N-Linked Oligosaccharides on Chondroitin 6-Sulfotransferase-1 Are Required for Production of the Active Enzyme, Golgi Localization, and Sulfotransferase Activity toward Keratan Sulfate*

Received for publication, January 6, 2006, and in revised form, April 19, 2006. Published, JBC Papers in Press, May 22, 2006, DOI 10.1074/jbc.M600140200

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We have shown previously that purified chondroitin 6-sulfotransferase-1 (C6ST-1) was a glycoprotein abundant in N-linked oligosaccharides and could sulfate both chondroitin (C6ST activity) and keratan sulfate (KSST activity); however, functional roles of the N-glycans have remained unclear. In the present study, we show essential roles of N-glycans attached to C6ST-1 in the generation of the active enzyme and in its KSST activity. Treatment with tunicamycin of COS-7 cells transfected with C6ST-1 cDNA totally abolished production of the active C6ST-1. A nearly complete removal of N-glycans of the recombinant C6ST-1 by peptide N-glycosidase F increased the C6ST activity but decreased the KSST activity. Among six potential N-glycosylation sites, deletion of the fourth or sixth site from the amino terminus inhibited production of the active C6ST-1, whereas deletion of the fifth site resulted in a marked loss of the KSST activity. Wild-type recombinant C6ST-1 showed a typical Golgi localization, whereas M-4 recombinant C6ST-1, in which the fourth N-glycosylation site was deleted, colocalized with calnexin, an endoplasmic reticulum-resident protein. Unlike wild-type recombinant C6ST-1, M-4 recombinant C6ST-1 showed a weak affinity toward wheat germ agglutinin and was converted completely to the nonglycosylated form by endoglycosidase H. These observations suggest that N-glycan attached to the fourth N-glycosylation site may function in the proper processing of N-glycans for the Golgi localization, thereby causing the production of the active C6ST-1, and that N-glycan attached to the fifth N-glycosylation site may contribute to the KSST activity of C6ST-1.

Chondroitin sulfate (CS) chains attached to various proteoglycans are composed of GlcAβ1–3GalNAcβ1–4 repeating...
found to be glycoproteins containing relatively abundant N-linked oligosaccharides. It has been reported that N-glycans contained in various glycoproteins are involved in protein folding, ER-associated protein degradation, intracellular trafficking, and production of functional proteins (21–25). We have previously shown that N-glycans attached to C6ST-1 are involved in the production and stability of the active enzyme (26). However, functional roles of N-glycans attached to these sulfotransferases has not been fully understood. In the present study, we examined the roles of N-glycans attached to C6ST-1 and found that N-glycans on these sulfotransferases has not been fully understood.

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### Materials

The following commercial materials were used. H$_2$SO$_4$ was from PerkinElmer Life Sciences; chondroitinase ACII, chondroitinase ABC, keratanase II, KS (bovine cornea), 2-acetamide-2-deoxy-3-O-β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose, and 2-acetamide-2-deoxy-3-O-β-D-gluco-4-enepyranosyluronic acid)-D-galactose were from Seikagaku Corp. (Tokyo, Japan); unlabelled PAPS, anti-FLAG monoclonal antibody, FLAG peptide, anti-FLAG mAb-conjugated agarose, pFLAG-CMV2, Dulbecco’s modified Eagle’s medium, fetal bovine serum, TLCK, TPCK, phenylmethlylsulfonyl fluoride, pepstatin A, and TRITC-conjugated anti-rabbit antibody were from Seikagaku Corp. (Tokyo, Japan). Materials—The following commercial materials were used. H$_2$SO$_4$ was from PerkinElmer Life Sciences; chondroitinase ACII, chondroitinase ABC, keratanase II, KS (bovine cornea), 2-acetamide-2-deoxy-3-O-β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose, and 2-acetamide-2-deoxy-3-O-β-D-gluco-4-enepyranosyluronic acid)-D-galactose were from Seikagaku Corp. (Tokyo, Japan); unlabelled PAPS, anti-FLAG monoclonal antibody, FLAG peptide, anti-FLAG mAb-conjugated agarose, pFLAG-CMV2, Dulbecco’s modified Eagle’s medium, fetal bovine serum, TLCK, TPCK, phenylmethlylsulfonyl fluoride, pepstatin A, and TRITC-conjugated anti-rabbit IgG goat antibody were from Sigma; peptide N-glycosidase F (PNGase F) and endoglycosidase H (Endo H) were from Roche Applied Science; Hidload Superdex 30 HR 16/60 and Fast Desalting Column HR 10/10, ECL detection kit, and Hyperfilm ECL were from Amersham Biosciences; WGA-agarose was from Vector Laboratories (Burlingame, CA); anti-calnexin C terminus rabbit polyclonal antibody was from Stressgen Biotechnology (Victoria, Canada); anti-α mannosidase II rabbit antiserum was from Abcam (Cambridge, UK); Alexa Fluor® 488-conjugated anti-mouse IgG goat antibody was from Molecular Probes, Inc. (Eugene, OR); Cellstain- Hoechst 33258 solution was from Dojindo Laboratories (Kumamoto, Japan). [35S]PAPS was prepared as described (27). Chondroitin (squinoid) skin was prepared as described (28).

