Human Corneal GlcNAc 6-O-Sulfotransferase and Mouse Intestinal GlcNAc 6-O-Sulfotransferase Both Produce Keratan Sulfate*

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Human corneal N-acetylglucosamine 6-O-sulfotransferase (hCGn6ST) has been identified by the positional candidate approach as the gene responsible for macular corneal dystrophy (MCD). Because of its high homology to carbohydrate sulfotransferases and the presence of mutations of this gene in MCD patients who lack sulfated keratan sulfate in the cornea and serum, hCGn6ST protein is thought to be a sulfotransferase that catalyzes sulfation of GlcNAc in keratan sulfate. In this report, we analyzed the enzymatic activity of hCGn6ST by expressing it in cultured cells. A lysate prepared from HeLa cells transfected with an intact form of hCGn6ST cDNA showed an activity of transferring sulfate to C-6 of GlcNAc of synthetic oligosaccharide substrates in vitro. When hCGn6ST was expressed together with human keratan sulfate Gal-6-sulfotransferase (hKSG6ST), HeLa cells produced highly sulfated carbohydrate detected by an anti-keratan sulfate antibody 5D4. These results indicate that hCGn6ST transfers sulfate to C-6 of GlcNAc in keratan sulfate. Amino acid substitutions in hCGn6ST identical to changes resulting from missense mutations found in MCD patients abolished enzymatic activity. Moreover, mouse intestinal GlcNAc 6-O-sulfotransferase had the same activity as hCGn6ST. This observation suggests that mouse intestinal GlcNAc 6-O-sulfotransferase is the orthologue of hCGn6ST and functions as a sulfotransferase to produce keratan sulfate in the cornea.

Keratan sulfate proteoglycan, the most abundant carbohydrate in the cornea, plays an important role in maintenance of corneal transparency (1, 2). Three acceptor proteins, lumican, keratan, and mimecan, have been reported to carry keratan sulfate via N-linked oligosaccharide in the cornea. The expression of these carrier proteins is regulated during eye development, suggesting the importance of keratan sulfate proteoglycans in corneal tissue (3–8).

Keratan sulfate consists of a linear poly-N-acetyllactosamine chain that carries sulfate residues on C-6 of GlcNAc and Gal. Because the sulfation of carbohydrates affects their biochemical characteristics, such as water solubility and electrical charge, this modification appears to be important for the function of keratan sulfate proteoglycans in the cornea. The importance of keratan sulfate sulfation in the cornea has been also suggested that lack of sulfation on keratan sulfate is a major cause of the hereditary eye disorder, macular corneal dystrophy (MCD)1 (1, 9, 10).

MCD patients show spotted opacity in the cornea, especially in the extracellular matrix of the stroma. The size of the opaque area increases progressively, and the patients require keratoplasty. By genetic linkage analysis, the critical region for MCD has been mapped to chromosome 16q22 (11–13). Previous reports indicated that the cornea of MCD patients synthesizes normal levels of poly-N-acetyllactosamine but does not contain keratan sulfate, suggesting that the sulfation step of keratan sulfate is impaired in MCD (9). One of the carbohydrate sulfotransferases, keratan sulfate Gal-6 sulfotransferase, has shown that the enzyme transfers sulfate to the Gal residue of poly-N-acetyllactosamine and keratan sulfate, but the gene encoding this sulfotransferase does not map to the MCD candidate region (14).

Several carbohydrate sulfotransferases have been identified through biochemical and functional genomic approaches (14–19). These sulfotransferases are highly homologous to each other, especially in the binding domains to the sulfate donor, PAPS (20, 21). We previously identified a carbohydrate sulfotransferase that maps to the critical MCD region by EST data base searches, and we designated it corneal GlcNAc 6-O-sulfotransferase (22). This protein, which is homologous to other carbohydrate sulfotransferases, is expressed in corneal cells. Several types of mutations, including deletion and missense mutations, were found in this gene in genomic DNAs derived from MCD patients, leading to identification of the causative gene of MCD. Here, we analyze the enzymatic activity of human corneal GlcNAc 6-O-sulfotransferase (hCGn6ST) using transfected HeLa cells, and we show that the enzyme transfers sulfate onto the C-6 of GlcNAc in a synthetic substrate and poly-N-acetyllactosamine. We also confirm that missense mutations in hCGn6ST found in MCD patients result in a failure of synthesizing highly sulfated keratan sulfate in the trans-

