornea. Studies of the lumican gene knockout mouse revealed the importance of this proteoglycan in organizing regular spacing and fibrillogenesis of collagen in the corneal stroma (8–10). Biochemical and electron microscopic analyses indicated that the protein moiety of lumican interacts with collagen, whereas keratan sulfate GAGs attached to lumican provide the water retention capability necessary for interfibrillar spacing (11, 12).

Keratan sulfate GAG is composed of repeating disaccharide units of (-3Galβ1–4GlcNAcβ1–), which is called poly-N-acetyllactosamine, with sulfate residues at the 6-O-position of GlcNAc and Gal (13, 14). Keratan sulfate GAG chain is also modified with fucose and sialic acid at GlcNAc and at the nonreducing terminus, respectively (15–18). Besides the cornea, keratan sulfate carbohydrate is also found in other tissues such as cartilage. Corneal keratan sulfates are highly sulfated long carbohydrates, which link to their core proteins via N-linkages. More than half of the total sulfated disaccharide units of keratan sulfate GAG are monosulfated disaccharide (-3Galβ1–4SO3−/H9252GlcNAcβ1–), and a majority of others are disulfated disaccharide units (-3SO3−/H9252Galβ1–4SO3−/H9252GlcNAcβ1–) (18, 19). Distribution of sulfate residues in a corneal keratan sulfate chain is not uniform. On the basis of analysis of keratanase digestion products, it was postulated (20) that chains are composed of a region of nonsulfated disaccharides near the linkage to protein, a central region of monosulfated disaccharides, and a stretch of disulfated disaccharides near the nonreducing terminus. Variety of the capping structure at the nonreducing terminus of corneal keratan sulfate is also reported (17, 19). By contrast, cartilage keratan sulfates have a shorter carbohydrate backbone than corneal keratan sulfate (13, 14). Cartilage keratan sulfates link to their core protein via O-linkages at serine or threonine residues. Fucosylation and siaylation ratios are higher in cartilage keratan sulfate than in corneal keratan sulfate (18). Because sulfation of carbohydrate greatly contributes to its hydrophilicity, the degree of sulfation of corneal GAGs is likely to affect corneal transparency by changing the water retention capacity of the corneal stroma. Indeed, several studies have reported the relationship between sulfation of keratan sulfate and the acquisition of corneal transparency during development (6, 21–24).

Four enzymes are responsible for production of keratan human corneal GlcNAc 6-O-sulfotransferase; MCD, macular corneal dystrophy; hIGn6ST, human intestinal GlcNAc 6-O-sulfotransferase; mIGn6ST, mouse intestinal GlcNAc 6-O-sulfotransferase; β1,3-N-acetylgalactosaminyltransferase-2; KSG6ST, keratan sulfate Gal 6-O-sulfotransferase; C2GnTI, core-2 N-acetylgalactosaminyltransferase-I; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; GlcNAc6ST-1, GlcNAc 6-O-sulfotransferase-I; HPLC, high pressure liquid chromatography; CHO, Chinese hamster ovary.
In Vitro Synthesis of Keratan Sulfate Disaccharide Units

sulfate carbohydrate: β1,3-N-acetylglucosaminyltransferase, β1,4-galactosyltransferase, GlcNAc 6-O-sulfotransferase and Gal 6-O-sulfotransferase. Two glycosyltransferases, both of which transfer GlcNAc or Gal to a nonreducing terminus of a carbohydrate core structure connected to carrier proteins such as lumican, are involved in elongation of the poly-N-acetyllactosamine backbone of keratan sulfate. The other two enzymes, which transfer sulfate to the 6-O-position of GlcNAc or Gal, are involved in the modification of poly-N-acetyllactosamine required to form keratan sulfate composed of both mono- and disulfated disaccharides. Previous reports have proposed a putative biosynthetic pathway of keratan sulfate carbohydrate (14, 20, 25, 26); however, no studies have firmly established such a pathway.

Previously, we reported that human corneal GlcNAc 6-O-sulfotransferase (hCGn6ST, also known as GlcNAc6ST-5 and GST4b), which is encoded by CHST7, is involved in production of corneal keratan sulfate and that lack of hCGn6ST activity in corneal cells results in a hereditary eye disease, macular corneal dystrophy (MCD) (27, 28). In human, there is a second homologous sulfotransferase called human intestinal GlcNAc 6-O-sulfotransferase (hIGn6ST or human GlcNAc6ST-3/GST4x), which is encoded by CHST5 (29). CHST5 is highly homologous to CHST7 in both coding and noncoding regions and is located next to CHST7 on human chromosome 16q22 (27, 30). Due to their homology, these two genes are probably the result of gene duplication during evolution. Unlike CHST7 and CHST5 in humans, the mouse genome only has one sulfotransferase gene, designated Chst5, which encodes mouse intestinal GlcNAc 6-O-sulfotransferase (mIGn6ST or mouse GlcNAc6ST-3/GST4b) and is an orthologue of human CHST5 and CHST7 (30).

