Differential Roles of Two N-Acetylgalactosaminyltransferases, CSGalNAcT-1, and a Novel Enzyme, CSGalNAcT-2

INITIATION AND ELONGATION IN SYNTHESIS OF CHONDROITIN SULFATE*

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By a tblastn search with β1,4-galactosyltransferases as query sequences, we found an expressed sequence tag that showed similarity in β1,4-glycosyltransferase motifs. The full-length complementary DNA was obtained by a method of 5'-rapid amplification of complementary DNA ends. The predicted open reading frame encodes a transmembrane protein comprising 543 amino acids, the sequence of which was highly homologous to a typical type II membrane protein. The predicted open reading frame encodes a protein mainly involved in the elongation of chondroitin sulfate, and showed much weaker activity toward glucuronic acid.

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3 The abbreviations used are: PG, proteoglycan; GAG, glycosaminoglycan; CS, chondroitin sulfate; HS, heparan sulfate; FGF, fibroblast growth factor; GlcA, glucuronic acid; Gal, galactose; Xyl, xylose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; GT, glucuronyltransferase; β4, β3, β2, or β1-galactosyltransferase; α4GlcNAcT, α1,4-N-acetylgalactosaminyltransferase; β4 or CSGalNAcT, β1,4- or CSβ1,3-galactosyltransferase; α4GlcAT, α1,4-N-acetylgalactosaminyltransferase; αGlcAT, α1,3-galactosyltransferase; CS, chondroitin; ORF, open reading frame; UDP, uridine diphosphate; pNP, para-nitrophenol; MES, 2-morpholinoethanesulfonic acid; ESI, electrospray ionization; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; HSase, heparinase; HPase, heparanase; CSase, chondroitinase; GADDH, glyceraldehyde-3-phosphate dehydrogenase; CHO, Chinese hamster ovary; mAb, monoclonal antibody; C4ST, chondroitin-4-sulfotransferase.
(GlcA(2S)β1–3GalNAc(6S)) and CS-E (GlcAβ1–3GalNAc(4S,6S)), promote the outgrowth of neurites in rat brain (10). These reports suggest that HS and CS have different functions. Thus, it is of interest to clarify the mechanism for the biosynthesis of HS and CS, the molecules responsible for these diverse biological phenotypes.

The initial stage in the biosynthesis of both CS and HS involves a linkage tetrasaccharide structure (GlcAβ1–4Galβ1–3Galβ1–4Xylβ1–), which binds covalently to serine residues of core proteins. To initiate the synthesis of HS, a GlcNAc residue is transferred to GlcA of the linkage tetrasaccharide with an α1–4 linkage. On the other hand, GalNAc is transferred to a same acceptor with a β1–4 linkage for the initiation of CS synthesis. Thus, because the acceptor substrate is identical, it is possible that the initiation enzyme for HS or CS synthesis compete with the acceptor substrate, i.e. the linkage tetrasaccharide bound to core proteins, in the cells. If this is the case, the initiation enzymes may play a key role in determining the species of the GAG chain, HS or CS, on the core proteins. After the initiation reaction, the addition of disaccharide units of GlcNAcβ1–4Galβ1–4 are repeated for elongation of the HS chain, whereas GalNAcβ1–4Galβ1–3 units are repeatedly added for polymerization of the CS chain.

For the synthesis of HS, five glycosyltransferases, EXT1, EXT2, EXT1L1, EXT1L2, and EXT3, were cloned and their products characterized. They retain conserved motifs of short amino acid stretches in their COOH terminus and belong to one family. The EXT1 and EXT2 genes, which have been identified as tumor suppressor genes and were implicated in hereditary multiple exostoses, were found to encode HS polymerases having the activity of both β1,4-galactosyltransferase (β4GalAT) and α1,4,N-acetylgalcosaminytransferase (α4GalNAcT) (11). Both enzymes are responsible for the elongation of HS chains. Three enzymes, EXT1L3–1, exhibit only α4GalNAcT activity, but their substrate specificities were different. EXT1L1 showed α4GalNAcT activity toward GlcA in elongation. EXT2L2 showed activity in initiation. EXT3L3 showed activity in both initiation and elongation for the synthesis of HS (12, 13).