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† The abbreviations used are: MCD, macular corneal dystrophy; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; EST, expression sequence tag; hCGn6ST, human corneal GlcNAc 6-O-sulfotransferase; miGn6ST, mouse intestinal GlcNAc 6-O-sulfotransferase; hGn6ST, human intestinal GlcNAc 6-O-sulfotransferase; hKSG6ST, human keratan sulfate Gal-6-O-sulfotransferase; GalNAc, N-acetyllactosamine; PBS, phosphate-buffered saline; GlcNAc(6S), GlcNAc-6-O-sulfate; PCR, polymerase chain reaction; DME, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HPLC, high pressure liquid chromatography; ORF, open reading frame.

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fected. Moreover, our results indicate that mouse intestine GlcNAc 6-O-sulfotransferase (mGn6ST), which has been identified to be a homologue of human intestine GlcNAc 6-O-sulfotransferase (hGn6ST) (18), has the same activity as hGn6ST, suggesting that mGn6ST is the mouse orthologue of hGn6ST and functions to produce keratan sulfate in the mouse cornea.

**EXPERIMENTAL PROCEDURES**

**Construction of Sulfotransferase Expression Vectors**—A nucleotide fragment of the hGn6ST ORF was obtained from previously reported cDNA (22) by PCR using the following primers: 5'-TTTAGGTGACACTATAG-3' (SP6 primer, Invitrogen). After digestion with SP6 and BglI sites of pcDNAHSH, which encodes a cleavable signal sequence (23) following polyhistidine, enterokinase cleavage site, and catalytic domain of hGn6ST. An expression vector encoding soluble hGn6ST was prepared as follows. A DNA fragment coding catalytic domain of hGn6ST was amplified from pcDNA-hGn6ST vector by PCR using the following primers: 5'-GGTAGATCTGCCAGGGCCCTCGTCCCCA-3' and 5'-GA-TTTAGGTGACACTATAG-3' (SP6 primer, Invitrogen). After digestion with BglI and XbaI (New England Biolabs, Beverly, MA), this amplicon was inserted into the BamHI-XhoI sites of pcDNAHSH, which encodes the signal sequence of human colony-stimulating factor and multi cloning site of EpiTagTM pcDNA3.1/His B (Invitrogen) in the multicloning site of EpiTagTM pcDNA3.1/His B (Invitrogen) in the pcDNAHSH-hGn6ST vector. The resultant expression vector, pcDNAHSH-hGn6ST, encodes a cleavable signal sequence (23) following polyhistidine, enterokinase cleavage site, and catalytic domain of hGn6ST.

An expression vector encoding R50C mutant hGn6ST was prepared as follows. A DNA fragment coding catalytic domain of hGn6ST, which contains R50C mutation, was amplified from an MCD patient (22) by PCR using the following primers: 5’-GGTAGATCTGCCAGGGCCCTCGTCCCCA-3’ and 5’-GA-TTTAGGTGACACTATAG-3’. The R50C mutation was introduced into the pcDNA-hGn6ST that was digested with AvaI and SfiI. Five expression vectors, each of which encodes a chGn6ST mutant, K174R, D203E, A211W, and E274K, were prepared with the same method described above except PCR primers (5’-GACGTGGTTTGATGCCTATCTGCCTTG-3’ and 5’-CGGCGCGCACCAGGTCCA-3’) and the restriction enzymes for replacement of 

**Enzymatic Activity of Corneal GlcNAc 6-O-Sulfotransferase**—An expression vector for intact hGn6ST was transfected into HeLa cells using Lipo-fectAMINE PLUS reagent (Life Technologies, Inc.). After incubation for 48 h in Dulbecco’s modified Eagle’s medium containing 10% of fetal bovine serum (DMEM, 10% FBS), the cells were washed with PBS, scraped, and collected into a 1.5-ml microfuge tube. The cells were again washed with PBS and suspended into cell lysis buffer (20 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl2, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 30 min, the sample was centrifuged at 9,000 × g for 10 min at 4 °C, and the supernatant was used as an enzyme fraction.