The fact that all three enzymes, hCGn6ST, hIGn6ST, and mIGn6ST, are highly homologous to each other suggests that they have similar biological function (30). However, in previous studies, we expressed each of these sulfotransferases in mammalian cells and found that cells expressing hCGn6ST and mIGn6ST, but not hIGn6ST, produce a carbohydrate structure that is recognized by an anti-keratan sulfate antibody (28). These observations strongly suggest that despite of the apparent structural homology of these sulfotransferases, hCGn6ST and mIGn6ST exhibit activities functionally distinct from that of hIGn6ST.

In this study, we analyzed the substrate specificity of hCGn6ST, hIGn6ST, and mIGn6ST in vitro using synthetic oligosaccharide substrates and found that hCGn6ST and mIGn6ST can transfer sulfate to longer oligosaccharide substrates with a poly-N-acetyllactosamine backbone. We also found that the keratan sulfate disulfate disaccharide unit can be synthesized by sequential enzymatic reactions of β1,4-galactosyltransferase-1 (β4Gal-T1), β1,3-N-acetylgalactosaminyltransferase-2 (β3Gn-T2), hCGn6ST, and keratan sulfate Gal 6-O-sulfotransferase (KSG6ST) in vitro. These results establish a biosynthetic pathway for the mono- and disulfated disaccharide sequences in corneal keratan sulfate.

**Experimental Procedures**

Construction of Expression Vectors for Sulfotransferases and a Glycosyltransferase—Mammalian expression vectors each encoding a full-length hCGn6ST, hIGn6ST, and mIGn6ST have been described previously (29). In this study, we analyzed the substrate specificity of hCGn6ST and found that hCGn6ST and mIGn6ST have been described previously (28). Expression vectors for CD34-IgG chimeric protein (pcDM8-CD34-IgG) (31) and for core-2 GlcNAc transferase-I (pcDNAI-C2GnTI) (32) were kindly provided by Dr. Minoru Fukuda (The Burnham Institute).

An expression vector designated pcDNA3.1-HSH, which consists of a DNA sequence encoding a cleavable signal sequence, polyhistidine, and enterokinase cleavage sequence at the multicloning site of pcDNA3.1/Hygro (Invitrogen) (33), was used to prepare soluble enzymes for this study. A DNA fragment encoding the catalytic domain of each sulfotransferase was amplified by PCR using a specific primer (see below) and the SP6 primer (Invitrogen) from the expression vector encoding the full-length cDNA (28). The amplified DNA fragment was digested by BglII and XbaI and inserted into the BamHI-XbaI site of pcDNA3.1-HSH. Primer sequences used for amplifying the catalytic domain of each sulfotransferase are as follows: for hCGn6ST, 5'-CAACAGGATCCATGTGGGACCATCGGCAACGCCAACAACCAGCCTGCTGCTGCTGCCCAACTTTGCACTGCTTCTTCGACTAGATCTGCCAG-TATTACCAATACACCTTTCT-3'; for hIGn6ST, 5'-CGAGGATCCCTCTACCTTGGGATGAT-3'; and 5'-GAATCTGCACAGGGATTAACACACTAAATACACCTTTCT-3' from human cornea cDNA (provided by Dr. Kohji Nishida, Department of Ophthalmology, Osaka University School of Medicine, Japan) and digested by BamHI and SalI. The digested product was cloned into the BamHI-XbaI site of pcDNA3.1-HSH.

An expression vector encoding soluble βGn-T2 was prepared as follows. A DNA fragment encoding the catalytic domain of βGn-T2 (34) was amplified by PCR using the primer pair 5'-CAAGGATCCGCTTCT-GAGAAGGAGACTACTCACCTCCCGC-3' and 5'-GAATCTGCACAGGGATTAACACACTAAATACACCTTTCT-3'. The amplified fragment was inserted by the TA cloning method (35) into the blunted EcoRI site of pcDNA3.1-A, which encodes a cleavable signal sequence followed by the IgG binding domain of Protein A (36).

A bacterial expression vector encoding hCGn6ST was prepared as follows. A DNA fragment encoding a polyhistidine tag and the enterokinase-cleavable sequence followed by the catalytic domain of hCGn6ST was prepared from pcDNA3.1-HSH-hCGn6ST (28) by NcoI-DraI digestion. The digested fragment was purified and inserted into the NcoI-blunted XhoI site of pET28a (+) (Novagen, Madison, WI). Metabolic Labeling and Carbohydrate Analysis of CHO Cells Transfected with Expression Vectors—An expression vector encoding each full-length sulfotransferase was co-transfected with pcDM8-CD34-IgG and with or without pcDNA1-C2GnTI into CHO cells using the LipofectAMINE PLUS reagent (BD Biosciences CLONTECH, Palo Alto, CA). After culturing the cells for 24 h in an minimal essential medium (Irvine Scientific, Santa Ana, CA) with 10% fetal calf serum, the medium was replaced with S-minimal essential medium (Invitrogen) including 10% dialyzed fetal calf serum and 0.1 mM [35S]sodium sulfate (PerkinElmer Life Sciences). Following incubation for 24 h, secreted CD34-IgG chimeric protein was isolated from the medium by Protein A-Sepharose beads (Sigma). The amount of isolated CD34-IgG from each transfecant was determined by Western blot analysis using monoclonal anti-human IgG-histidine-conjugated anti-human IgG antibody. Equal amounts of [35S]sulfate-labeled CD34-IgG, which were produced by transfected cells, were subjected to SDS-PAGE followed by fluorography.