Very recently, three glycosyltransferases, chondroitin synthase (CSS) (14), CS glucuronosyltransferase (CSGlcAT) (15) and CSGalNAcT (16, 17), which are involved in the synthesis of CS, thase (CSS) (14), CS glucuronyltransferase (CSGlcAT) (15) and very recently, three glycosyltransferases, chondroitin synthase (CSS) (14), CS glucuronosyltransferase (CSGlcAT) (15) and CSGalNAcT (16, 17), which are involved in the synthesis of CS, thase (CSS) (14), CS glucuronyltransferase (CSGlcAT) (15) and.cs 3GalT-6, and GlcAT-I Fused with FLAG Peptide—

**Differential Roles of Two CSGalNAcTs**

**Isolation of Human CSGalNAcT cDNA**—We performed a tblastn search of the GenBank™ data base using β1,4-galactosyltransferase motifs as queries and identified an expressed sequence tag with GenBank™ accession number NM_018589, which contained a partial open reading frame (ORF), but showed high homology to the carboxyl-termina lregion of CSGalNAcT-1. An additional search of the Human Genome Project data base revealed that the genome sequence with GenBank™ accession number NT_008776 was identical to the expressed sequence tag. To obtain the complete ORF, the 5′-rapid amplification of complementary DNA (cDNA) ends method was employed using a Mar-
chased from the Peptide Institute Inc. (Osaka, Japan). The Cy5 (Amer-
sham Biosciences)-labeled Xyl-peptide was incubated with 5 μl of
three glycosyltransferases, β4GalT-7, β3GalT-6, and GlcAT-1, and 1 mm
 donor substrates, UDP-Gal and UDP-GlcA, at 37 °C for 16 h as de-
scribed in detail previously (17). A 50 mM MES buffer (pH 6.5) contain-
ing 0.1% Triton X-100, 1 mM UDP-Gal, 1 mM UDP-GlcA, 10 mM MnCl2,
and 500 μM Xyl-bikunin peptide was used for the reaction. The glyco-
syltransferases for the synthesis of the tetrasaccharide-bikunin peptide
were inactivated by heating at 95 °C for 5 min. Then, the reaction
mixture was filtrated with an Ultrafree-MC column (Millipore), and a 10-μl aliquot was incubated with 1 nm donor substrate, UDP-GalNAc,
and each CSGalNAcT at 37 °C for 8 h for the assay of initiation activity
of each CSGalNAcT. The reaction products of CSGalNAcTs were fil-
trated with an Ultrafree-MC column and a 10-μl aliquot was subjected
to reversed-phase high performance liquid chromatography on an ODS-
80T's QA column as described above.

Determination of Products by CSGalNAcT-2 with Mass Spectrometry
(MS)—An additional peak detected by reversed-phase chromatography
was isolated and analyzed by an electrospray ionization (ESI) or ma-
trix-assisted laser desorption/ionization-time of flight (MALDI-TOF)
MS (ESI-MS, esquire3000plus, and MALDI-TOF-MS, Reflex IV; Bruker
Daltonics, Billerica, MA). Then, 25 μl of product was dissolved in 5 μl
of H2O, and 45 μl of 0.1% formic acid and 50 μl of methanol were added.
The product solution was infused at a rate of 3 μl/min with a capillary
voltage of 3 kV. The hypothetical product peaks obtained by ESI-MS
were analyzed by ESI tandem mass spectrometry (ESI-MSMS). These
analyses were done in both positive-ion and negative-ion ESI modes.
For MALDI-TOF MS analysis, 10 μl of product was dried, dissolved
in 1 μl of H2O, and applied.