Soluble hGn6ST was prepared as follows. The expression vector pcDNAHSH-hGn6ST was transfected into HeLa cells as described above. Following 24 h of incubation in DMEM, 10% FBS, the medium was replaced with S-MEM (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum and [35S]sodium sulfate (PerkinElmer Life Sciences) at a concentration of 100 μCi/ml. After a 24-h incubation, cells were washed with PBS, scraped, and collected into a 1.5-ml microfuge tube. The cells were washed again with PBS and extracted with 500 μl of chloroform/methanol (2:1). The cell pellets were washed with 200 μl of methanol and digested in 200 μl of 0.1 mM Tris- HCl, pH 8.0, 1 mM CaCl2 with 20 μl of 1 mg/ml Pronase (Calbiochem). After an overnight incubation at 37 °C, 20 μl of freshly prepared Pronase (1 mg/ml) were added and incubated overnight at 37 °C. The digested mixture was boiled for 5 min to stop the reaction. After phenol and chloroform extraction, the sample in the aqueous phase was subjected to Sephadex G-50 column chromatography (1 × 45 cm, equilibrated with 0.1 mM NH4HCO3). The carbohydrate fraction that eluted in the void volume was collected, desalted by Sephadex G-25 gel filtration (1 × 30 cm, equilibrated with 7% 1-propanol/water), and lyophilized. The sample was dissolved in 150 μl of water and analyzed for [35S] radioactivity.

**Enzymatic and Chemical Cleavages of Carbohydrates**—Purified oligosaccharides (each 5000 cpm) produced by *in vitro* sulfotransferase reaction were digested with 10 milliunits of β-N-acetylglucosaminidase A from human placenta (Sigma), which cleaves and release both GlcNAc and GlcNAc6S from non-reducing terminal of carbohydrate, in 20 μl of 25 mM sodium citrate buffer, pH 3.5 and 100 μa galactosidase for overnight at 37 °C. The digested samples were boiled for 5 min and were analyzed by HPLC.

Metabolically labeled carbohydrate samples (each 5 × 106 cpm) produced from transfected HeLa cells were digested with 250 milliunits of keratanase from *Pseudomonas sp.* (Calbiochem) in 90 μl of 50 mM Tris-HCl, pH 7.4. After overnight incubation at 37 °C, the samples were boiled for 5 min to stop the reaction and applied to a column (1 × 45 cm) of Sephadex G-50 equilibrated with 0.1 mM NH4HCO3. Each 400 μl of fraction was collected in a tube. Ten μl of each fraction were used to count [35S] radioactivity, and the remainder of the fraction was desalted and lyophilized.

To remove sialic acid, 0.1 volume of 0.1 N HCl was added to the sample and incubated at 90 °C for 1 h. The reaction was stopped by addition of 2 N NaOH. After digestion with Apo and SfiI, the sample was desalted and lyophilized. After dissolving in water, the sample was subjected to column chromatography.

**Column Chromatography**—A Sephadex G-50 column (1 × 45 cm) was used for gel filtration chromatography. The column was equilibrated and eluted with 0.1 N NH4HCO3. Fractions of 300 μl were collected, and [35S] radioactivity was determined by scintillation counting.

A Whatman Partisol SAX-10 column (4.6 mm × 25 cm) was used for HPLC analysis. This column was equilibrated with 5 mM KH2PO4. The elution conditions were as described previously (14, 27). In brief, the column was eluted with 5 mM KH2PO4 isocratically for the samples produced by β-N-acetylglucosaminidase A treatment. For the samples produced by keratanase treatment, the column was eluted with 5 mM KH2PO4 followed by a 20-min gradient from 5 to 250 mM KH2PO4. The flow rate was 1 ml/min. Fractions of 0.5 min were collected, and [35S] radioactivity was determined by scintillation counting.

**Western Blot Analysis**—Transfected cells were transferred to a 1.5-ml tube by scraping and washed in PBS. The cells were suspended into 800 μl of TKMS buffer (20 mM Tris-HCl, pH 7.6, 25 mM KCl, 2.5 mM MgCl2, 0.25 mM sucrose, 1 mM phenylmethylsulfonyl fluoride) and lysed by 5 times freeze/thaw cycles. After centrifugation at 9,000 × g for 10 min at 4 °C, the protein content was washed with TKMS, and supernatant was collected and concentrated by lyophilization. The sample was centrifuged at 9,000 × g for 10 min at 4 °C and the supernatant was collected as a membrane fraction.