To remove N-linked glycans from isolated CD34-IgG, the protein was precipitated once with acetone and dissolved in 1× sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 1.4 mM EDTA, pH 8.0, 40 mM dithiothreitol (DTT) (sodium dithiothreitol)). After adjusting the concentration of CD34-IgG to 1.25 µl of the solution was treated with 1 unit of N-glycosidase F (Roche Diagnostics) in 25-µl reactions including 50 mM NaPO₄, pH 7.5, and 0.75% Triton X-100. Following incubation for 3 h at 37 °C, the sample was subjected to SDS-PAGE followed by fluorography.

Preparation of Soluble Enzymes—Concentrated cultured medium from transfected HeLa cells was used as an enzyme source of sulfotransferases and N-acetylgalactosaminyltransferase. An expression vector for soluble enzyme was transfected into HeLa cells as described above. After culturing transfected cells for 48 h in an minimal essential medium with 10% fetal calf serum, the medium was replaced with OPTI-MEM (Invitrogen) and incubated for 24 h at 37 °C. The medium was recovered and concentrated by Microcon YM-30 (Millipore Corp., Bedford, MA). After the addition of an equal volume of glycerol, the concentrated medium was stored at −20 °C. To measure the quantity of soluble polyhistidine-tagged sulfotransferase in the concentrated medium, polyhistidine-tagged hCGn6ST was prepared in a large quantity from a bacterial culture, purified, and used as a standard. Briefly, competent BL21(DE3) bacteria (Novagen) were transformed with the expression vector, pET28a-hCGn6ST, and the recombinant transformants were cultured in Luria broth medium, as recommended by the manufacturer. Polyhistidine-tagged hCGn6ST was purified from an inclusion body fraction of the bacterial lysate by a nickel column (Ni²⁺-nitritrocatic acid; Qiagen, Valencia, CA), SDS-PAGE and Coomassie Blue staining indicated that 75.2% of protein components in the bound fraction were hCGn6ST, which was used as a standard. For calculation of the amount of sulfotransferase in the
enzyme fraction expressed by HeLa cells, an aliquot of each enzyme fraction of hCGn6ST, was incubated at 37 °C overnight. The carbohydrate product (3000 cpm of product IV and 1500 cpm of product V) was incubated in a 30-μl reaction mixture containing 50 mM imidazole-HCl, pH 6.8, 10 mM MnCl₂, 5 mM 5′-AMP, 20 mM NaF, 100 nCi of [35S]PAPS (PerkinElmer Life Sciences), 2.25 nmol of substrate, and 1 μl of enzyme fraction at 37 °C for 1 h. After adding 1 ml of water to stop the reaction, the product was purified by a reverse phase C18 column (High Load C18 column, Alltech Associates, Deerfield, IL), and incorporation of [35S]sulfate by the substrate was determined by scintillation counting.

Sequential Enzymatic Reactions for Production of Keratan Sulfate in Vitro—A synthetic carbohydrate substrate GlcNAcβ1–6Manα1–6Manβ1-octyl was labeled with [35S]sulfate by incubating 11.25 nmol of the substrate in a 75-μl reaction mixture containing 50 mM imidazole-HCl, pH 6.8, 10 mM MnCl₂, 2 mM 5′-AMP, 20 mM NaF, 1.76 μCi of [35S]PAPS (PerkinElmer Life Sciences), and 5 μl of the enzyme fraction of hCGn6ST at 37 °C overnight. The product was purified by passage through a C18 column and then lyophilized and subjected to HPLC. A Whatman Partisil SAX-10 column (4.6 mm × 25 cm) was used for HPLC analysis (40). This column was equilibrated with 75% acetonitrile in H₂O. The samples were eluted under the following conditions: 75% acetonitrile/H₂O for 5 min; 1 μl KH₂PO₄, 75% acetonitrile for 30 min; and a gradient from 5 μl KH₂PO₄, 75% acetonitrile to 10 μl KH₂PO₄, 75% acetonitrile over 25 min followed by 10 μl KH₂PO₄, 75% acetonitrile for 15 min. The flow rate was 1 ml/min, and 1-ml fractions were collected. [35S]radioactivity of eluates was monitored by scintillation counting. The identified reaction product was purified by a C18 column, lyophilized, and dissolved in 100 μl of water.

Next, the sulfated substrate was treated with βGal-TI as follows. The reaction condition basically followed a previous description (37). In brief, 100 μl of a reaction mixture including 50 mM HEPES-KOH, pH 7.5, 14 mM MnCl₂, 1 mM UDP-Gal (Sigma), 10 units of calf intestine alkaline phosphatase (New England Biolabs, Beverly, MA), 50 μl (1.18 × 10⁶ cpm) of the purified product (product I in Fig. 4), and 10 milliliters of bovine milk βGal-TI (Sigma) was incubated at 37 °C overnight. The product was purified by a C18 column, lyophilized, and subjected to HPLC analysis in the manner described above.