In Vivo CS/HS Synthesis on a Syndecan-4/FGF-1 Chimera Pro-
ten—For the expression in CHO-K1 cells, the full-length cDNAs of
CSGalNAcT-1 and CSGalNAcT-2 were amplified by PCR with primers
CSGalNAcT-1, 5′-CCCAAGCTTATGATGTTGCTGTCGGGGCCT-3′
and 5′-GCTCTAGACTGTTTTTGGTCTCCTCTG-3′, and
CSGalNAcT-2, 5′-CCCAAGCTTATAAAGTTGCTGTTGAGCCTG-
A-3′ and 5′-GCTCTAGATCAACACACGTTAGTCGGTTG-3′. The
amplified fragments were inserted into pcDNA3.1/ Hygromycin (+) (+)
(pcDNA3.1/Hygro) (Invitrogen) after HindIII and Xbal digestion. The
resulting plasmid, pcDNA3.1/HygroCSGalNAcT-1, CSGalNAcT-2, and
the pcDNA3.1/Hygro expression vector were transfected into CHO-K1
cells harboring PMEMneo-PG-FGF-1 that were expressing the synde-
can-4/FGF-1 chimera protein (PG-FGF-1) using LipofectAMINE 2000
(Invitrogen) according to the manufacturer’s instructions. Medium con-
ditioned by the CHO transfected containing the secreted PG-FGF-1
protein with GAGs was collected. Fractionation of PG-FGF-1 using a
DEAE-Sepharose column (Amersham Biosciences) and endoglycosi-
dases: hepatine (HSase), heparanase (HPase), and chondroitinase
ABC (CSase) (Seikagaku Corp.) digestion and Western blotting with
monoclonal antibody against FGF-1 (mAb1) (20) were performed as
described previously (21). The PG-FCF-P and bands digested by glycosi-
dases were quantified by densitometric scanning of the digitized image
using NIH Image (version 1.60) software (National Institutes of Health,
Bethesda, MD).

Quantitative Analysis of CSGalNAcT-1 and CSGalNAcT-2 Tran-
scripts in Human Tissues by Real-time PCR—For quantification of
the two CSGalNAcT transcripts, we employed the real-time PCR method,
as described in detail previously (18, 22, 23). Marathon Ready™ cDNAs
derived from various human tissues and cells were purchased from
Clontech. Standard curves for the endogenous control, glyceraldehyde-
3-phosphate dehydrogenase (GAPDH) cDNAs, were generated by serial
dilution of a pCR2.1 (Invitrogen) DNA containing the GAPDH cDNA.
The Ontario Cancer Institute–funded probes for CSGalNAcT-1 and
CSGalNAcT-2 and the probe for GAPDH were as follows. The forward
primer for CSGalNAcT-1 was 5′-GACT-
TCATCAATATGAGGTTGCT-3′, the reverse primer, 5′-GTCGG-
TACCATGATGGTCTGCT-3′, and the probe, 5′-ACCTTTATCGC
CAATTCTG-3′ with a minor groove binder (24). The forward primer for
CSGalNAcT-2 was 5′-CTGACATCTTGGTGATGGTCACT-3′, the
reverse primer, 5′-ACCTTTATCCGAACCTTACGTTGCT-3′, and the
probe, 5′-ACCTTTATCCGAACCTTACGTTGCT-3′ with a minor groove
binder. PCR products were continuously measured with an ABI PRISM 7700
Sequence Detection System (Applied Biosystems, Foster City, CA).
The relative amount of each CSGalNAcT transcript was normalized to the
amount of GAPDH transcript in the same cDNA.

RESULTS

Determination of Nucleotide and Amino Acid Sequence of
CSGalNAcT-2—We determined a novel full-length cDNA se-
quence by the 5′-rapid amplification of cDNA ends method and
registered it in the GenBank™ data base with accession num-
ber AB079252. The nucleotide sequence and the putative
amino acid sequence are shown in Fig. 1. This cDNA sequence
consisted of a 254-bp 5′-untranslated region, 1629-bp coding
region, and 1791-bp 3′-untranslated region that contained a
poly-A tail (Fig. 1). Although the original expressed sequence
tag was obtained in a GenBank™ data base search with β1,4-
glycosyltransferase motifs as query sequences, the full-length
ORF sequence was identified as highly homologous to CSGal-
NAcT as previously reported by us (17). We designated this
novel cDNA as CSGalNAcT-2, and renamed the previous CSGal-
NAcT, CSGalNAcT-1. An alignment of CSGalNAcT-1 and
CSGalNAcT-2 is shown in Fig. 2. A hydropathy profile of the
putative amino acid sequence based on Kyte and Doolittle
hydrophobicity plots indicates that the ORF of CSGalNAcT-2
encodes a typical type II membrane protein, which is consistent
with the topology of other glycosyltransferases, with a cytoplas-
ic tail of 14 amino acids, a transmembrane domain of 20
amino acids, and a large catalytic portion of 508 amino acids.
CSGalNAcT-2 contains a DXD motif, which is conserved in
many glycosyltransferases and functions as a key sequence for
divalent cation binding, and a GWGGED motif, which is highly
conserved among some of the βGalT family.

