Enzymatic Synthesis of Chondroitin with a Novel Chondroitin Sulfate N-Acetylgalactosaminyltransferase That Transfers N-Acetylgalactosamine to Glucuronic Acid in Initiation and Elongation of Chondroitin Sulfate Synthesis*

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We found a novel glycosyltransferase gene having a hypothetical β1,4-galactosyltransferase motif (GenBank™ accession number AB081516) by a BLAST search and cloned its full-length open reading frame using the 5’-rapid amplification of cDNA ends method. The truncated form was expressed in insect cells as a soluble enzyme. It transferred N-acetylgalactosamine, not galactose, to para-nitrophenyl-β-glucuronic acid. The N-acetylgalactosamine-glucuronic acid linkage has been identified only in chondroitin sulfate; therefore, we examined its chondroitin elongation and initiation activities. N-Acetylgalactosaminyltransferase activity was observed toward chondroitin poly- and oligosaccharides, chondroitin sulfate oligosaccharides, and linkage tetrasaccharide (GlcA-Gal-Gal-4Gal-Xyl-4), and the chondroitin polysaccharide and linkage tetrasaccharide were better acceptor substrates than the others. Northern blot analysis and quantitative real-time PCR analysis revealed that its 4-kb transcripts were highly expressed in thyroid and placenta, although they were ubiquitously expressed in various tissues and cells. These results suggest that this enzyme has N-acetylgalactosaminyltransferase activity in both the elongation and initiation of chondroitin sulfate synthesis. Furthermore, we performed enzymatic synthesis of chondroitin pentasaccharide in vitro. In one tube reaction with four enzymes, β1,4-galactosyltransferase-VII, β1,3-galactosyltransferase-VI, glucuronyltransferase-I, and this enzyme, and a synthetic xylose-peptide acceptor, the structure GalNAc-GlcA-Gal-Gal-Xyl-peptide was constructed. This is the first report of a chondroitin pentasaccharide constructed with recombinant glycosyltransferases in vitro.

To date, seven members of the human β1,4-galactosyltransferase (β4Gal-T) family have been identified. β4Gal-T1 to -T6 exhibit activity to transfer galactose (Gal) to N-acetylgalactosamine (GlcNAc) with a β1,4-linkage on N- and O-glycans and glycolipids (1–4). β4Gal-T7 synthesizes the Galβ1–4Xyl structure, which occurs in the linkage portion of glycosaminoglycans (GAGs) (5, 6), β4Gal-T1, one of the best-understood glycosyltransferases (7), was crystallized, and its structure was analyzed (8–10). An acid-rich short sequence, WGGGDD, which is shared by the six β4Gal-T members, has been recognized to contain the catalytic general base of the family. In the case of human β4Gal-Ts, the motif is rather long, WG-WGGEDDD, which is conserved among all the family members except for β4Gal-T7. Another motif, DXD, which is widely conserved among many glycosyltransferases, binds to divalent cations, such as Mn2+.

Chondroitin sulfate (CS) proteoglycans are found on cell surfaces and in the extracellular matrix of various kinds of human cell. They consist of the GAGs and their core proteins, to which CS is bound covalently. The CS proteoglycans distribute in various tissues, especially brain, tendon, skin, muscle, endothelial and epithelial cells, and contribute to the physical strength of tissues, cell adhesion, signal transduction, and so on (11–14).

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Chondroitin has the structure (GlcAβ1–3GalNAcβ1–4) on the linkage tetrasaccharide (GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–O-protein). The glycosyltransferases, such as glucuronoltransferase-I (GlcAT-I), β1,3-galactosyltransferase-VI (β3Gal-T6), β4Gal-T7, and xylosyltransferase, for the linkage have been identified (5, 6, 15–17). Recently, chondroitin synthase (CSS) has been cloned and identified (18). This enzyme shows activities of β1,3-glucuronoyltransferase (β3GlcAT) and β1,4-N-acetylgalactosaminyltransferase (β4GalNAc-T), although its chondroitin polymerization activity has not been detected in vitro. We recently cloned and identified CSSGlA-T, which acts as β3GlcAT in the elongation of CS (19). Both CSS and CS-GlcA-T have β3GlcA-T activities, however, their acceptor specificities differ. CSSGlA-T prefers shorter acceptors than CSS; therefore, we propose that CSSGlA-T and CSS function at different stages of the synthesis in the stage of CS, i.e. the former at an early stage and the latter at a late stage.