The obtained product was subsequently treated with an enzyme fraction of human β3Gn-T2. The reaction mixture (150 μl) containing 100 mM HEPES-KOH, pH 7.5, 20 mM MnCl₂, 5 mM UDP-GlcNAc (Sigma), 45 μl (3.35 × 10⁵ cpm) of the purified product (product II in Fig. 4), and 75 μl of a reaction mixture containing 50 mM HEPES-KOH, pH 7.5, 14 μM MgCl₂, 20 mM NaF, 1 mM PAPS (Sigma), 60 μl (10080 cpm) of the purified product (product IV in Fig. 4), and 10 μl of enzyme fraction of KSG6ST was incubated at 37 °C overnight. The reaction product was purified by a C18 column, lyophilized, and subjected to HPLC analysis as described above.

The product was further treated with an enzyme fraction of KSG6ST. A 50-μl reaction mixture containing 50 mM imidazole-HCl, pH 6.8, 10 mM MnCl₂, 2 mM 5′-AMP, 20 mM NaF, 1 mM PAPS (Sigma), 30 μl (61000 cpm) of the purified product (product III in Fig. 4), and 5 μl of enzyme fraction of hCGn6ST, was incubated at 37 °C overnight. The reaction product was purified by a C18 column, lyophilized, and subjected to HPLC analysis as described above.

Keratanase Treatment of Reaction Products—To determine the carbohydrate structure, reaction products (products IV and V in Fig. 4) were treated with keratanase, which recognizes (SO₄)₂-GlcNAcβ1–3Galβ1–4GlcNAc structure and digests Galβ1–4GlcNAc linkages (41). The carbohydrate product (3000 cpm of product IV and 1500 cpm of product V) was incubated in a 30-μl reaction mixture containing 50 mM Tris-HCl, pH 7.4, and 125 milliliters of keratanase from Pseudomonas sp. (Calbiochem) at 37 °C for 4 h. The digested product was purified by a C18 column and subjected to HPLC followed by scintillation counting.

FIG. 1. Sulfation of CD34-IgG by hCGn6ST, hIGn6ST, and mIGn6ST in CHO cells. CHO cells were co-transfected with expression vectors for CD34-IgG (lanes 1–8), for sulfotransferases (mock, lanes 1 and 2; hCGn6ST, lanes 3 and 4; hIGn6ST, lanes 5 and 6; mIGn6ST, lanes 7 and 8), and for C2GnTI (lanes 2, 4, 6, and 8). The transfected cells were metabolically labeled with [35S]sulfate, and the CD34-IgG produced was isolated and analyzed by fluorography. Fluorograms represent the pattern of intact (A) and N-glycanase-treated (B) CD34-IgG. The arrows indicate the migration position of CD34-IgG.

RESULTS

hCGn6ST Produces Sulfated N- and O-Glycans in Cultured Cells—In a previous study, we found that hCGn6ST and mIGn6ST, but not hIGn6ST, are required for the production of keratan sulfate (28), although the amino acid sequences of all three enzymes are highly homologous to each other. To elucidate the carbohydrate similarities and differences in those sulfotransferases, we analyzed the enzymatic activity of recombinant sulfotransferases in vitro and in vivo. First, we expressed the sulfotransferases in CHO cells and analyzed the production of sulfated carbohydrates. We used CD34-IgG as an acceptor protein because this protein carries both N- and O-glycans (31), and we used CHO cells as hosts because they have no core-2 N-acetylgalcosaminyltransferase activity and produce predominantly N-glycans (32). When an expression vector carrying C2GnTI is transfected into CHO cells, the cells produce both N- and O-glycans (32). Mock-transfected CHO cells showed a negligible level of sulfated carbohydrate on CD34-IgG, which is produced by the endogenous sulfotransferase (Fig. 1A, lanes 1 and 2). On the other hand, cells transfected with any of the sulfotransferase expression vector produced significant amounts of CD34-IgG with sulfated glycans (Fig. 1A, lanes 3–8). The transfected cells expressing hCGn6ST produced sulfated N-glycans on CD34-IgG (Fig. 1A, lane 3), and these sulfated carbohydrates were released from the protein by N-glycanase digestion (Fig. 1B, lane 3). We also found that hCGn6ST-expressing CHO cells produced large amounts of sulfated O-glycans resistant to N-glycanase digestion (Fig. 1, A and B, lane 4). These results indicate that hCGn6ST can transfer sulfate on both N- and O-glycans in vivo. Almost identical results were obtained for mIGn6ST (Fig. 1, A and B, lanes 7 and 8), suggesting that mIGn6ST has a similar preference for carbohydrate substrates as hCGn6ST. Unlike these two enzymes, hIGn6ST-expressing CHO cells produced sulfated O-glycans but almost no sulfated N-glycans on CD34-IgG (lanes 5 and 6), suggesting that hIGn6ST mostly functions in the production of sulfated O-glycans in vivo.