A genome sequence identical to that of the CSGalNAcT-2
cDNA was found in a genome clone (GenBank™ AC011890),
which is localized on chromosome 10. The genomic structure of
the CSGalNAcT-2 gene was determined to be composed of at
least 7 exons by comparison between the cDNA and genome
sequences (Fig. 1). The exon/intron junctions of CSGalNAcT-2
were identical to those of CSGalNAcT-1 (data not shown).

Determination of CSGalNAcT-2 Activity in Elongation and
Initiation of Chondroitin Poly- and Oligosaccharides—The
truncated soluble forms of CSGalNAcT-1 and CSGalNAcT-2
were expressed in insect cells and employed for all experi-
ments. On Western blotting using anti-FLAG antibody, each
enzyme was detected as a single band corresponding to the
predicted size (Fig. 3B). An additional band appeared by
Coomassie staining at ~50 kDa shared by all lanes (Fig. 3A),
however, the protein recovered from mock transfectants
showed no activity for any donors and acceptor substrates (data
not shown). At first, we screened the transfer activity of
CSGalNAcT-2 using nine donor substances and multiple monosac-
charide-pNP acceptors. In screening of donor and acceptor sub-
strates, CSGalNAcT-2 transferred a GalNAc residue to GlcA-
pNP, however, no activity was observed with other
combinations of donor and acceptor substrates (data not shown).
It was identified in a previous report that CSGalNAc-
T-1 is β4GalNAcT, which transfers GalNAc to GlcA for ini-
tiation and elongation in the synthesis of CS (17). So far, the
linkage of GalNAc with GlcA has been identified only in CS. We
considered that CSGalNAcT-2 was also a GalNAcT involved in
the synthesis of CS, and performed further analysis using CS-
related substrates as acceptors.

Two kinds of GalNAc-GlcA linkages are known in CS, one in
its polymer structure (3GalNAcβ1–4GlcAβ1–3) and the
other between the polymer CS and the linkage tetrasaccharide
(GlcAβ1–3Galβ1–3Galβ1–4Xyl). At first, chondroitin was util-
ized as an acceptor substrate to examine the elongation activity
of CSGalNAcT-2. As shown in Fig. 3C, CSGalNAcT-2 ap-
parently transferred GlcNAc to chondroitin to produce an
additional peak (indicated by an arrow in Fig. 3C) as a reaction
product. This peak was isolated and identified to have GalNAc
on its nonreducing terminus with chondroitinase ACII treat-
ment (data not shown), the method used having been described
in a previous study (17). Second, a linkage tetrasaccharide

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(GlcAβ1–3Galβ1–3Galβ1–4Xylβ-O-methoxyphenyl) synthesized chemically was utilized as an acceptor substrate to identify the initiation activity of CSGalNAcT-2. As shown in Fig. 3D, the peak P appeared at a 30.3-min retention time in addition to the acceptor substrate peak (peak S) at 31.1 min. Peaks S and P were isolated by reversed-phase chromatography and their molecular weights were determined by MALDI-TOF MS. Peak S gave a molecular mass of 779.14 m/z, the same as that of the linkage tetrasaccharide with Na+/H11001 (Fig. 3E). Peak P gave two peaks of 982.28 and 998.25 m/z as shown in Fig. 3F. The molecular mass of 982.28 or 998.25 m/z was the same molecular weight as a GalNAc-added linkage tetrasaccharide-O-methoxyphenyl with Na+/H11001 or with K+/H11001, respectively. Moreover, the peak P was identified to have GalNAc at its nonreducing terminus with chondroitinase ACII treatment (data not shown). These results suggested that CSGalNAcT-2 has two GalNAcT activities, i.e. elongation of chondroitin and initiation of the CS synthesis by transferring GalNAc to the linkage tetrasaccharide.

Comparison of Acceptor Substrate Specificity between CSGalNAcT-1 and CSGalNAcT-2—In view of these results, CSGalNAcT-2 was suggested to be GalNAcT involved in the synthesis of CS as well as CSGalNAcT-1. To distinguish the functions of the two CSGalNAcTs, we compared GalNAcT activity toward GlcA found at the nonreducing terminus of various kinds of
The amino acid sequences of CSGalNAcT-1 and CSGalNAcT-2 were aligned using GENETYX. The DXD motif and the β1,4-galactosyltransferase motif were boxed. The putative transmembrane domains are underlined. The DXD motif and the β1,4-galactosyltransferase motif were boxed.