CS consists of many kinds of sulfated glucuronic acid (GlcA) and N-acetylgalosamine (GalNAc) (20). The C2 and C3 positions of GlcA and the C4 and C6 positions of GalNAc are the sites possibly sulfated by the respective sulfotransferases. There are reports that the sulfation is related to the elongation and termination of the CS chain. Using bovine serum, UDP-GalNAc and various kinds of sulfated (GlcAβ1–3GalNAcβ1–4)n, 6-O- or 4-O-sulfated GalNAc in the acceptor stimulates the GalNAc-T activity, whereas terminal 3-O-sulfated GlcA or penultimate 4,6-O-disulfated GalNAc inhibits the activity (21). Gundlach and Conrad (22) demonstrated acceptor substrate specificity to GlcA-T and GalNAc-T in a microsomal preparation from chick embryo chondrocytes using even- and odd-numbered oligosaccharides prepared from chondroitin, CSA, CS-C, and hyaluronic. They showed that oligosaccharides prepared from CS-C and chondroitin were active as acceptors for both enzymes, whereas oligosaccharides from CS-A and hyaluronan were mostly inactive. In the case of CSSGlA-T, the sulfated oligosaccharides prepared from CS-C made better substrates than non-sulfated oligosaccharides (19).

The progress made with the human genome project and other databases, such as expressed sequence tags (ESTs) and full-length complementary DNAs (cDNAs), has enabled us to search for novel genes that are homologous to known genes. We performed a search for novel genes that are homologous to known genes. We searched these databases utilizing the amino acid sequences of the β4Gal-T family while paying particular attention to the existence of a trans-membrane domain and conserved motif regions, DXD and GWGGED, as query sequences. In this study, we focused on a novel gene, which possesses the GWGGED motif and has elongation and initiation activities in the synthesis of CS, although its cloning and a brief characterization were reported while we were preparing this report (23). Finally, we show that the synthesis of chondroitin pentasaccharide is possible using the three reported enzymes and this novel enzyme in vitro.

**Experimental Procedures**

**Isolation of a Novel Human CSGalNAc-T cDNA**—We performed a BLAST search of the EST databases using amino acid sequences of the cloned human β1,4-galactosyltransferases (β4Gal-T) as queries and found a novel EST (GenBankX3 accession number AK055514) that possesses the GWGGED motif for β4Gal-T. To open the reading frame sequence, we performed the 5′-rapid amplification of cDNA ends (5′-RACE) technique with a Marathon-Ready cDNA amplification kit (Clontech, Palo Alto, CA). The 5′ sequence was extended by two-step 5′-RACE. Two reverse primers, 5′-ACCTATATTGATAGTGTCGACC-3′ for the first PCR and 5′-GTCGGCAAGACGACGACGTCAT-3′ for the nested PCR, were employed for the first-step extension. The second-step 5′-RACE was performed by two reverse primers 5′-TCTCGACGCTCTCCTGAGC-3′ and 5′-ATCTGCGCCGTCTCGAGCTGCTA-3′.

**Construction and Purification of CSGalNAc-T Protein Fused with Flag Peptide**—The putative catalytic domain of the enzyme (amino acids 37–532) was expressed as a secreted protein fused with a Flag peptide in insect cells according to the instruction manual of GATEWAY Cloning Technology (Invitrogen, Groningen, Netherlands). A DNA fragment of 1–1.6 kb was ampliﬁed by PCR using the Ready cDNA derived from human bone marrow (Clontech), as a template, and two primers, 5′-GGGAGCAATTGAGTACAAAAGGCACGCTTCAGATGACGACGTTGA-3′ and 5′-GGGAGCACCAGTCCGCTGCTGCTGCTGCT-3′. The ampliﬁed fragment was inserted into pBEBP to create the expression vector pBEBP-CSGalNAc-T as described previously (24). The CSGalNAc-T catalytic domain was expressed in Sf21 insect cells. A 50 ml volume of culture medium was mixed with anti-FLAG M1 antibody resin (Sigma, St. Louis, MO). The protein-resin mixture was washed twice with 50 ml TBS (50 ml Tris-HCl, pH 7.4, and 150 ml NaCl) containing 1 ml CaCl2 and suspended in 100 µl of each of the assay buffers described below.