Proteins in the membrane fraction were precipitated by cold acetone and dissolved in 1% SDS. Fifty μg of membrane proteins from each transfected cell were separated by SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane by electroblotting.
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RESULTS

Enzymatic Activity of hCGn6ST—hCGn6ST has high homology to human and mouse intestinal GlcNAc 6-O-sulfotransferases and belongs to the carbohydrate sulfotransferase family (22) (Fig. 1). All sulfotransferases in this family have an activity that transfers sulfate to C-6 of GlcNAc, Gal, or GalNAc. Furthermore, mutations in the gene encoding hCGn6ST cause MCD, a hereditary eye disease in which patients lack sulfated keratan sulfate in the cornea and serum (22). Therefore, we hypothesized that hCGn6ST is a sulfotransferase that catalyzes sulfation of the C-6 of GlcNAc in keratan sulfate. To examine the enzymatic activity of hCGn6ST, HeLa cells were transfected with expression vectors harboring cDNAs encoding intact or soluble hCGn6ST, and enzyme fractions were prepared for analyzing of sulfotransferase activity. In vitro analysis showed that the enzyme fraction from intact hCGn6ST DNA transfectant has sulfotransferase activity that transfers sulfate from PAPS to a substrate, GlcNAc 6-O-sulfotransferase activity that transfers sulfate to C-6 of GlcNAc, Gal, or GalNAc.

We next analyzed the structure of sulfated carbohydrates produced by transfected cells with sulfotransferases. HeLa...
cells were transfected with expression vectors harboring cDNAs encoding hCGn6ST and a sulfotransferase, hKSG6ST, that transfers sulfate onto C-6 of Gal in keratan sulfate (14). The transfected cells were metabolically labeled with [35S]sulfate, and carbohydrates isolated from those cells were analyzed by keratanase treatment. Keratanase recognizes the disaccharide repeat of keratan sulfate that consists of unsulfated Gal connected to sulfated GlcNAc and cleaves the Galβ1–4GlcNAc(6S) linkage (30). A carbohydrate with the same backbone as keratan sulfate but with sulfate on C-6 of Gal or no sulfate on GlcNAc cannot be digested by keratanase. Carbohydrates isolated from HeLa cells transfected with the pcDNA3 vector alone or with pcDNA3-hKSG6ST were resistant to keratanase treatment (Fig. 3). This finding is consistent with the substrate specificity of keratanase and indicates that HeLa cells express no endogenous sulfotransferases that produce keratan sulfate. On the other hand, keratanase treatment of samples from HeLa cells transfected with pcDNA3-hCGn6ST produced fragments that eluted in fractions 57–72 from a Sephadex G-50 column (Fig. 3). Carbohydrates from the hCGn6ST-expressing cells digested with keratanase were separated into two populations, fractions 63–66 and 68–72, by Sephadex G-50 chromatography. Keratanase-sensitive carbohydrates were also found in cells co-transfected with pcDNA3-hCGn6ST and pcDNA3-hKSG6ST. However, the resultant carbohydrate fragments by keratanase digestion were seen as one broad peak, in contrast to the two peaks observed in hCGn6ST-expressing cells following the same chromatography (Fig. 3). These results suggest that hCGn6ST has activity that transfers sulfate to C-6 of GlcNAc on poly-N-acetyllactosamine chains resulting in production of keratan sulfate. The data also suggest that co-expression of hCGn6ST and hKSG6ST in HeLa cells produces highly sulfated keratan sulfate with sulfate residues not only on GlcNAc but also on C-6 of Gal, making it less sensitive to keratanase.