Specific Activity of Sulfotransferases on Synthetic Substrates—Next we analyzed substrate specificity of all three sulfotransferases using synthetic carbohydrates (Fig. 2, bottom). As a source of sulfotransferase, we used concentrated culture medium of HeLa cells expressing soluble forms of sulfotransferases. The results of the sulfation analysis and the calculated relative enzymatic activity for substrates are shown in Fig. 2 and Table I, respectively. We found that all three
FIG. 2. Sulfation of synthetic carbohydrate substrates by hCGn6ST, hIGn6ST, and mIGn6ST in vitro. Each bar indicates incorporation of [35S]sulfate per 1 h per 1-μl enzyme fraction of each sulfotransferase into synthetic substrates at 37 °C. Data presented are the average of duplicated experiments. Open and hatched bars indicate the results using enzyme fractions derived from mock-transfected cells and transfected cells expressing soluble hCGn6ST (A), hIGn6ST (B), and mIGn6ST (C). The carbohydrate structure of each substrate is shown at the bottom.
soluble sulfotransferases have activity for synthetic carbohydrates and that hCGn6ST has the strongest activity among the sulfotransferases tested for all of the reactive substrates (Fig. 2 and Table I). We also calculated the specific activity of each sulfotransferase for a carbohydrate substrate. Purified hCGn6ST protein from bacterial cells was used to determine the quantity of sulfotransferase in the reaction mixture (see “Experimental Procedures”). By this analysis, we calculated the specific activity of hCGn6ST, hGn6ST, and mGn6ST for the trisaccharide substrate, GlcNAc1,6-Man1,6-Man1,6-octyl, as 73.5, 1.69, and 20.2 pmol/h/μg enzyme, respectively (Table I).

All of the soluble sulfotransferases have activity for short carbohydrate substrates, which have GlcNAc on their nonreducing terminus, such as GlcNAcβ1–6-Manα1–6-Manβ1-octyl (Fig. 2, substrate 1) and GlcNAcβ1–6(Galβ1–3)GalNAcα1-octyl (substrate 11). These sulfotransferases have no activity for carbohydrates that have internal GlcNAc but no nonreducing terminal GlcNAc in the structure (substrates 2, 6, and 10), indicating that these sulfotransferases only transfer sulfate on GlcNAc at the nonreducing terminus of carbohydrate.

A trisaccharide substrate with a core-2 O-glycan structure is the most suitable substrate for all the sulfotransferases (substrate 11). This finding is consistent with results obtained in vivo (Fig. 1), which indicates that all of the sulfotransferases have higher enzymatic activity for O-glycans than for N-glycans on CD34-IgG. Although core-2 carbohydrate is the preferable substrate for hCGn6ST and mGn6ST, all of the carbohydrates with nonreducing terminal GlcNAc can be utilized as substrates for hCGn6ST and mGn6ST. A nonreducing terminal GlcNAc connected to mannose by β1–2 linkage was a poorer acceptor for these two enzymes than one connected to mannose by β1–6 linkage (substrates 1 and 5). Interestingly, a biantenary substrate, which has two nonreducing terminal GlcNAc residues by β1–2 and β1–6 linkages (substrate 7), has much less substrate activity for hCGn6ST and mGn6ST than a monoantennary substrate with a nonreducing terminal GlcNAc by β1–6 linkage (substrate 1). We also found that a biantenary substrate that has a GlcNAc by β1–6 linkage and a Galβ1–4GlcNAc by β1–2 linkage (substrate 9) has less sulfotransferase activity for the sulfotransferases than a monoantennary substrate, GlcNAcβ1–6Manα1–6Manβ1-octyl (substrate 1). These results indicate that the carbohydrate on the β1–2 branch hinders sulfation of GlcNAc on the β1–6 branch by the sulfotransferases. On the other hand, the substrate activities of a monoantennary GlcNAcβ1–2Manα1–6Manβ1-octyl (substrate 5) and a biantenary GlcNAcβ1–2(Galβ1–4GlcNAcβ1–6)Manα1–6Manβ1-octyl (substrate 8) for both hCGn6ST and mGn6ST are almost equivalent, suggesting that the sulfation of a GlcNAc residue linked to the mannose core via β1–2 linkage is not affected by the presence of another carbohydrate residue on β1–6 linkage. Notably, hCGn6ST and mGn6ST utilized carbohydrates with extended GlcNAcβ1–3Gal repeats (substrates 3 and 4) as their substrates, and the length of the GlcNAcβ1–3Gal repeat did not affect the activity of the enzymatic reaction. These results suggest that hCGn6ST and mGn6ST can transfer sulfate onto the nonreducing terminal GlcNAc of a poly-N-acetyllactosamine structure.