**Construction of β3Gal-T, βGal-T6, and GlcAT-I Fused with Flag Peptides**—The putative catalytic domain of β3Gal-T, βGal-T6, and GlcAT-I was expressed as a secreted protein fused with Flag peptide in insect cells or COS-1 cells. For β3Gal-T, 5′-GGGAGCAATTGAGTACAAAAGGCACGCTTCAGATGACGACGTTGA-3′ and 5′-GGGAGCACCAGTCCGCTGCTGCTGCTGCT-3′ were used as PCR primers. To amplify the GlcAT-I gene, two primers, 5′-GGGAGCAATTGAGTACAAAAGGCACGCTTCAGATGACGACGTTGA-3′ and 5′-GGGAGCACCAGTCCGCTGCTGCTGCTGCT-3′ were utilized. These glycosyltransferases were expressed using the GATEWAY system as described. For β3Gal-T, a PCR fragment with 5′-CGGGATCC- GAGCCCCAGGACCCGAGCCGATG-3′ and 5′-GCTCTAGAGGGCG- GGGGGTGAAAGCGTGTTC-3′ was introduced into the BamHI and sites of pFLAG-CMV-1 (Sigma) and expressed in COS-1 cells.

**Assay of CSGalNAc-T Activity with High Performance Liquid Chromatography**—To determine the enzymic activity, UDP-galactose (UDP-Gal) and UDP-N-acetylgalactosamine (UDP-GalNAc) (Sigma) were utilized as donor substrates. UDP-[3H][H]GalNAc (7.0 Ci/mmol) was from PerkinElmer Life Science (Boston, MA). For acceptor substrates, Gal-αumarin (pNP), GlcA-βpNP, GlcNAc-benzyl, GlcNAc-αpNP, GlcNAc-βpNP, glucose-αpNP, glucose-βpNP, GlcA-βpNP, fucose-αpNP, mannose-αpNP, xylose-(Xyl)-αpNP, and Xyl-βpNP were purchased from Calbiochem (La Jolla, CA) and Sigma. Chondroitin and chondroitin sulfate (CS-A, -B, -C, and -D) were purchased from Seikagaku Corporation (Tokyo, Japan). Chondroitin hexasaccharide and linkage tetrasaccharide methoxyphenyl were kindly provided by Seikagaku Corporation (Tokyo, Japan). Oligosaccharides of CS and chondroitin were prepared as previously described (22). A Xyl-peptide (VLPQEEEGS(-Xyl)GGGQLVT) was obtained from the Peptide Institute Inc. (Osaka, Japan).

For the reaction of Gal-T assay, a 14 m M Hepes buffer (pH 7.4) containing 250 µM UDP-Gal, 12.5 m M MnCl2 and 500 µM of each acceptor substrate was used. For the GalNAc-T assay, a 50 m M MES buffer (pH 6.5) containing 0.1% Triton X-100, 12.5 m M UDP-Gal, 12.5 m M MnCl2, and 500 µM of each acceptor substrate was used. A 10 µl volume of enzyme solution for 20 µl of each reaction mixture was added, and the solution was incubated at 37 °C for 2 h for Gal-T and 16 h for GalNAc-T.