### Functional Role of N-Glycans Attached to C6ST-1

| TABLE 1
| Nucleotide sequences of oligonucleotide primers used for introduction of mutation by site-directed mutagenesis |
|-----------------|-----------------|-----------------|
| Alterations in amino acids | Codon changes | Nucleotide sequences of primers |
|-----------------|-----------------|-----------------|
| N63S | AAC to AGC | CTAATTTTTGTCGAGATCGCAGCGACACTGGACCAGCCCCTGAC-3’ (42-mer) |
| N74S | AAT to AGT | CACACGGCTTCCTATCGAGAAGTGGCAGCATCTCCTTTGACTCC-3’ (36-mer) |
| N96S | AAC to AGC | GCCGGCGGTCCAGCCGACCATGGAAGTGGCAGCATCTCCTTTGACTCC-3’ (36-mer) |
| N250S | AAC to AGC | CACACGGCTTCCTATCGAGAAGTGGCAGCATCTCCTTTGACTCC-3’ (36-mer) |
| N413S | AAC to AGC | GCCGGCGGTCCAGCCGACCATGGAAGTGGCAGCATCTCCTTTGACTCC-3’ (36-mer) |
| N457S | AAC to AGC | GCCGGCGGTCCAGCCGACCATGGAAGTGGCAGCATCTCCTTTGACTCC-3’ (36-mer) |

### EXPERIMENTAL PROCEDURES

Amplification was carried out by 35 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 5.5 min. The second PCR was carried out using oligonucleotides mC6ST-1-F2 (GAGAATTCCGATGGAGAAAAAGACTCGC) and mC6ST-1-R2 (GCAGACGTTCATCGATGCCAAGAACTGGTGCC) as primers and the first PCR mixture as the template. At the 5’-ends of oligonucleotides mC6ST-1-F2 and mC6ST-1-R2, restriction enzyme recognition sites were introduced, an EcoRI site for mC6ST-1-F2 and a HindIII site for mC6ST-1-R2. Amplification was carried out by 35 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 3 min 10 s. The reaction products were subjected to agarose gel electrophoresis. The amplified DNA band was cut out, and the DNA fragment was recovered from the gel, digested with HindIII and EcoRI, and subcloned into these sites of pFLAG-CMV-2 plasmid. Recombinant mC6ST-1 was expressed in COS-7 cells as a fusion protein with FLAG peptide and was affinity-purified as described below. Side-directed mutagenesis was carried out by PCR using a QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer’s instructions. Putative N-glycosylation sites, the Asn-X-(Ser/Thr) motif, in which X is any amino acid except for Pro, were disrupted by substituting asparagine (Asn$^63$, Asn$^74$, Asn$^96$, Asn$^{250}$, Asn$^{413}$, and Asn$^{457}$) with serine. The nucleotide sequences of all of the mutated cDNAs were confirmed by the sequencing. The sequences of all of the PCR primers used for the site-directed mutagenesis are shown in Table 1. All of the mC6ST-1 constructs have a FLAG tag at the N-terminal end.

**Cell culture and transfection of COS-7 or Chinese Hamster Ovary Cells**—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 50 μg/ml streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum. CHO-K1 cells were cultured in F-12 medium containing the antibiotics and fetal bovine serum as above. These cells were grown at 37 °C in 5% CO$_2$, 95% air. The plasmids prepared as above were transfected into COS-7 cells or CHO-K1 cells by the lipofection method using TransFast reagent (Promega) under serum-free conditions according to the manufacturer’s instructions. At 48 h after the addition of cDNA, the transfectants were extracted with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.2, 10 mM
MgCl₂, 2 mM CaCl₂, 0.5% (v/v) Triton X-100, 20% (v/v) glycerol, and protease inhibitors (50 μM TLCK, 30 μM TPCK, 300 μM phenylmethylsulfonyl fluoride, and 3 μM pepstatin A) for 30 min on a rotatory shaker. The lysates were clarified by centrifugation at 10,000 × g for 10 min, and the supernatants were applied to an anti-FLAG mAb-conjugated agarose column. The absorbed materials were eluted with a buffer containing FLAG peptide under the conditions recommended by the manufacturer. The purified protein solution (1.5 ml) was used to determine C6ST and KSST activity. Under the standard assay conditions, C6ST activity of the affinity-purified wild-type recombinant C6ST-1 ranged from 100 to 500 pmol/min/ml among different preparations. The KSST activity of each preparation was ~30% of C6ST activity.