Fig. 2. Multiple alignment of amino acid sequences of CSGalNAcT-2 and CSGalNAcT-1 by GENETYX. Introduced gaps are shown with hyphens. The putative transmembrane domains are underlined. The DXD motif and the β1,4-galactosyltransferase motif are boxed. Identical amino acids are shown with asterisks.

Comparison of Initiation Activity with Linkage Tetrasaccharide Peptide between Two CSGalNAcTs—We performed an in vitro enzymatic synthesis of a chondroitin pentasaccharide-bikunin peptide (VLPQEEEGS*GGGQLVT) in the presence of CSGalNAcT-1 and -2. The value of this relative activity was consistent with that of the CSGalNAcT-2 product. The peak of the reaction product is indicated with an arrow. D, linkage tetrasaccharide-0-methoxyphenyl was used as an acceptor, and the product was analyzed by reversed-phase chromatography. Each peak of substrate (peak S) and reaction product (peak P) was isolated and subjected to MALDI-TOF-MS analysis (E and F).

Change of GAG Composition of PG-FGF-1 by CSGalNAcT-1—Previously, the gene construct ———chondroitin-related acceptor substrates. The amount of enzyme was estimated by Western blotting, and approximately equal amounts of enzyme were used for the GalNAcT reaction. The results are summarized in Table I. Regarding the elongation activity, CSGalNAcT-2 utilized chondroitin polysaccharide as an acceptor more than any other substrate examined, and showed higher levels of enzymatic activity than CSGalNAcT-1 toward all substrates except for the linkage tetrasaccharide (Table I). Furthermore, CSGalNAcT-2 showed remarkably strong activity, compared with CSGalNAcT-1, toward sulfated substrates such as CS poly- and oligosaccharides (Table I). CS-A and CS-B, both of which are sulfated at position C-4 of GalNAc, were better substrates for CSGalNAcT-2 than CS-C, which is sulfated at position C-6 of GalNAc and CS-D, which is sulfated at C-6 of GalNAc and C-2 of GlcA (Fig. 4). CSGalNAcT-2 exhibited much stronger activity toward the longer oligosaccharides, prepared from chondroitin and CS, than toward the shorter ones. These results strongly indicated that CSGalNAcT-2 is much more active in the elongation of chondroitin and CS than CS CSGalNAcT-1.

Regarding the initiation activity, CSGalNAcT-1 preferred the linkage tetrasaccharide as substrate and showed a much higher level of activity for the linkage tetrasaccharide than did CSGalNAcT-2. This indicated that CSGalNAcT-1 is the enzyme mainly responsible for the initiation of chondroitin and CS synthesis, not for elongation. To examine whether two GalNAcTs show a synergistic effect for initiation and elongation activities, two enzymes were mixed in the enzyme reaction in vitro. The value of this relative activity was consistent with that of the CSGalNAcT-2 product (Fig. 5B). The peak of the reaction product was indicated with an arrow. D, linkage tetrasaccharide-0-methoxyphenyl was used as an acceptor, and the product was analyzed by reversed-phase chromatography. Each peak of substrate (peak S) and reaction product (peak P) was isolated and subjected to MALDI-TOF-MS analysis (E and F).

were employed, and the incubation was terminated before a large amount of acceptor substrate remained. As seen in Fig. 5, B and C, the reaction product (Peak P) appeared at 30.1 min with CSGalNAcT-1 or -2, respectively. Peak P was identified as GalNAc-GlcA-Gal-Gal-Xyl-bikunin peptide in a previous study (17). The peak area of the CSGalNAcT-1 product (Fig. 5B) was 3-fold larger than that of the CSGalNAcT-2 product (Fig. 5B). The value of this relative activity was consistent with that toward the methoxyphenyl-linkage tetrasaccharide in Table I, i.e. the activity of CSGalNAcT-1 was 2.3-fold that of CSGalNAcT-2. These results indicated that the presence of bikunin peptide does not influence the relative activity of the two CSGalNAcTs in vitro.