After the incubation, the mixture was filtrated with an Ultrafilter-NC column (Millipore, Bedford, MA), and a 10 µl aliquot was subjected to reverse-phase high performance liquid chromatography (HPLC) on an ODS-80Ts QA column (4.6 × 250 mm, Tosoh, Tokyo, Japan). 0.1% TFA/H2O was used as a running solution, and the products were eluted with a 0–15% (for GlcA-βpNP or 7–10% (for linkage tetrasaccharidemethoxyphenyl) acetonitrile gradient at a flow rate of 1.0 ml/min at 50 °C. For glycosylated peptides, H2O containing 0.1% TFA and 21% acetonitrile was used as the running solution. An ultraviolet spectrophotometer (absorbance at 210 nm), SPD-10A VP (Shimadzu, Kyoto, Japan) was used for detection of the peaks. For the analysis of glycosylated peptide, labeling was carried out with Cy5 (Amersham Biosciences), and fluorescence was detected with a fluorescence detector, RF-10A XL (Shimadzu). For the analysis of elongation activity, a CSGalNAc-T was immobilized on a 100 µg of chondroitin or CS and 40,000–55,500 dpm of UDP-[3H]GalNAc was used. The reaction mixture was incubated at 37 °C, the reaction mixture was filtrated and fractionated with a G2500PW column (Tosoh, Tokyo, Japan) or a Superdex 30-pg column (Amersham Biosciences). The radioactivity of each fraction was monitored by liquid scintillation spectrophotometry.

**Determination of Products by Mass Spectrometry Using CSGalNAc-T**—An additional peak obtained by reverse-phase chromatography...
MALDI-TOF MS, 10 pmol of product was dried, dissolved in 1 l of 0.1% formic acid and 50 l of H2O, and 45 l of fH2O, and 45
Daltonics, Billerica, MA). The product (25 pmol) was dissolved in 5 l of methanol were added.

The product solution was infused at a rate of 3 l/min with CDP-Star and Hyperfilm ECL (Amersham Biosciences) were used as probes for Northern blot analysis. The probe was amplified by PCR with 5'-TATCT-3 and 5'-GTC-GACTTCATCAATATAGGTGGGTTTGAT-3 as primers. Human Multiple Tissue northern Blot III was purchased from Clontech. For the labeling and detection of probe, an AlkPhos Direct Labeling and Detection System (Promega) was utilized.

When this peak was subjected to further analysis by ESI-MS/MS, new peaks appeared at 203.91 and 298.97 m/z. The former peak had exactly the same molecular weight as GlcA-GalNAc-Np (517.19 m/z), and it produced GlcA-Np without a water molecule. ESI-MS in the negative-ion mode revealed that the product could be GalNAc-GlcA-Np (m/z 517.19). Possible N-glycosylation sites are indicated by arrowheads.

RESULTS

Comparison of Amino Acid Sequences between CSGalNAc-T and Known β1,4-Glycosyltransferases—While we were preparing this report, a paper on the cloning and characterization of CSGalNAc-T was published (23). The nucleotide and putative amino acid sequences of the clone we obtained were the same as those of Uyama et al.’s cDNA clone (GenBank™ accession numbers AB081516 and AB071403). This enzyme maintained local homology with βGal-T1 in the putative catalytic domain as shown in Fig. 1, however, no remarkable homology with βGal-T4 was found (data not shown) (29). A DxD motif, which is conserved in many glycosyltransferases and functions as a key sequence for divalent cation binding, existed in the putative catalytic domain. Another motif, GWGRED, which is a key sequence for divalent cation binding, existed in the putative catalytic domain as shown in Fig. 1, however, no remarkable homology with βGal-T4 was found (data not shown) (29). 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product from GlcA/H9252

Reversed-phase HPLC analyses of the CSGalNAc-T NA

product. The chondroitin (open box) and CS-C (closed box) were used as acceptor substrates of CSGalNAc-T. The purified reaction mixtures were applied to a G2500PW column with 0.2 m NaCl as a running buffer at a flow rate of 0.6 ml/min. The activity of each labeled fraction (340 l each) was quantified by liquid scintillation spectrophotometry. The fractions containing the CSGalNAc-T product are indicated by an arrow.

FIG. 3. Determination of chondroitin and CS polymer elongation activity of CSGalNAc-T. The chondroitin (open box) and CS-C (closed box) were used as acceptor substrates of CSGalNAc-T. The purified reaction mixtures were applied to a G2500PW column with 0.2 m NaCl as a running buffer at a flow rate of 0.6 ml/min. The activity of each labeled fraction (340 l each) was quantified by liquid scintillation spectrophotometry. The fractions containing the CSGalNAc-T product are indicated by an arrow.

Enzymatic Synthesis of Chondroitin with CSGalNAc-T

Two kinds of Gal-N-acetylglucosaminyltransferase, CSGalNAc-T.