Treatment with Tunicamycin—To determine the effects of tunicamycin, tunicamycin was added to the culture medium after transfection at the final concentrations indicated in Fig. 1, and the culture was continued for a further 48 h. The cellular extracts were prepared as described above.

Western Blot Analysis—SDS-PAGE was carried out according to Laemmli (30). The affinity-purified proteins before or after PNGase F or Endo H digestion as described below were boiled in the sample buffer with 10% (w/v) 2-mercaptoethanol and then separated by 10% gels. Proteins were electrophoretically transferred to Hybond ECL® membranes (Amersham Biosciences) at 60 V for 4 h. For immunoblotting, nonspecific binding sites were blocked with 5% (w/v) skim milk in TBST (20 mM Tris-HCl, pH 7.6, 130 mM NaCl, and 0.1% (v/v) Tween 20) at 4 °C for 16 h. The FLAG-tagged recombinant proteins were stained with an anti-FLAG M2 mAb at a 1:10,000 dilution. The membranes (Amersham Biosciences) were run in every gel. Protein bands on the Western blot detected by anti-FLAG antibody. To determine C6ST activity in the presence of KS, the 35S-labeled glycosaminoglycans were digested with chondroitinase ABC, and the resulting unsaturated disaccharides were separated by paper chromatography, as described above. The radioactivity of the ethanol-soluble fractions was determined.

Digestion with PNGase F, Endo H, Chondroitinase ACII, Chondroitinase ABC, and Keratanase II—The affinity-purified recombinant C6ST-1 with or without mutations was precipitated with 3 volumes of ethanol containing 1.3% (w/v) potassium acetate. The precipitates were dissolved in 4 μl of buffer 1 (0.5% SDS, 0.15 M Tris-HCl, pH 7.8) for PNGase F digestion or 4 μl of buffer 2 (0.1 M sodium acetate, pH 5.2, 0.05% SDS, 0.2 M 2-mercaptoethanol) for Endo H digestion and heated at 100 °C for 2 min. After cooling, 4 μl of buffer 3 (3.2 mM phenylmethylsulfonyl fluoride, 4% Nonidet P-40, and 32 mM EDTA, pH 8.0) including 0.5 units of PNGase F or 0.5 units of Endo H were added to the mixture and incubated at 37 °C for 18 h. After digestion, the samples were subjected to SDS-PAGE as described above. Digestion of the recombinant protein with PNGase F under nondenaturing conditions was carried out at 37 °C in the buffer used for the extraction of the cells as described above. Digestion with chondroitinase ACII or chondroitinase ABC was carried out for 4 h at 37 °C in the reaction mixture containing, in a final volume of 25 μl, 1.25 μmol of Tris acetate buffer, pH 7.5, 2.5 μg of bovine serum albumin, and 30 milliunits of chondroitinase ACII or chondroitinase ABC. Digestion with keratanase II was carried out for 24 h at 37 °C in the reaction mixture containing, in a final volume of 50 μl, 0.005 units of keratanase II and 2.5 μmol of acetate buffer, pH 6.5 (33, 34).

Immunocytochemistry—COS-7 cells were plated on the coverslips in 35-mm culture dishes at a density of 1.0 × 10⁵ cells/dish. Cells were transfected as above. At 24 h after transfection, cells were fixed by ice-cold methanol for 30 min. After washing with phosphate-buffered saline (PBS) several times, the cells were blocked with 0.1% Tween 20 in PBS (PBST) containing 5% BSA. After washing with PBS, the cells were incubated with a mixture of anti-FLAG monoclonal antibody (1:500 dilution in 5% BSA-PBST) and anti-calnexin C terminus rabbit polyclonal antibody.
antibody (1:200 dilution in 5% BSA-PBST) or a mixture of anti-FLAG monoclonal antibody (1:500 dilution in 5% BSA-PBST) and anti-β1/2mannosidase II rabbit antiserum (1:200 dilution in 5% BSA-PBST) for 90 min. They were then washed with PBS and further incubated with Alexa Fluor 488-conjugated anti-mouse IgG goat antibody (1:400 dilution in 5% BSA-PBST) and TRITC-conjugated anti-rabbit IgG goat antibody for 1 h. To stain the nucleus, Hoechst 33258 was added to the second antibody solution at a final concentration of 5 µg/ml. The cells were washed with PBS, mounted in glycerol containing Mowiol 4–88 (Calbiochem) and 1% n-propyl gallate (Sigma), and observed by an Olympus fluorescence microscope.