Change of GAG Composition of PG-FGF-1 by CSGalNAcT-1 or CSGalNAcT-2 Transfection—Previously, the gene construct encoding a chimera protein (PG-FGF-1) consisting of syndecan-4 and FGF-1 was transfected into CHO-K1 cells to express the chimera protein (21). Syndecan-4 is a well analyzed proteoglycan possessing chains of HS and/or CS. The GAG compo-
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Comparison of acceptor specificity between two CSGalNAcTs

Values represent averages ± S.D. of triplicate experiments.

| Acceptor substrate       | Activity (pmol/ml medium/h) |
|--------------------------|----------------------------|
|                          | CSGalNAcT-2    | CSGalNAcT-1    |
| Chondroitin Polyssaccharide | 25.6 ± 3.2     | 16.0 ± 2.0     |
| Oligosaccharide CH14     | 2.1 ± 0.5      | 0.7 ± 0.2      |
| CH12                    | 3.7 ± 0.2      | 0.6 ± 0.3      |
| CH10                    | 1.9 ± 0.4      | 0.4 ± 0.2      |
| CH8                     | 1.7 ± 0.3      | 0.3 ± 0.2      |
| CH6                     | 0.9 ± 0.4      | 0.2 ± 0.2      |
| Chondroitin sulfate Polyssaccharide CS-A | 13.9 ± 0.2 | 0.5 ± 0.3 |
| CS-B                    | 8.5 ± 0.5      | 0.7 ± 0.1      |
| CS-C                    | 2.8 ± 0.2      | 1.2 ± 0.2      |
| CS-D                    | 3.8 ± 0.3      | 0.8 ± 0.1      |
| Oligosaccharide CS14    | 8.5 ± 1.5      | 1.8 ± 0.3      |
| CS12                    | 7.9 ± 0.1      | 1.6 ± 0.4      |
| CS10                    | 6.1 ± 1.3      | 2.1 ± 0.7      |
| CS8                     | 3.4 ± 0.3      | 1.7 ± 0.4      |
| CS6                     | 0.8 ± 0.1      | 0.9 ± 0.1      |
| Linkage tetrasaccharide | 8.0 ± 1.6      | 23.5 ± 2.2     |

Fig. 4. Determination of chondroitin and CS polymer elongation activity of CSGalNAcT-2. Chondroitin (open circle), CS-A (closed circle), CS-B (open square), CS-C (closed square), and CS-D (open triangle) were used as acceptor substrates. The fractions containing the CSGalNAcT product are indicated by an arrow.

Fig. 5. Comparison of initiation activity between two CSGalNAcTs. A, the GlcA-Gal-Xyl-bikunin peptide (peak S) was synthesized enzymatically with three glycosyltransferases, β4GalT-7, β3GalT-6, and GlcAT-1, and detected by a reversed-phase chromatography. The product (peak S) was used as an acceptor substrate for CSGalNAcT-2 (B) or CSGalNAcT-1 (C). The products (peak P) were identified as described previously (17).
Fig. 6. Change of GAG composition in Syndecan-4/FGF-1 (PG-FGF-1) secreted by CHO-K1 cells transfected with the CSGalNAC-T2 or CSGalNAC-T1 genes. PG-FGF-1 recovered from the culture medium of CHO-K1 cells transfected with the mock vector (lanes 1–4), the CSGalNAC-T2 gene (lanes 5–8), and the CSGalNAC-T1 gene (lanes 9–12) were resolved by SDS-PAGE and immunoblotted with mAb1. Aliquots of the sample were subjected to endoglycosidase digestion before being subjected to SDS-PAGE. PG-FGF-1 was left untreated (lanes 1, 5, and 9), or digested with CPhase (lane 2, 6, and 10), with a mixture of HPase and HSase (lane 3, 7, and 11) or with a mixture of all enzymes (lane 4, 8, and 12). Positions of molecular mass standards are indicated in kilodaltons. The density of each band was measured with a film exposed for 2 min.

Fig. 7. Quantitative analysis of CSGalNAcT-2 and CSGalNAcT-1 transcripts in human tissues by real-time PCR. Standard curves for CSGalNAcTs and GAPDH were generated by serial dilution of each plasmid DNA. The expression level of the CSGalNAcT-2 (open bars) and CSGalNAcT-1 (closed bars) transcripts was normalized to that of the GAPDH transcript, which was measured in the same cDNAs. Data were obtained from triplicate experiments and are given as the mean ± S.D. PBMC, peripheral blood mononuclear cell.