Determination of CSGalNAc-T Activity in Elongation of Chondroitin Poly- and Oligosaccharides—Two kinds of GalNAc-GlcA linkages are known in CS, one in its polymer structure (3GalNAcβ1–4GlcAβ1–), and the other between the polymer CS and a linkage tetrasaccharide (GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–O-methoxyphenyl) that was chemically synthesized. As shown in Fig. 4, a peak (P) appeared at 28.9 min (Fig. 4B) in addition to the acceptor substrate peak (S) at 29.7 min (Fig. 4, A and B). The peaks S and P were isolated by reversed-phase chromatography and identified with MALDITOF MS. The peak S gave a molecular mass of 779.20 m/z, the same as that of the linkage tetrasaccharide (Fig. 4C). The peak P gave two peaks of 982.23 and 1004.21 m/z as shown in Fig. 4D. The peaks of 982.23 and 1004.21 m/z were the same molecular weight as GalNAc-linkage tetrasaccharide-O-methoxyphenyl and GalNAc-linkage tetrasaccharide-O-methoxyphenyl with Na+, respectively. Moreover, the peak P was identified to have GalNAc on its non-reducing terminus with chondroitinase ACII treatment (data not shown). These results demonstrated that CSGalNAc-T acts to initiate chondroitin sulfate synthesis by transferring GalNAc to GlcA at the non-reducing terminus of the linkage tetrasaccharide.

Acceptor Substrate Specificity of CSGalNAc-T—The CSGalNAc-T activity toward various kinds of acceptors is summarized in Table I. CSGalNAc-T preferred the best linkage tetrasaccharide of all substrates examined. Regarding elongation activity, chondroitin was the preferred acceptor substrate, whereas the sulfated CS polysaccharides were an unsuitable substrate for CSGalNAc-T. The activity levels for the longer oligosaccharide prepared from chondroitin and CS were higher than those for the shorter ones, although they were rather low.

Tissue Distribution of CSGalNAc-T Transcripts—To determine the size of the CSGalNAc-T transcripts, Northern blot analysis was performed. As shown in Fig. 5A, the transcript was ~4 kb in length. We did not observe any other bands except the 4-kb band. Its expression was found in all tissues examined, however, the levels differed among tissues. The expression levels of CSGalNAc-T in various tissues and cell lines, as determined by quantitative real-time PCR, were shown as the relative amount versus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts (Fig. 5, B and C). The transcripts were highly expressed in thyroid and placenta, although they were ubiquitously expressed in other tissues. However, their expression levels were extremely low in almost all cell lines examined.
Enzymatic Synthesis of Chondroitin Pentasaccharide in Vitro—We performed an in vitro enzymatic synthesis of a chondroitin pentasaccharide, a foundation structure of CS, by employing four glycosyltransferases and synthetic Xyl-peptide (VLPQEEEGS(Xyl)GGGQLVT; N-terminal sequence of bikunin) (Fig. 6). As shown in Fig. 6B, when three known glycosyltransferases, /H92524Gal-T7, /H92523Gal-T6, and GlcAT-I, the specificities of which for the GAG synthesis were already reported, were utilized in the enzyme reaction, three additional peaks were detected by reversed-phase chromatography. Before this experiment, we had confirmed that each peak, P1, P2, and P3, is a sequential product of /H92524Gal-T7, /H92523Gal-T6, and GlcAT-I, respectively (data not shown). Each peak was identified to be Gal-Xyl-peptide (P1), Gal-Gal-Xyl-peptide (P2), or GlcA-Gal-Gal-Xyl-peptide (P3) by the position of control oligosaccharide-peptides, which were prepared by a sequential enzyme reaction using each of the three enzymes as illustrated in Fig. 6D. When CSGalNAc-T was added to the reaction mixture in addition to the three enzymes, a novel peak (P4) appeared (Fig. 6C) at 35.0 min. Chondroitinase ACII treatment of P4 resulted in the apparent digestion of P4, indicating that it has a GalNAc1–4GlcA linkage in its structure (data not shown).

These results demonstrated that the chondroitin pentasaccharide with the peptide can be synthesized in vitro, using three known specific enzymes and one novel glycosyltransferase, CSGalNAc-T.