RESULTS

Effects of Tunicamycin on the Formation of the Active C6ST-1

To determine the effects of N-glycans on the generation of the active C6ST-1, we cultured COS-7 cells transfected with C6ST-1 cDNA in the medium containing various concentrations of tunicamycin. In the presence of 0.05 µg/ml tunicamycin, only a protein band of 54 kDa that corresponds to the core protein without N-glycans was detected (Fig. 1B, lane 4), indicating that attachment of N-glycans to C6ST-1 was totally abolished. Under such conditions, C6ST activity was lost (Fig. 1A). These results indicate that attachment of N-glycans is essential for the generation of the active C6ST-1. In the presence of 0.01 µg/ml tunicamycin, C6ST activity was fully retained, whereas the expressed protein exhibited multiple discrete bands on the Western blot (Fig. 1B, lane 3). These discrete protein bands are thought to correspond to different glycoforms of C6ST-1 bearing different numbers of N-glycans, because these protein bands were converted to the nonglycosylated form after the PNGase F digestion (data not shown). Partial loss of N-glycans in the presence of 0.01 µg/ml of tunicamycin may not affect the production of the active C6ST-1 significantly.

Treatment of the Recombinant C6ST-1 with PNGase F—To obtain information about the effect of N-glycans attached to C6ST-1 on the enzyme activity, we digested the recombinant C6ST-1 with PNGase F under the nondenaturation conditions and determined C6ST activity (Fig. 2A, open bar) and KSST activity (Fig. 2A, closed bar) of the preincubated proteins was determined. The recombinant protein was analyzed by Western blot after incubation in the absence (B, lane 1) or presence (B, lane 2) of PNGase F. Sizes of the prestained molecular size standards are indicated at the left, and the core protein of FLAG-C6ST-1 is indicated by an arrowhead.
digestion, the glycosylated protein bands were detected as a single band at 67 kDa with a diffused band larger than 67 kDa (Fig. 2B, lane 1). These protein bands disappeared after PNGase F digestion, and a main protein band of 54 kDa and a faint smear larger than 54 kDa appeared (Fig. 2B, lane 2). Because the molecular mass of the recombinant C6ST-1 calculated from the cDNA is 53996, the main protein band detected after PNGase F digestion should correspond to the core protein without N-glycans, indicating that N-glycans attached to the recombinant C6ST-1 appeared to be removed nearly completely by PNGase F digestion under the non-denaturation conditions. On the other hand, C6ST activity was not decreased but rather increased after PNGase F digestion (Fig. 2A). These results suggest that N-glycans attached to C6ST-1 are not necessarily required for C6ST activity if the active enzyme is once generated. We previously reported that C6ST-1 could transfer sulfate not only to chondroitin but also to KS. Unlike C6ST activity, the activity toward KS (KSST activity) was decreased after PNGase F digestion (Fig. 2). Unlike C6ST activity, the activity toward KS (KSST activity) was decreased after PNGase F digestion (Fig. 2). The roles of N-glycans on KSST activity will be discussed below.

**Effects of Deletion of One N-Glycosylation Site**—There are six potential N-glycosylation sites in C6ST-1, Asn63, Asn74, Asn96, Asn250, Asn413, and Asn457. To evaluate contribution of the individual N-glycan on these sites to the generation of the active C6ST-1, we constructed expression vectors in which the Asn residue of each N-glycosylation site was mutated to Ser and transfected into COS-7 cells. In Fig. 3, structures and names of these mutant cDNAs are shown. The C6ST activity of the expressed proteins was determined after the FLAG affinity purification (Fig. 4A, open bars). A marked decrease in the C6ST activity was observed in M-4 and M-6 recombinant proteins. When the recombinant proteins were detected with the anti-FLAG antibody after PNGase F digestion, only a protein band of 54 kDa was detected in every mutant recombinant protein at the same density as that of the wild-type recombinant protein (Fig. 4C), indicating that the introduction of mutation into any N-glycosylation site did not cause degradation of the recombinant proteins or alteration in the expression level of these proteins. Before PNGase F digestion, broad protein bands were detected at the migration position larger than 67 kDa in wild-type, M-1, M-2, M-3, and M-5 mutant proteins in addition to a main band of 67 kDa. The broad protein bands were not observed in M-4 and M-6 mutant protein (Fig. 4B), suggesting that the degree of microheterogeneity of N-glycans may be decreased in M-4 and M-6 mutant proteins. When the recombinant proteins were detected with the anti-FLAG antibody after Endo H digestion, the broad protein bands were still observed in wild-type, M-1, M-2, M-3, and M-5 mutant protein in addition to the 54-kDa band that corresponded to the core protein of C6ST-1. On the other hand, only the 54-kDa band was detected in M-4 and M-6 mutant proteins (Fig. 4D). Essentially the same results were obtained when CHO-K1 cells were used (data not shown). Thus, the decrease in C6ST activity of
M-4 and M-6 mutant proteins appears to be accompanied with the incomplete processing of N-glycans.