The substrate specificity of hGn6ST is remarkably different from that of the other two sulfotransferases (Fig. 2B). hGn6ST utilized shorter carbohydrates as preferable substrates (substrates 1, 5, 7, 8, and 11) but had very little activity on longer carbohydrates that have more than one unit of GlcNAcβ1–3Gal disaccharide (substrates 3, 4, 13, and 14). This result suggests that hGn6ST does not play a role for the production of keratan sulfate because keratan sulfate consists of GlcNAcβ1–3Gal repeats. Interestingly, hGn6ST has higher enzymatic activity for GlcNAcβ1–2Manα1–6Manβ1-octyl (substrate 5) than for GlcNAcβ1–6Manα1–6Manβ1-octyl (substrate 1), whereas hCGn6ST and mGn6ST have the opposite preference for these substrates. We also found that hGn6ST has almost the same sulfotransferase activity for a monoantennary GlcNAcβ1–
2Manα1–6Manβ1-octyl (substrate 5) and two biantennary substrates, GlcNAcβ1–2(GlcNAcβ1–6)Manα1–6Manβ1-octyl (substrate 7) and GlcNAcβ1–2Galβ1–4GlcNAcβ1–6)Manα1–6Manβ1-octyl (substrate 8). This finding provides further evidence that the presence of carbohydrate on β1–6 branch does not affect sulfation of GlcNAc on β1–2 branch by hIGn6ST.

Based on these results, we concluded that hCGn6ST and mIGn6ST can produce sulfated poly-N-acetyllactosamine carbohydrate that will be processed to keratan sulfate, on both N- and O-glycans, whereas hIGn6ST is only active on short carbohydrates such as core-2 trisaccharide.

**In Vitro Synthesis of Keratan Sulfate Disaccharide by Sequential Treatment by Glycosyltransferases and Sulfotransferases**—Since hCGn6ST functions in the production of keratan sulfate (28) and transfers sulfates to nonreducing terminal GlcNAc (Fig. 2A), we hypothesized that sulfation of the GlcNAc residue of keratan sulfate by hCGn6ST is coupled to elongation of the poly-N-acetyllactosamine chain by β1,3-N-acetylgalactosaminyltransferase and β1,4-galactosyltransferase (Fig. 3). To test this hypothesis, we determined whether keratan sulfate is produced in *vitro* using sulfotransferases and glycosyltransferases. For sulfotransferases, we used hCGn6ST and human keratan sulfate Gal 6-O-sulfotransferase (KSG6ST). For a β1,4-galactosyltransferase, we used bovine milk β4Gal-T1, which is the orthologue of human β4Gal-T1. As a β1,3-N-acetylgalactosaminyltransferase, we chose human β3Gn-T2 because it is ubiquitously expressed in human tissues and has the highest N-acetylgalactosaminyltransferase activity among enzymes tested (34). First we produced a radiolabeled monosulfated trisaccharide, [35S]SO3−GlcNAcβ1–6Manα1–6Manβ1-octyl by treating substrate 1 in Fig. 2 with hCGn6ST and a sulfating donor, [35S]PAPS. The reaction mixture was then subjected to SAX-10 HPLC (Fig. 4A). We pooled the fractions of the product (product I in Fig. 4A), which were used as a substrate for the next step.

Next we treated product I with β4Gal-T1 and a Gal donor, UDP-Gal, and analyzed the carbohydrate structure by HPLC (Fig. 4B). The product (product II in Fig. 4B) was eluted later than that of starting material (product I in Fig. 4A) and was slightly later than the position of a monosulfated tetrasaccharide marker (Fig. 4, arrow b), suggesting that product II is an isomer of [35S]SO3−Galβ1–4GlcNAcβ1–6Manα1–6Manβ1-octyl. This product II was treated with β3Gn-T2 and a GlcNAc donor, UDP-GlcNAc, and the reaction product was subjected to HPLC (Fig. 4C). In the HPLC profile, we found that half of the substrate is modified to a product (product III) that has a later retention position (fractions 32–35 in Fig. 4C) by β3Gn-T2 treatment. We again treated product III with hCGn6ST and a sulfotransferase, and separated the reaction product by HPLC (Fig. 4D). The retention position of the product (product...
IV in Fig. 4D) was greatly delayed relative to the starting material (product III in Fig. 4C), suggesting that product IV is a sulfated version of product III. To determine the carbohydrate structure of product IV, we tested the susceptibility of product IV to keratanase. Keratanase is an endoglycosidase that recognizes SO\textsubscript{4}–GlcNAcβ1–3Galβ1–4GlcNAc and digests Galβ1–4GlcNAc linkage (41). Additional sulfate on Gal and/or lack of sulfate on GlcNAc impairs the sensitivity to keratanase. HPLC analysis showed an identical retention time for the digestion product (Fig. 5A, closed square) as a carbohydrate marker, [\text{35S}SO\textsubscript{4}–GlcNAcβ1–6Manβ1–2Manβ1-octyl] (Fig. 5A, marker a), indicating that keratanase treatment released one SO\textsubscript{4}–GlcNAcβ1–3Gal unit from product IV and produced a carbohydrate structure identical to product I. Thus, we concluded that the carbohydrate structure of product IV is SO\textsubscript{4}–GlcNAcβ1–3Galβ1–4(\[\text{35S}SO\textsubscript{4}–GlcNAcβ1–6Manβ1–6Manβ1-octyl. Since the structure of product IV has an elongated GlcNAcβ1–3Gal unit on product I and this elongation step has been processed by β4Gal-TI and β3Gn-T2, we concluded that the carbohydrate structures of products II and III are Galβ1–4(\[\text{35S}SO\textsubscript{4}–GlcNAcβ1–6Manβ1–6Manβ1-octyl and GlcNAcβ1–3Galβ1–4(\[\text{35S}SO\textsubscript{4}–GlcNAcβ1–6Manβ1–6Manβ1-octyl, respectively.