DISCUSSION

We found that a novel enzyme, CSGalNAc-T, exhibited GalNAc-T activity toward GlcA. From its acceptor specificity, it was suggested that CSGalNAc-T is involved in both the elongation and initiation of CS synthesis. While we were preparing this report, a paper describing the cloning and characterization of the same enzyme, CSGalNAc-T, was published (23). However, the activity toward the linkage tetrasaccharide was not described by these authors. Here, we demonstrate an apparent initiation and elongation activity, in addition, we examined in greater detail the substrate specificity toward various acceptors. We also demonstrate the following points, which Uyama et al. did not detect or examine: 1) CSGalNAc-T showed apparent initiation activity toward the linkage tetrasaccharide. 2) Regarding the elongation activity, CSGalNAc-T preferred chondroitin to CS. No species of CS, CS-A, -B, -C, or -D, was a suitable acceptor for CSGalNAc-T. 3) The activity was dependent on the length of oligosaccharide chain as an acceptor. 4) Finally, we could construct a chondroitin pentasaccharide on the peptide using the four enzymes, including CSGalNAc-T, by synthesis in vitro.

Because CSGalNAc-T contained the /H92524Gal-T motif (GWGGED) in its putative catalytic domain, we considered at first that it would exert /H92524Gal-T activity. Unexpectedly, it transferred GalNAc, not Gal, to GlcA-pNp. This enzyme is the first example demonstrating that the GWGGED motif is shared not only by /H92524Gal-T but also by /H92524GalNAc-T. Based on the crystallized structure of bovine /H92524Gal-T1, it is proposed that the Trp residue at position 314 is necessary to lock with a donor substrate, UDP-Gal, with its strict specificity. The Gly313 residue is proposed to be also important for the rotation of Trp314 to maintain Gal-T activity (9). However, many members of the UDP-GalNAc-polypeptide N-acetylgalactosaminyltransferase (pp-GalNAc-T) family, for which a donor substrate is UDP-GalNAc, possess the WGGE sequence in their catalytic domains, but not the GWGGED sequence (30–34), whereas

| Acceptor substrate          | Activity (pmol/ml medium/h) |
|-----------------------------|-----------------------------|
| Chondroitin Polysaccharide  | 15.9                        |
| Oligosaccharide             |                             |
| CH14                        | 0.6                         |
| CH12                        | 0.5                         |
| CH10                        | 0.3                         |
| CH8                         | 0.2                         |
| CH6                         | 0.1                         |
| Chondroitin sulfate Polysaccharide | 0.5, 0.8, 1.3, 0.8         |
| CS-A                        | 1.8                         |
| CS-B                        | 1.5                         |
| CS-C                        | 1.9                         |
| CS-D                        | 1.6                         |
| Linkage tetrasaccharide     | 22.5                        |

FIG. 4. Determination of initiation activity of CSGalNAc-T. The linkage tetrasaccharide-O-methoxyphenyl (A) was incubated with CSGalNAc-T and UDP-GalNAc, and the product (B) was analyzed by reversed-phase chromatography. Both the substrate (indicated as S) and additional peak (indicated as P) were collected and identified by MALDI-TOF-MS (C and D).
CSGalNAc-T possesses the GWGGED motif. Thus, we speculate that the first Gly and Trp residues in the GWGGED motif do not determine the specificity for a donor substrate. The product of CSGalNAc-T was GalNAc-GlcA, which has been identified only in CS. It was predicted that CSGalNAc-T is the enzyme involved in the synthesis of CS. Chondroitin has the structure of disaccharide units in the polymerized chain, \((\text{GalNAc} \rightarrow \text{GlcA})_n\), and the linkage tetrasaccharide \((\text{GlcA} \rightarrow \text{Gal} \rightarrow \text{GlcA} \rightarrow \text{Gal})_n\). In the present study, we defined the ability of CSGalNAc-T to make both linkages as elongation and initiation activity. CSS was reported to have elongation activity but did not exhibit initiation activity (18). Regarding the elongation activity of CSS, it could efficiently transfer GalNAc to chondroitin polysaccharide but not to CS polysaccharide. This is similar to the CSGalNAc-T activity.