To confirm that hCGn6ST produces keratan sulfate, we analyzed the carbohydrate structure of keratanase-digested fragments obtained from cells transfected with pcDNA3-hCGn6ST. Two carbohydrate fractions (pools I and II in Fig. 3) were analyzed by Sephadex G-50 gel filtration and SAX-10 anion exchange HPLC. By HPLC analysis, the carbohydrate in pool I was eluted at the 12.5-min retention position (Fig. 4A). This retention time was identical to a carbohydrate standard, GlcNAc(6S)/β1–3Gal, that was prepared from bovine corneal keratan sulfate by keratanase treatment (30). In contrast, carbohydrate in pool II did not elute at the position of known standards. We further analyzed its carbohydrate structure by mild acid and exo-β-galactosidase treatment. The carbohydrate in pool II was cleaved by mild acid treatment that releases sialic acid from carbohydrate chains (Fig. 5B). The de-sialylated carbohydrate was further digested by exo-β-galactosidase (Fig. 5C). This product, which was derived from pool II by de-sialylation and de-galactosylation, eluted at the 12.5-min retention position that was identical to the carbohydrate in pool I and the standard monosulfated disaccharide by SAX-10 HPLC (Fig. 4C). From these results, we conclude that the carbohydrate in pool II originated from sulfated poly-N-acetyllactosamine chains, which have sulfate residue on C-6 of GlcNAc, with a sialylgalactose on their non-reducing terminal. These findings indicate that hCGn6ST has sulfotransferase activity that transfers sulfate to C-6 of GlcNAc in poly-N-acetyllactosamine and results in production of keratan sulfate.

Co-expression of hCGn6ST and hKSG6ST Produces Highly Sulfated Keratan Sulfate—hKSG6ST transfers sulfate to the
C-6 of Gal in keratan sulfate (14), and HeLa cells expressing both hCGn6ST and hKSG6ST produced sulfated carbohydrate less sensitive to keratanase treatment than carbohydrate produced in cells expressing hCGn6ST alone (Fig. 3). Therefore, we assumed that co-expression of both hKSG6ST and hCGn6ST results in highly sulfated keratan sulfate. To confirm this hypothesis, we compared the reactivity of carbohydrates from transfected cells to the mouse monoclonal antibody, 5D4, that detects keratan sulfate in a variety of tissues (29, 31). The minimum epitope recognized by this antibody is a linear pentasulfated hexasaccharide (32). A longer epitope reacts with this antibody more effectively than the shorter one (32), and 5D4 does not recognize desulfated keratan sulfate (33, 34). First, we performed Western blot analysis against proteoglycans prepared from HeLa cells transfected with the expression vectors. The SDS-PAGE pattern of proteins stained by Coomassie Blue showed no differences among these transfected HeLa cells (Fig. 6A, lanes a–d). However, the 5D4 antibody detected proteoglycans extracted only from hCGn6ST/hKSG6ST co-expressing cells (Fig. 6A, lane h). Furthermore, immunostaining of transfected HeLa cells by the 5D4 antibody gave positive signals only when wild type hCGn6ST and hKSG6ST were co-expressed but not when mutant forms of hCGn6ST plus hKSG6ST were co-expressed (Fig. 7). These indicate that missense mutations of hCGn6ST found in MCD patients result in a failure of synthesizing highly sulfated keratan sulfate and suggest that lack of sulfation on GlcNAc in keratan sulfate leads to the MCD phenotype.
transfected HeLa cells with anti-keratan sulfate antibody. Keratan sulfate production in transfected HeLa cells was analyzed by Western blotting (A) and immunostaining (B). A, HeLa cells were transfected with pcDNA3 (lanes a and c), pcDNA3-hCGn6ST (lanes b and f), pcDNA3-hKSG6ST (lanes e and g), and both pcDNA3-hCGn6ST and pcDNA3-hKSG6ST vectors (lanes d and h). Membrane protein fractions were prepared from those transfectants and separated by SDS-PAGE followed by Coomassie Blue staining (lanes a–d) or Western blotting using an anti-keratan sulfate antibody, 5D4 (lanes e–h). B, transfected cells were analyzed by immunocytochemistry using the 5D4 antibody. Each panel shows HeLa cells transfected with pcDNA3 (lane a), pcDNA3-hCGn6ST (lane b), pcDNA3-hKSG6ST (lane c), and both pcDNA3-hCGn6ST, and pcDNA3-hKSG6ST (lane d). Bar in lane a is 20 μm.

hCGn6ST in the human cornea (22), suggesting that the mIGn6ST plays the same role as hCGn6ST does, and is involved in the production of keratan sulfate in the mouse cornea.