Fig. 8. A schematic diagram of catalytic activities of glycosyltransferases involved in the synthesis of HS and CS. The scale of the letters of enzyme names indicates the strength of activity. Xylosyltransferase (XT) (41, 42), β1,4-galactosyltransferase VII (βGalT7) (43, 44), β1,3-galactosyltransferase VI (βGalT6) (45), and β1,3-glucuronolactonetransferase I (GlcAT-I) (46) work sequentially for the synthesis of linkage tetrasaccharide on syndecan-4. In the case of HS/HP synthesis, EXT1 and EXT2, which are HS polymerases having both β1,4-glucuronolactonetransferase and α1,4-N-acetylgalactosaminyltransferase activities (11), and EXT1L, EXT2L, and EXT3L, which are HS α1,4-N-acetylgalactosaminyltransferases (12, 13), participate. In the case of CS/DS synthesis, CSS, which is CS synthase having both β1,4-galactono- and β1,4-α-acetylgalactosaminyltransferase activities (14), and CS β1,3-glucuronolactonetransferase (CSGlcAT) (15) and CS β1,4-acetylgalactosaminyltransferase 1 and 2 (CSGlNAcT-1 and CSGlNAcT-2) (17) participate.

DISCUSSION

The investigation of CS has progressed to the relationship between its structural properties including saccharide composition and sulfation, and its biological function. However, the mechanisms of its biosynthesis, such as the number of enzymes participating and their roles, still remained to be elucidated. To date, three glycosyltransferases, CSS (14), CSGlcAT (15), and CSGlNAcT-1 (16, 17), which participate in the biosynthesis of CS, have been reported. In this study, a novel enzyme CSGalNAcT-2 was found to be a fourth member involved in the synthesis, and identified to be the second GalNAcT transferring a GalNAc to GlcA.

The amino acid sequence of CSGalNAcT-2 is highly homologous to that of CSGalNAcT-1. The β4GT motif (GWGGE) and DXD sequence, which is a divalent cation binding motif, are conserved with a good alignment (Fig 2). Six β4GalTs, β4GalT1 to -6, possess a WGGGEDD sequence as the motif (25–28), whereas both of the CSGalNAcTs possess KGWGEDVH in place of WGGGEDD. Very recently, Ramakrishnan et al. (29) reported that Tyr289 of bovine β4GalT1 is essential for its donor binding based on its crystallized structure. The elimination of a hydrogen bond by mutating the Tyr289 residue to Leu, Ile, or Asn enhances the GalNAcT activity in place of the GaT activity. In the case of β4GalNAcT of Caenorhabditis elegans, the amino acid at the same position is Ile (47). In the primary sequence of CSGalNAcT-1 and -2, it was difficult to find the amino acid residue corresponding to the position of Tyr289 on β4GalT1 because of a gap in the amino acid alignment between β4GalT1 and CSGalNAcTs. However,
CSGalNAcT-2 has an almost identical sequence to that of CSGalNAcT-1 around this region that probably determines the donor substrate specificity. Therefore, the βGalNAcT activity of CSGalNAcT-2 was easily predicted before the experiments. In fact, CSGalNAcT-2, as well as CSGalNAcT-1, exhibited GalNAcT activity in both the initiation and elongation of the CS synthesis in vitro (Table I). However, CSGalNAcT-2 showed remarkably different activity from CSGalNAcT-1, i.e. much stronger activity toward chondroitin and CS substrates, particularly CS-A and CS-B, than CSGalNAcT-1 (Table I and Fig. 4). Sulfation of chondroitin is important to exhibit various biological functions. Sulfation at position C-4 of GalNAc is directed by chondroitin-4-O-sulfotransferase-1 (C4ST-1) (30), C4ST-2 (31), and C4ST-3 (32), that at C-6 of GalNAc by chondroitin-6-O-sulfotransferase-1 (C6ST-1) (33) and C6ST-2 (34), and that at C-2 of GlcA by uronyl 2-sulfotransferase (35) in the GalNAc-GlcA backbone. It is still unclear whether the sulfation occurs after the synthesis of the long chondroitin chain or the sulfation and GalNAc-GlcA polymerization occur simultaneously. The preference of CSGalNAcT-2 for sulfated substrates indicates that the elongation of sulfated chondroitin is directed by CSGalNAcT-2, not by CSGalNAcT-1. CSGalNAcT-2 may be responsible for the elongation of polymers that are simultaneously coupled with sulfation.