We further treated product IV with KSG6ST and PAPS to test whether this enzyme transfers sulfate onto product IV. The retention position of the product following KSG6ST treatment was greatly shifted to a later fraction (Fig. 4E), indicating that the product received a sulfate residue by the enzyme treatment. We also treated the carbohydrate component of product V with keratanase; however, keratanase did not change the retention position of the product (Fig. 5B), indicating that product V is resistant to keratanase. Since the addition of sulfate on Gal makes the carbohydrate resistant to keratanase, we concluded that the carbohydrate structure of product V is a trisulfated pentasaccharide, SO\textsubscript{4}–GlcNAcβ1–3(\[\text{35S}SO\textsubscript{4}–Galβ1–4(\[\text{35S}SO\textsubscript{4}–GlcNAcβ1–6Manβ1–6Manβ1-octyl. The results of sequential enzymatic reactions shown in Fig. 4 indicate that the sulfated carbohydrate can be processed to keratan sulfate disaccharide in vitro by four enzymes: β4Gal-TI, β3Gn-T2, hCGn6ST, and KSG6ST. Since the nonreducing terminal carbohydrate structure of product IV is identical to that of product I, it is very likely that the sulfation and elongation steps are repeatable by the three enzymes, hCGn6ST, β1,4-galactosyltransferase, and β1,3-N-acetylgalcosaminyltransferase. This idea is consistent with the proposed biosynthetic pathway of keratan sulfate, which is shown in Fig. 3. The sulfated poly-N-acetyllactosamine chain may be produced by two glycosyltransferases and GlcNAc 6-O-sulfotransferase in a cooperative manner. Sulfation of Gal residues by Gal 6-O-sulfotransferase, which may take place later than the production of the GlcNAc-sulfated poly-N-acetyllactosamine, completes the keratan sulfate GAG synthesis.

DISCUSSION

In previous studies, we found that the loss of enzymatic activity of hCGn6ST in corneal cells causes a hereditary eye disease, MCD (27), and that hCGn6ST is required for the production of keratan sulfate GAGs (28). We also found that miGn6ST, which is an orthologue of both hCGn6ST and hGn6ST, has similar activity as hCGn6ST, but not hGn6ST, in the production of keratan sulfate GAGs in the mouse cornea (28).

Keratan sulfate GAGs are found in the cornea as well as in the other tissues such as cartilage. Corneal keratan sulfate is linked to carrier proteins in N-linked manner, whereas cartilage keratan sulfate is attached to proteins largely via O-linkages (13, 14). By metabolic labeling of a glycoprotein produced by sulfotransferase-expressing CHO cells, we found that both hCGn6ST and miGn6ST contribute to the processing of sulfated N- and O-glycans (Fig. 1). This result is consistent with our previous hypothesis that hCGn6ST and miGn6ST function in the production of keratan sulfate in both the cornea and cartilage (27, 28). By contrast, hIGn6ST activity resulted only in the production of sulfated O-glycans on a carrier protein (Fig. 1). Experiments to test the substrate specificity of hIGn6ST in vitro also showed that it has the highest activity in the presence of core-2 oligosaccharide, whereas it shows minimal activity for short N-linked type oligosaccharides (Fig. 2B). Several studies have reported that hGn6ST has sulfotransferase activity for core-2 oligosaccharide and produces L-selectin ligand carbohydrate structures (42, 43). Our results support this possibility. On the other hand, Uchimura et al. (43) reported that hGn6ST has no activity for GlcNAcβ1–2Man and GlcNAcβ1–6ManOMe substrates. By contrast, we found that hGn6ST has activity for GlcNAc, which linked to Manα1–6Manβ1-octyl structures via β1–2 and β1–6 linkage (Fig. 2B). It is possible that hGn6ST recognizes trisaccharide but not disaccharide structures as substrates.

The present study also revealed that hIGn6ST exhibits very low sulfotransferase activity on longer carbohydrate substrates (Fig. 2B and Table I). Thus, hIGn6ST showed significantly reduced sulfotransferase activity toward substrates with one additional unit of GlcNAcβ1–3Gal (substrates 3 and 13 in Fig. 2) less than short carbohydrate substrates (substrates 1 and 11), supporting our hypothesis that hIGn6ST is not required for production of keratan sulfate GAGs (28). Based on currently available data, hGn6ST may only contribute to L-selectin ligand production (42, 43) (Fig. 2B and Table I). We have also found that an MCD patient lacking both the hIGn6ST coding region and the putative gene regulatory region of hCGn6ST shows no phenotype other than corneal opacity typical of MCD (27), suggesting that the biological function of hGn6ST can be compensated by other sulfotransferases. Since sulfotransferases such as GlcNAcβ6ST-1 (also known as GST2) and L-selectin ligand sulfotransferase/high-endothelial cell specific GlcNAc 6-O sulfotransferase (also known as GlcNAc6ST-2 and GST3) can produce L-selectin ligand structures (31, 42–45), it is likely that lack of hGn6ST is compensated by sulfotransferases with similar activity to hGn6ST.