**DISCUSSION**

In this report, we demonstrate that hCGn6ST transfers sulfate onto C-6 of GlcNAc in poly-N-acetyllactosamine chain catalyzing the synthesis of keratan sulfate. So far, eight GlcNAc 6-O-sulfotransferases have been cloned and characterized (16–18, 22, 27, 35, 36). Prior to the molecular characterizations of these sulfotransferases, biochemical analyses suggested that a GlcNAc 6-O-sulfotransferase adds sulfate only to the non-reducing terminal GlcNAc of carbohydrate chains (37). Uchimura et al. (16, 27) reported that both GlcNAc 6-O-sulfotransferase-1 and -4 (also named chondroitin 6-O-sulfotransferase-2) transfer sulfate to the non-reducing terminal but not to internal GlcNAc. Therefore, it is possible that hCGn6ST also transfers sulfate onto C-6 of non-reducing terminal GlcNAc during keratan sulfate chain synthesis. Previous reports suggested that sulfation of GlcNAc residues in keratan sulfate is coupled to the elongation step of carbohydrate chain synthesis (38–40), supporting our hypothesis.

Another keratan sulfate sulfotransferase, hKSG6ST, transfers sulfate onto the C-6 of Gal in keratan sulfate. Since hKSG6ST preferentially adds sulfate to a Gal residue adjacent to the sulfated GlcNAc in poly-N-acetyllactosamine chain, the sulfation of GlcNAc 6-O-sulfotransferase activity is the major cause of decreases in keratan sulfate production in cultured chick corneal stromal cell (41). GlcNAc 6-O-sulfotransferase is thought to be a critical enzyme in keratan sulfate biosynthesis. Indeed, we found mutations in CHST6, the gene encoding hCGn6ST, in MCD patients who produce no keratan sulfate in their cornea and serum (22). It is therefore likely that the sulfation of GlcNAc in keratan sulfate takes place during the elongation of the poly-N-acetyllactosamine chains. The sulfation of GlcNAc may be required for the sulfation of Gal by hKSG6ST.

By structural analysis, we found that HeLa cells expressing hCGn6ST produced sulfated poly-N-acetyllactosamine that has sulfate on GlcNAc and a sialylgalactose on its non-reducing terminal. Huckerby et al. (42) found a keratan sulfate structure in which the non-reducing terminal is capped by sialic acid in bovine cartilage. This is identical structure to that we found. Oebein et al. (40), however, reported a biantennary complex type structure that has a sialylgalactosyl N-acetyllactosamine

**FIG. 6.** Western blot analysis and immunocytochemistry of transfected HeLa cells with anti-keratan sulfate antibody. Keratan sulfate production in transfected HeLa cells was analyzed by Western blotting (A) and immunostaining (B). A, HeLa cells were transfected with pcDNA3 (lanes a and c), pcDNA3-hCGn6ST (lanes b and f), pcDNA3-hKSG6ST (lanes e and g), and both pcDNA3-hCGn6ST and pcDNA3-hKSG6ST vectors (lanes d and h). Membrane protein fractions were prepared from those transfectants and separated by SDS-PAGE followed by Coomassie Blue staining (lanes a–d) or Western blotting using an anti-keratan sulfate antibody, 5D4 (lanes e–h). B, transfected cells were analyzed by immunocytochemistry using the 5D4 antibody. Each panel shows HeLa cells transfected with pcDNA3 (lane a), pcDNA3-hCGn6ST (lane b), pcDNA3-hKSG6ST (lane c), and both pcDNA3-hCGn6ST, and pcDNA3-hKSG6ST (lane d). Bar in lane a is 20 μm.

**FIG. 7.** Immunocytochemistry of HeLa cells transfected with mutant hCGn6STs. A vector expressing wild type (a) or missense mutants of hCGn6ST (b, R50C, c, K174R, d, D203E, e, R211W, f, A217T, and g, R274K) was transfected together with pcDNA3-hKSG6ST into HeLa cells. After transfection, cells were stained with the 5D4 antibody. Bar in a is 20 μm.
and sulfated poly-N-acetyllactosamine without sialylation on its non-reducing terminal in pig corneal keratan sulfate. This discrepancy may be due to tissue differences because corneal cells produce N-linked keratan sulfate proteoglycans rather than the O-linked type found in cell types such as cartilage. HeLa cells transfected with hCGn6ST cDNA may produce O-linked keratan sulfate proteoglycan, similar to cartilage tissue.