Sulfation affects enzyme activity for chondroitin chain elongation and termination. CSGalNAc-T partially purified from bovine serum was examined for its activity toward various sulfated chondroitins (21). It showed higher levels of activity when 6-O- or 4-O-sulfated GalNAc in the acceptor was utilized, whereas terminal 3-O-sulfated GlcA or penultimate 4,6-O-disulfated GalNAc inhibits the activity. The CS-A or -C polysaccharide used in this study contains a large amount of 4-O- or 6-O-sulfated GalNAc, respectively, and CS-D mainly contains 2-O-sulfated GlcA and 6-O-sulfated GalNAc residues. CS-B contains a large amount of dermatan sulfate with 4-O-sulfation.

FIG. 5. Northern blot analysis and quantitative real-time PCR analysis of CSGalNAc-T transcript in human tissues and cell lines. A, the 5′-untranslated region (695 bp) was amplified and used as a probe for Northern blot analysis. The position of a 4.4-kb marker is indicated with an arrow. B and C, standard curves for CSGalNAc-T and GAPDH were generated by serial dilution of each plasmid DNA. The expression level of the CSGalNAc-T transcript was normalized to that of the GAPDH transcript, which was measured in the same cDNAs. Data were obtained from triplicate experiments and are indicated as the mean ± S.D. PBMC, peripheral blood mononuclear cells; GOTO and SCCH-26, neuroblastomas; T98G and U251, glioblastomas; SW1736, thyroid cancer; HL-60, promyelocytic leukemia; Numaliga, B cell lymphoma; Daudi, B cell (Burkitt’s) lymphoma; U937, monocytic leukemia; K562, erythroid leukemia; U266, myeloma; Colo-205, HCT15, LSB, and SW480, colorectal cancers; MKN45 and KATO III, stomach cancers, G-361, melanoma; HepG2, hepatocarcinoma; Capan-2, pancreas cancer; PA-1, ovary teratocarcinoma.

FIG. 6. Enzymatic synthesis of chondroitin pentasaccharide. A, the synthetic Xyl-peptide was detected by a reversed-phase chromatography. The acceptor substrate was incubated with three glycosyltransferases, βGal-T7, βGal-T6, and GlcAT-I (B) or four glycosyltransferases, βGal-T7, βGal-T6, GlcAT-I, and CSGalNAc-T (C) and analyzed by the chromatography. For the reaction buffer 50 mM MES (pH 6.5) was employed. Each peak was identified by the control oligosaccharide-peptide, which was prepared by the sequential enzyme reaction (D). S, Xyl-peptide; P1, Gal-Xyl-peptide; P2, Gal-Gal-Xyl-peptide; P3, GlcA-Gal-Xyl-peptide; P4, GalNAc-GlcA-Gal-Gal-Xyl-peptide.
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sulfated GalNAc. However, these acceptors obtained from natural materials are mixtures of sulfated GalNAc and GlcA residues and may contain unexpected sulfated sugars that inhibit the elongation activity of CSGalNAc-T. Another possibility is that a different CSGalNAc-T functions in sulfated chondroitin elongation. In fact, we have found another candidate in a data base, temporarily named CSGalNAc-T2, which has a highly homologous sequence to CSGalNAc-T. CSGalNAc-T preferred pentasaccharide of CS on a peptide, using a set of recombinant enzymes, all recombinant enzymes prepared in a soluble form were active in synthesizing the pentasaccharide on the peptide, the sequence of which is the N-terminus of bikunin, a Kunitz-type proteinase inhibitor. It is concluded that CS is synthesized on a serine residue in this region (43). The peptide-sugar chain synthesized enzymatically in vitro would be a very useful tool to investigate which amino acid residues are recognized by each enzyme for CS and HS synthesis and which amino acid residues or sulfations determine the classification of CS and HS. It is also a valuable acceptor substrate to synthesize a complex structure of CS using additional glycosyltransferases and sulfotransferases. This is the first time a chondroitin pentasaccharide has been constructed using recombinant glycosyltransferases in vitro.

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Enzymatic Synthesis of Chondroitin with a Novel Chondroitin Sulfate N-Acetylgalactosaminyltransferase That Transfers N-Acetylgalactosamine to Glucuronic Acid in Initiation and Elongation of Chondroitin Sulfate Synthesis

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