Regarding the initiation activity, CSGalNAcT-1 exhibited much stronger GalNAcT activity toward the linkage tetrasaccharide than CSGalNAcT-2. In a previous study, we reported that CSGalNAcT-1 has effective initiation activity, i.e. it transferred GalNAc to the linkage tetrasaccharide conjugated with a bikunin peptide (GlcA-Gal-Gal-Xyl-bikunin peptide) in vitro (17). The initiation activity of CSGalNAcT-1 relative to CSGalNAcT-2 was 2.3- and 3.0-fold higher toward methoxypentyl-linkage tetrasaccharide and tetrasaccharide-bikunin peptide, respectively. The presence of a peptide backbone did not affect the relative initiation activity. The in vitro assay system using the syndecan-4/FGF-1 chimera protein (PG-FFG-1) demonstrated that CSGalNAcT-1 exhibits initiation activity for syndecan-4 by competing with the synthesis of HS (Fig. 6). Syndecan-4 is a complex-type PG that has four putative GAG attachment sites, and both CS and HS can bind to these sites (36). In our construction of PG-FFG-1, a part of syndecan-4 by competing with the synthesis of HS composition of PG-FFG-1 as demonstrated in our previous study (21). In this assay system, we identified an increase in CS on PG-FFG-1 only in CSGalNAcT-1-transfected CHO-K1 cells, not in CSGalNAcT-2 transfectants. The increase of CS is probably directed by the initiation activity of CSGalNAcT-1. This in vitro observation is consistent with previous results in vitro of the effective initiation activity of CSGalNAcT-1 as we reported (17). Taken together with the previous findings in vitro, the in vivo results of the present study strongly support that CSGalNAcT-1 is the enzyme most responsible for the initiation of CS synthesis in the cells.

On the other hand, CSGalNAcT-2 transfected into CHO-K1 cells showed no increase in CS, although it had some initiation activity in vitro toward the linkage tetrasaccharide. This contradiction between the in vitro and in vivo activities of CSGalNAcT-2 suggests that the initiation of CS synthesis might be controlled by some unknown mechanisms. One possibility is the effect of sulfation on the linkage tetrasaccharide. In vertebrates, CS is specifically sulfated at position C-6 of the inner Gal (Galβ1–4) and at C-4 and C-6 of the outer Gal (Galβ1–3) in the linkage tetrasaccharide (37, 38). These sulfations might influence the CSGalNAcTs in terms of recognition. Another possibility is the effect of the peptide sequence of the core protein on the enzyme recognition. A currently proposed GAG attachment motif is the Ser-Gly-Ser-Gly sequence and surrounds acidic amino acids (39, 40). CSGalNAcT-2 might recognize CS binding peptide sequences of core proteins other than syndecan-4.

The tissue distribution of transcripts for three glycosyltransferases, i.e. CSS, CSGlcAT, and CSGalNAcT-1, have been reported to be ubiquitously expressed in all tissues examined (14–17). CSGalNAcT-2 also showed a ubiquitous expression like the others. However, characteristic of that CSGalNAcT-2 was a high level of expression in the small intestine, leukocytes, and spleen, whereas the others are not so highly expressed in these tissues. Hiraoa et al. (31) reported that the transcripts of C4ST-1 and C4ST-2, which produce CS-A, are also highly expressed in the small intestine, spleen, and leukocytes. In contrast, C6ST-2, which produces CS-C, is expressed significantly in the spleen, but not so in small intestine (34). The preference of CSGalNAcT-2 for CS-A and CS-B to CS-C and CS-D, as demonstrated in Table I, led us to speculate of a cooperative CS synthesis by CSGalNAcT-2 and C4STs in the cells. The similar profile of the tissue distribution of CSGalNAcT-2 to that of C4STs further suggested this cooperative synthesis. In the case of HS, it has been proposed that EXT1 and EXT3 are involved in the initiation of the synthesis (12, 13), however, the exact role of each enzyme in vitro remains to be elucidated.

Finally, the catalytic activities of each enzyme involved in the synthesis of HS and CS are schematically summarized in Fig. 8. Four glycosyltransferases responsible for the synthesis of the linkage tetrasaccharide have been cloned and analyzed (41–46). Using these enzymes, it is now possible to synthesize enzymatically the linkage tetrasaccharide bound to the peptide (17). The initiation of the CS chain is also feasible using CSGalNAcT-1 (17). Three enzymes, i.e. CSS, CSGlcAT, and CSGalNAcT-2, have been identified as involved in the polymerization of the CS chain. In addition to the four enzymes, there still may be unknown enzymes, which remain to be cloned and analyzed, for the synthesis of the CS.

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