In the present study, we confirmed that missense mutations in CHST6 found in MCD patients abolish the sulfotransferase activity of the encoded protein (Fig. 7). It is possible that the mutations cause rapid degradation or intracellular mislocalization of the protein instead of functional inactivation. However, since all of the missense mutations examined, except for A217T, substituted the amino acids conserved among carbohydrate sulfotransferases (Fig. 1), it is likely that these residues are necessary for hCGn6ST enzymatic activity. These amino acids may also be important for other carbohydrate sulfotransferases. Structure analysis (20, 21) suggested such conserved motifs form binding domains for a sulfate donor, PAPS, and site-directed mutagenesis of the PAPS-binding motifs of HKN-1 sulfotransferase result in marked decreases in its enzymatic activity (26). Therefore, the mutations found in MCD patients are likely to cause inactivation of the enzyme rather than protein degradation or mislocalization.

The A217T missense mutation does not occur in motifs conserved among sulfotransferases (Fig. 1B). Because hCGn6ST with this mutation had no activity for keratan sulfate production (Fig. 7), and mIGn6ST, which has the same enzymatic activity as hCGn6ST, conserves the alanine residue at the identical position, this amino acid must be important for sulfotransferase activity. A217 may be required for recognition of specific carbohydrate structure for a substrate.

mIGn6ST is highly homologous to hIGn6ST, whose substrate specificity is not known (18). In this report, we demonstrate that mIGn6ST, but not hIGn6ST, has the same enzymatic activity as hCGn6ST (Fig. 8). The expression pattern of mIGn6ST mRNA (Fig. 9c) is also similar to that of hCGn6ST in corneal cells (22), suggesting that mIGn6ST is the mouse orthologue of hCGn6ST. In humans, genes encoding hCGn6ST and hIGn6ST are homologous not only in the coding region but also in the 5′- and 3′-flanking sequences (22), suggesting that the two genes may have been produced by gene duplication. We found no other mouse EST with homology to hCGn6ST higher than that of hIGn6ST in the GenBank EST data base (release 120). It is possible that the mouse genome encodes only one GlcNAc 6-O-sulfotransferase gene involved in keratan sulfate production and that during evolution the gene was duplicated to produce CHST5 and CHST6, each of which encodes hIGn6ST and hCGn6ST, respectively, in the human genome. Sequence information of the flanking regions of Chst5, which encodes mIGn6ST protein, is required to confirm this hypothesis. Because the specific carbohydrate substrate of hCGn6ST has not been identified, it is not known whether mIGn6ST has an additional enzymatic activity that is similar to hIGn6ST activity. It is possible that the mouse sulfotransferase has dual enzymatic activities and the human homologues recognize each specific substrate.

In this report, we demonstrate that hCGn6ST and mIGn6ST have enzymatic activity that produces keratan sulfate in cooperation with hKS6ST. The Chst5 knockout mouse may show a phenotype similar to MCD patients and is likely to be a useful animal model for further studies of the corneal dystrophy.

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Fig. 8. Immunocytochemistry of HeLa cells transfected with human and mouse IGN6STs. The expression vector pcDNA3 (a and e), pcDNA3-hCGn6ST (b and f), pcDNA3-hIGn6ST (c and g), and pcDNA3-mIGn6ST (d and h) were transfected together with (e–h) or without (a–d) pcDNA3-hKS6ST into HeLa cells. Transfectants were stained with the anti-keratan sulfated antibody 5D4. Bar in a is 20 μm.

Fig. 9. Immunohistochemistry and in situ hybridization of keratan sulfate and mIGn6ST mRNA in the mouse cornea. Serial sections of corneal tissues were sequentially analyzed by immunohistochemistry (a and b) and in situ hybridization (c and d). The clefts in the stroma are artifacts produced during tissue processing. Specimens were stained with (a) or without (b) the 5D4 antibody for immunohistochemistry and were stained with mIGn6ST antisense (c), or a sense (d) probe for in situ hybridization. ep, epithelium; st, stroma; en, endothelium. Bar in d is 50 μm.
Human Corneal GlcNAc 6-O-Sulfotransferase and Mouse Intestinal GlcNAc 6-O-
Sulfotransferase Both Produce Keratan Sulfate

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