WGA-Agarose Chromatography—The Western blot analysis of the recombinant proteins described above suggest that structural alterations may have arisen in N-glycans of M-4 and M-6 mutant proteins. To confirm such a possibility, we examined the affinity of the recombinant proteins to WGA-agarose. We previously reported that C6ST-1 secreted to the culture medium of chick embryo chondrocytes was bound to WGA-agarose and was eluted with a buffer containing GlcNAc. As shown in Fig. 5A, the wild-type recombinant protein was bound completely to the WGA-agarose and eluted with 0.5 M GlcNAc as observed in the purified chick C6ST-1. As shown in Fig. 5D, the pass-through fractions without C6ST activity contained a 54-kDa protein corresponding to the core protein, whereas the fractions eluted with 0.5 M GlcNAc contained a 67-kDa protein and diffused bands larger than 67 kDa. These observations clearly indicate that the active C6ST-1 bears N-glycans that were processed to the forms with affinity toward WGA. In contrast, when M-4 mutant protein was applied to the WGA-agarose column, more than 50% of the C6ST activity was recovered in the pass-through fractions (Fig. 5B), indicating that structures of N-glycans of M-4 mutant protein should be altered so that the affinity to WGA-agarose became lower. M-6 mutant protein behaved similarly to the wild-type recombinant proteins, albeit the proportion of the activity recovered in the pass-
Functional Role of N-Glycans Attached to C6ST-1

Effects of Deletion of 3–6 N-Glycosylation Sites—To clarify the minimal requirement of N-glycans for the production of C6ST activity, N-glycans attached to C6ST-1 were deleted in several combinations (Fig. 6). It is evident from the Western blot shown in Fig. 6B that each N-glycosylation site from N-4 to N-6 was actually glycosylated. N-4 and N-6 site were glycosylated because both M-12356 (lane 3) was larger than either M-12356 (lane 4) or M-12345 (lane 5); the N-5 glycosylation site was glycosylated because M-123 (lane 2) was larger than M-1235 (lane 3). C6ST activities of M-12356, M-12345, and M-123456 were not detected at all, whereas C6ST activity of M-1235 was 25% of C6ST activity of the wild-type recombinant C6ST-1 (Fig. 6A, open bar), indicating that the existence of N-glycans on both the N-4 and N-6 glycosylation sites is essential and sufficient for the production of the active C6ST-1.

As described above, N-glycans of M-4 or M-6 mutant recombinant proteins were completely removed by Endo H digestion, although some part of the N-glycans on the wild-type recombinant protein forming the larger smear band remained intact after Endo H digestion. These observations raised a possibility that the N-glycan attached to the N-4 or N-6 glycosylation site itself might be processed to an Endo H-resistant form. Alternatively, N-glycosylation of the N-4 or N-6 glycosylation site might influence the processing of N-glycans attached to the N-glycosylation sites other than the N-4 or N-6 site. To determine which possibility is the case, we examined the Western blot before (Fig. 6B) or after (Fig. 6D) Endo H digestion. No broad protein bands were observed in M-1235 (lane 3), M-12356 (lane 4), and M-12345 (lane 5) before or after Endo H digestion. These results support the latter idea that N-glycosylation of N-4 or N-6 site may affect the processing of the other N-glycans.

Effects of N-Glycans Attached to N-4 and N-6 Glycosylation Sites on the Intracellular Localization—It has been observed in various glycoproteins that intracellular localization of glycoproteins depended on the presence or absence of N-glycans (35–37). N-Glycans attached to the N-4 or N-6 glycosylation site of C6ST-1 may also affect the proper intracellular localization of C6ST-1 and thereby contribute to the production of the active C6ST-1. To investigate such a possibility, we stained the cells transfected with wild-type, M-4, or M-6 cDNA with anti-FLAG mAb (Figs. 7 and 8). The wild-type recombinant protein showed condensed perinuclear localization characteristic of Golgi-resident proteins and colocalized through fractions was slightly larger than that of the wild-type recombinant protein (Fig. 5C).
well with the Golgi marker protein, mannosidase II (Fig. 7, a–d) but only partially overlapped with the ER marker protein, calnexin (Fig. 8, a–d). On the other hand, M-4 mutant protein showed diffused localization around the nucleus and only partially colocalized with the Golgi marker protein (Fig. 7, e–h), but colocalized well with the ER marker protein (Fig. 8, e–h). These observations imply that the wild-type recombinant C6ST-1 may be localized at the Golgi apparatus, but the major part of the M-4 mutant protein may still be located at the ER.