By analyzing the substrate specificity of sulfotransferases, we found that miGn6ST has remarkably similar substrate specificity to hCGn6ST but not to hIGn6ST (Fig. 2). Previous
In vitro expression experiments indicated that mIGn6ST is the orthologue of hCGn6ST, since both enzymes have substrate specificity, such as preference for a nonreducing terminal GlcNAc linked to mannos via β-1–2 linkage (Fig. 2B, substrates 5, 7, and 8) and has probably lost enzymatic activity for poly-N-acetyllactosamine chains.

In this and previous studies, we hypothesize that the sulfation of GlcNAc residues on keratan sulfate is coupled to elongation of the poly-N-acetyllactosamine backbone (28) (Fig. 3). In this study, we tested sequential treatment of a carbohydrate substrate with soluble candidate enzymes for production of keratan sulfate GAGs (Fig. 4). Sulfated carbohydrate has been utilized for elongation of the N-acetyllactosamine backbone by a β1,4-galactosyltransferase and a β1,3-N-acetylgalactosaminyltransferase (Fig. 4, B and C), indicating that the presence of a sulfate residue on the 6-O-position of GlcNAc does not affect elongation of poly-N-acetyllactosamine chains. The chain length of poly-N-acetyllactosamine also does not affect sulfation of nonreducing terminal GlcNAc by hCGn6ST (Fig. 2A, substrates 3 and 4). Because the carbohydrate structure of the nonreducing terminus of product IV is identical to that of product I (Fig. 4), the enzymatic reactions of extension and sulfation of poly-N-acetyllactosamine can be repeated by these three enzymes, as illustrated in Fig. 3. KSG6ST has sulfotransferase activity for both the nonreducing terminus and internal Gal residues of keratan sulfate, and that also has preference for Gal adjacent to sulfated GlcNAc (40, 46). We also confirmed that sulfation of internal Gal in a carbohydrate chain is accomplished by KSG6ST (Fig. 4E). Thus, we conclude that sulfation of Gal in keratan sulfate is not coupled with the elongation step but occurs during and/or after production of GlcNAc-sulfated poly-N-acetyllactosamine carbohydrate (Fig. 3). Since the sulfation degree of Gal is lower than that of GlcNAc in a keratan sulfate GAG chain, the Gal sulfation step seems to be independent from the GlcNAc sulfation step in keratan sulfate biosynthesis (13, 14, 40, 47), and this is consistent with our hypothesis. We also found that an N-acetylgalactosaminyltransferase cannot transfer GlcNAc to sulfated Gal at the nonreducing terminus, indicating that sulfation of Gal residues cannot be coupled with the chain elongation of keratan sulfate GAGs. It is possible that sulfation of the terminal Gal blocks further elongation of keratan sulfate GAG chain, as suggested by an earlier study (20).

Previously, we found that mRNA encoding hCGn6ST and mIGn6ST is present in human and mouse corneal tissues (27, 28). Since hCGn6ST has been involved in production of keratan sulfate GAGs in vivo and in vitro (28) (Figs. 2 and 4 and Table I), it is evident that hCGn6ST is the sulfotransferase that transfers sulfation to GlcNAc of poly-N-acetyllactosamine in the cornea. KSG6ST may be responsible for sulfation of Gal residues of corneal keratan sulfate, because mRNA encoding the enzyme is expressed in chick cornea (40). Since β3GnT-2 has the highest GlcNAc transferase activity for Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–3Galβ1–4Glc, which has an elongated poly-N-acetyllactosamine structure, among enzymes tested in the previous study (34), we speculate that β3GnT-2 functions in the synthesis of corneal keratan sulfate. β3GnT-2 mRNA is ubiquitously expressed in human tissues and is therefore probably expressed in the cornea (34). There is no information on a candidate β1,4-galactosyltransferase involved in production of corneal keratan sulfate to date. Since human genome sequence is available and several studies have reported nucleotide sequences and substrate specificities of human β1,4-galactosyltransferases (39, 48–54), analyses of tissue distribution and biological function of β1,4-galactosyltransferases in vivo and in vitro should identify the β1,4-galactosyltransferases responsible for keratan sulfate biosynthesis in the cornea.

In the present study, we established a role for hCGn6ST in sulfation of GlcNAc in keratan sulfate GAGs. We also demonstrated the production of highly sulfated keratan sulfate in vitro. Keratan sulfate proteoglycan is important for maintaining both extracellular matrix organization and corneal transparency. Further studies defining the corneal keratan sulfate biosynthetic pathway are necessary to determine how corneal transparency develops and is maintained.

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Enzymatic Synthesis in Vitro of the Disulfated Disaccharide Unit of Corneal Keratan Sulfate

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