The intracellular localization of M-6 mutant protein was very similar to that of the wild-type recombinant C6ST-1, although the overlap with mannosidase II (Fig. 7, i–l) and with calnexin (Fig. 8, i–l) was slightly less clear than that of the wild-type recombinant C6ST-1 and M-4 mutant protein, respectively.

Effects of N-Glycans on KSST Activity—We previously found that purified C6ST-1 transferred sulfate not only to position 6 of the GalNAc residue of chondroitin (C6ST activity) but also to position 6 of the Gal residue of KS (KSST activity) (18, 32). We confirmed that the FLAG-tagged recombinant C6ST-1 also exhibited both C6ST activity and KSST activity (Fig. 9). The sulfated product obtained from chondroitin was degraded by chondroitinase ABC but not by keratanase II (Fig. 9, A–C), whereas the sulfated product obtained from KS was degraded by keratanase II but not by chondroitinase ABC (Fig. 9, D–F).

As shown in Fig. 2A, KSST activity of the wild-type recombinant C6ST-1 was decreased after PNGase F digestion, suggesting that the presence of some N-glycans may be required for the KSST activity. To elucidate the contribution of each N-glycan to KSST activity, we compared KSST activity of the mutant recombinant proteins in which one of the N-glycosylation sites was mutated (Fig. 4A, closed bar). KSST activity of M-4 and M-6 mutant proteins were almost lost as observed in C6ST activity. Unlike C6ST activity, KSST activity of M-5 mutant protein was...
markedly decreased compared with the activity of the wild-type recombinant protein. Thus, it may be possible that the observed decrease in KSST activity after PNGase F digestion shown in Fig. 2A might be due mainly to removal of the \( N \)-glycan attached to the N-5 glycosylation site of C6ST-1. To determine the importance of the \( N \)-glycan attached to the N-5 glycosylation site in KSST activity, we compared KSST activity among various mutant C6ST-1s in which 3–6 \( N \)-glycosylation sites were deleted (Fig. 6A, closed bar). As observed in C6ST activity, KSST activity of M-12356, M-12345, and M-123456 were not detected at all. KSST activity of M-123 was 33% of KSST activity of the wild-type recombinant C6ST-1 (Fig. 6), whereas KSST activity of M-1235 was almost lost. These results clearly indicate that \( N \)-glycan attached to the N-5 glycosylation site was essential for the KSST activity. To consider the reason why M-5 mutant recombinant protein exhibited low KSST activity, we determined the inhibitory effects of KS on C6ST activity (Fig. 10). M-5 mutant protein was inhibited by KS more strongly than the other recombinant proteins; in the presence of 7 nmol (as glucosamine) of KS, C6ST activity of M-5 mutant protein decreased to 20% of the control, whereas C6ST activity of wild-type and other mutant proteins were not affected at all, and in the presence of 14 nmol of KS, C6ST activity of M-5 mutant protein was less than 6% of control, whereas wild type and the mutant proteins other than M-5 mutant still retained about 50% of C6ST activity. These observations suggest that apparent low KSST activity of M-5 mutant recombinant protein may be attributable to the augmented sensitivity of M-5 mutant recombinant protein to the substrate inhibition.

### Effects of Deletion of \( N \)-Glycans Attached to the Recombinant C6ST-1 on the Kinetics Parameters for C6ST Activity and KSST Activity

To evaluate the effects of \( N \)-glycans on C6ST activity and KSST activity of the recombinant C6ST-1 quantitatively, we determined the \( K_m \) and \( V_{max} \) of the mutant recombinant C6ST-1 for PAPS and for each acceptor (Table 2). In C6ST activity, both M-4 and M-6 mutant proteins showed the lower \( V_{max} \) values for both PAPS and chondroitin than the wild-type recombinant C6ST-1, one-tenth for PAPS and one-twentieth for chondroitin. On the other hand, values of the \( K_m \) of M-4 and M-6 mutant proteins for both PAPS and chondroitin were smaller than the values of the wild-type recombinant C6ST-1, although the degree of the change in the \( K_m \) was more modest than that observed in the \( V_{max} \). Thus, the observed decrease in C6ST activity of M-4 and M-6 mutant proteins appears to be attributable mainly to the decreased \( V_{max} \) values for PAPS and chondroitin. On the other hand, the value of the \( V_{max} \) of M-5 mutant protein for chondroitin was larger than the values of the wild-type recombinant C6ST-1. In KSST activity, M-5 mutant protein showed the much lower \( V_{max} \) values for both PAPS and chondroitin than the wild-type recombinant C6ST-1. The \( K_m \) values of M-5 mutant protein for both PAPS and KS were slightly

### TABLE 2

The \( K_m \) and \( V_{max} \) of the wild-type and mutant recombinant C6ST-1

| Recombinant proteins | C6ST activity PAPS | Chondroitin PAPS | Chondroitin KS | KSST activity PAPS | KSST activity KS |
|----------------------|---------------------|------------------|----------------|-------------------|-----------------|
|                      | \( K_m \) \( \mu \) | \( V_{max} \) \( \mu \) | \( K_m \) \( \mu \) | \( V_{max} \) \( \mu \) | \( K_m \) \( \mu \) | \( V_{max} \) \( \mu \) |
| Wild-type            | 1.10                | 1.00             | 1.46           | 1.00              | 3.08            | 1.00            |
| M-4                  | 0.34                | 0.11             | 0.76           | 0.04              | ND              | ND              |
| M-5                  | 4.17                | 3.74             | 1.44           | 1.62              | 2.90            | 0.09            |
| M-6                  | 0.40                | 0.13             | 1.03           | 0.05              | ND              | ND              |

\( \mu \) and ND, not determined.

10. M-5 mutant protein was inhibited by KS more strongly than the other recombinant proteins; in the presence of 7 nmol (as glucosamine) of KS, C6ST activity of M-5 mutant protein decreased to 20% of the control, whereas C6ST activity of wild-type and other mutant proteins were not affected at all, and in the presence of 14 nmol of KS, C6ST activity of M-5 mutant protein was less than 6% of control, whereas wild type and the mutant proteins other than M-5 mutant still retained about 50% of C6ST activity. These observations suggest that apparent low KSST activity of M-5 mutant recombinant protein may be attributable to the augmented sensitivity of M-5 mutant recombinant protein to the substrate inhibition.

### Functional Role of \( N \)-Glycans Attached to C6ST-1

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**TABLE 2**

The \( K_m \) and \( V_{max} \) of the wild-type and mutant recombinant C6ST-1

Sulfotransferase activities were assayed using chondroitin or KS as described under "Experimental Procedures," except that the concentration of each acceptor and PAPS was varied. The \( V_{max} \) values were expressed as the ratio to the \( V_{max} \) of the wild-type recombinant protein.
lower than those of the wild-type recombinant C6ST-1. The low KSST activity of M-5 mutant protein could also be attributable to the decreased $V_{\text{max}}$ values for both PAPS and KS.

**DISCUSSION**

In this report, we examined functional roles of N-glycans attached to C6ST-1. Treatment of COS-7 cells transfected with C6ST-1 cDNA with tunicamycin abolished both N-glycosylation of the recombinant C6ST-1 and production of the active enzyme. On the other hand, C6ST activity of the recombinant C6ST-1 was not decreased but rather increased by PNGase F digestion under the conditions where N-glycans of the recombinant C6ST-1 were nearly completely removed. These observations indicate that N-glycans of C6ST-1 are essential for the generation of the active C6ST-1 in the cells but not required for the C6ST activity itself after the formation of the active protein. Among six potential N-glycans, N-glycans attached to N-4 and N-6 glycosylation sites appeared to be necessary and sufficient for production of the active C6ST-1, because deletion of N-4 or N-6 glycosylation site resulted in marked decrease in C6ST activity, and M-1235 mutant protein still retained 25% of the C6ST activity of the wild-type recombinant C6ST-1.

The effects of N-glycans on KSST activity showed somewhat different aspects from the effects on C6ST activity. KSST activity of the recombinant C6ST-1 was decreased by the PNGase F digestion. When the N-5 glycosylation site was deleted, C6ST activity was hardly affected, but a marked loss of the KSST activity was observed. Unlike C6ST activity, KSST activity of M-5 mutant protein almost disappeared. These results indicate that KSST activity of the recombinant C6ST-1 appears to be supported by the presence of N-glycan attached to the N-5 glycosylation site in addition to N-glycans attached to the N-4 and N-6 glycosylation sites. At present, the mechanism by which N-glycan attached to the N-5 glycosylation site could stimulate KSST activity is not clear. It has been shown that, in the corneal KS used for the substrate, almost all GlcNAc residues and about a half of the Gal residues are sulfated at position 6 (38). Because C6ST-1 transfers sulfate to position 6 of the Gal residue of KS, the corneal KS contains structures acting as both the substrate and the reaction product of C6ST-1. The structure corresponding to the reaction products in which Gal(6SO4) is abundantly contained may inhibit the sulfotransferase activity of C6ST-1. The presence of N-glycan at the N-5 glycosylation site might block the binding of Gal(6SO4) residues of KS and thereby relieve the inhibition by KS. In Fig. 10, we showed that the degree of inhibition of C6ST activity by KS was higher in M-5 mutant protein than in the wild-type recombinant C6ST-1. The observed low KSST activity of M-5 mutant protein may be due partly to the increased inhibition by KS used for the acceptor.

In C6ST-1, N-glycan attached to the N-4 glycosylation site appears to be involved in the Golgi localization, because M-4 mutant protein colocalized with calnexin, an ER-resident protein, although the wild-type recombinant C6ST-1 showed a typical Golgi localization (Figs. 7 and 8). M-4 mutant protein was shown to bear no Endo H-resistant glycans (Fig. 4), suggesting that N-4 glycan may be required for the interaction with ER-resident chaperons, such as calnexin, to form the correctly folded protein. Deletion of N-4 glycan may thus result in the accumulation of the misfolded protein in the ER, thereby inhibiting processing of the N-glycans to the Endo H-resistant form that occurs in the Golgi apparatus. To clarify the functional role of N4-glycan in the folding of the C6ST-1, structural characterization of N-4 glycan should be important. On the other hand, M-6 mutant protein also had no Endo H-resistant glycans, although intracellular localization of M-6 mutant protein was similar to that of wild-type recombinant C6ST-1. M-6 mutant protein may be located in the Golgi subcompartment different from the subcompartment where wild-type recombinant protein resides, and hence processing of the N-glycans to the Endo H-resistant form might be ablated. It remains to be studied whether the Golgi subcompartments where M-6 mutant protein and wild-type recombinant protein reside are the same or different.

On Western blot, the wild-type protein showed a 54-kDa core protein band, a 67-kDa slightly broad band, and higher smeared ones. In Fig. 4, the higher smeared bands appeared to correlate to C6ST activity, suggesting that the smeared bands correspond to the active enzyme. To determine if such a possibility is the case, we analyzed fractions eluted from the WGA-agarose by Western blot (Fig. 5D). However, because the 67-kDa band and the higher smeared one behaved identically in WGA-agarose and both peaked at the peak position of C6ST activity, it remains unclear whether the smeared bands correspond to the active enzyme.

At present, it is not clear whether C6ST-1 is relevant to the sulfation of KS in vivo. In the brain of C6ST-1-deficient mice, synthesis of KS does not appear to be affected, because the staining pattern of the brain tissues of the mice with 5D4 antibody could not be distinguished from that of the wild mice (13). Because 5D4 antibody is able to stain the highly sulfated KS (39, 40), involvement of C6ST-1 in the synthesis of the lower sulfated KS would not be thoroughly excluded. Functional roles of N-5 glycan on C6ST-1 in the synthesis of KS in vivo remain to be studied.

M-4 and M-6 mutant proteins were devoid of the Endo H-resistant N-glycans, whereas N-4 and N-6 glycans themselves seem not to be the Endo-H-resistant forms, because M-12356 and M-123455 mutant proteins were both sensitive to Endo-H. It is thus possible that N-4 and N-6 glycans may affect the processing of the other N-glycans by an as yet undefined manner. We have previously reported that deletion of the N-glycan attached to the C-terminal region of C4ST-1 also affected microheterogeneity of N-glycans attached to different sites of C4ST-1 (26). There may exist a common mechanism by which proper processing of N-glycans is influenced by the presence or absence of one N-glycan.

We have shown previously that KS Gal-6-sulfotransferase transfers sulfate to position 6 of the Gal residue in KS (41). As far as we know, only two sulfotransferases, C6ST-1 and KS Gal-6-sulfotransferase, are able to sulfate position 6 of the Gal residue in KS. ClustalW alignment of these genes showed that the N-glycosylation site corresponding to the N-4 site is the only one that is conserved between these genes and that the amino acid sequence around the N-4 site is well conserved between these proteins (Fig. 11), suggesting that...
the N-glycan attached to the conserved N-glycosylation site of KS Gal-6-sulfotransferase may play an important role as observed in C6ST-1.

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Functional Role of N-Glycans Attached to C6ST-1

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