Identification of Chondroitin Sulfate Glucuronyltransferase as Chondroitin Synthase-3 Involved in Chondroitin Polymerization

CHONDROITIN POLYMERIZATION IS ACHIEVED BY MULTIPLE ENZYME COMPLEXES CONSISTING OF CHONDROITIN SYNTASE FAMILY MEMBERS

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Recently, we demonstrated that chondroitin polymerization is achieved by any two combinations of human chondroitin synthase-1 (ChSy-1), ChSy-2 (chondroitin sulfate synthase 3, CSS3), and chondroitin-polymerizing factor (ChPF). Although an additional ChSy family member, called chondroitin sulfate glucuronyltransferase (CSGlca-T), has been identified, its involvement in chondroitin polymerization remains unclear because it possesses only glucuronyltransferase II activity responsible for the elongation of chondroitin sulfate (CS) chains. Herein, we report that CSGlca-T exhibits polymerization activity on α-thrombomodulin bearing the truncated linkage region tetrasaccharide through its interaction with ChSy-1, ChSy-2 (CSS3), or ChPF, and the chain length of chondroitin formed by the co-expressed proteins in various combinations is different. In addition, ChSy family members co-expressed in various combinations exhibited distinct but overlapping acceptor substrate specificities toward the two synthetic acceptor substrates, GlcUAβ1–3Galβ1-O-naphthalenemethanol and GlcUAβ1–3Galβ1–O-C2H4NH-benzyloxycarbonyl, both of which share the disaccharide sequence with the glycosaminoglycan-protein linkage region tetrasaccharide. Moreover, overexpression of CSGlca-T increased the amount of CS in HeLa cells, whereas the RNA interference of CSGlca-T resulted in a reduction of the amount of CS in the cells. Furthermore, the analysis using the CSGlca-T mutant that lacks any glycosyltransferase activity but interacts with other ChSy family members showed that the glycosyltransferase activity of CSGlca-T plays an important role in chondroitin polymerization. Overall, these results suggest that chondroitin polymerization is achieved by multiple combinations of ChSy-1, ChSy-2, CSGlca-T, and ChPF and that each combination may play a unique role in the biosynthesis of CS. Based on these results, we renamed CSGlca-T chondroitin synthase-3 (ChSy-3).

Chondroitin sulfates (CSs) are universally ubiquitous molecules distributed on cell surfaces and in extracellular matrices (1–4). CS is a linear, sulfated polysaccharide composed of repeating disaccharide units consisting of alternating uronic acid (GlcUA) and GalNAc residues and synthesized as a proteoglycan bound to specific Ser residues in the core protein (1–4). Compelling evidence has shown that CS-proteoglycans play crucial roles in a number of physiological phenomena, such as cell adhesion, morphogenesis, neural network formation, and cell division (5, 6). Therefore, an understanding of CS synthesis and its regulatory mechanism underlying diverse CS functions is essential.

The biosynthesis of CS is initiated by the addition of Xyl to specific serine residues in the core protein, followed by the sequential addition of two Gal residues and a GlcUA residue, forming the tetrasaccharide linkage structure GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1–O-Ser. Each transferring reaction is catalyzed by the corresponding glycosyltransferase. Then chondroitin polymerization with alternating GalNAc and GlcUA takes place, forming the repeating disaccharide region. Then a number of sulfotransferases modify the chondroitin backbone with sulfate at specific positions, resulting in the structural diversity of CS (7).

To date, six homologous glycosyltransferases, all of which are probably responsible for CS biosynthesis, have been cloned. We and others have revealed four chondroitin-synthetizing enzymes: chondroitin synthase-1 (ChSy-1), chondroitin syn-

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thase-2 (ChSy-2)/chondroitin sulfate synthase-3 (CSS3), and chondroitin GalNac transferases 1 and 2 (8–12). ChSy-1 and ChSy-2 show dual glycosyltransferase activities of GlcUA transferase II (GlcAT-II) and GalNac transferase II (GalNacT-II), which are responsible for synthesizing the repeating disaccharide units of CS, whereas chondroitin GalNac transferases 1 and 2 catalyze chain initiation and elongation, exhibiting activities of N-acetylgalactosaminyltransferase I (GalNAcT-I) and GalNacT-II (9–12). In addition, chondroitin sulfate GlcUA transferase (CSGlcA-T) has been identified by others (13).

Previously, we revealed that chondroitin polymerization could be demonstrated in vitro when ChSy-1 was co-expressed with chondroitin-polymerizing factor (ChPF), which shows a weak yet significant homology to ChSy-1 (14, 15). Although ChPF has little glycosyltransferase activity, co-expression of ChPF and ChSy-1 resulted in a marked augmentation of not only the glycosyltransferase activity but also the polymerase activity of ChSy-1 (14). In addition, co-expressed ChSy-2 (CSS3) and ChSy-1 or ChSy-2 (CSS3) and ChPF showed chondroitin polymerase activities (16). Thus, chondroitin polymerization was achieved by any two combinations of ChSy-1, ChSy-2 (CSS3), and ChPF. Although an additional ChSy family member, called CSGlcA-T, has been identified, the involvement of CSGlcA-T in chondroitin polymerization remains unclear, because it possesses only glucuronyltransferase II activity responsible for the elongation of CS chains (13). Herein, we report that CSGlcA-T exhibits polymerization activity onto α-thrombomodulin bearing the truncated linkage region tetrasaccharide through its interaction with ChSy-1, ChSy-2 (CSS3), or ChPF. Based on these results, we renamed CSGlcA-T chondroitin synthase-3 (ChSy-3).

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-[U-14C]GlcUA (285.2 mCi/mmol) and UDP-[3H]GalNac (10 Ci/mmol) were purchased from PerkinElmer Life Sciences. Unlabeled UDP-GlcUA and UDP-GalNAc were obtained from Sigma. Chondroitin (a chemically desulfated derivative of whale cartilage chondroitin sulfate A) and *Arthrobacter aurescens* chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku Corp. (Tokyo, Japan). Purified α-thrombomodulin (α-TM) (17) was provided by the research institute, Dai-ichi Pharmaceutical Co. (Tokyo, Japan), and contained a linkage region tetrasaccharide, GlcUAβ1-3Galβ1-3Galβ1-4Xyl (18). GlcUAβ1-3Galβ1-O-naphthalenemethanol (NM) and GlcUAβ1-3Galβ1-O-C2H4NH-Cbz were chemically synthesized (19). Superdex™ Peptide HR10/30 and Superdex™ 200 10/300 GL columns were obtained from Amersham Biosciences.

**Construction of a Soluble Form of CSGlcA-T (ChSy-3)—**The cDNA fragment of a truncated form of CSGlcA-T (ChSy-3), lacking the first 57 N-terminal amino acids, was amplified by PCR with KIAA1402 cDNA obtained from the Kazusa DNA Research Institute (Chiba, Japan) as a template using a 5'-primer (5'-GAAGATCTAGGCTCGCTAGACAAAG-3') containing an in-frame BglIII site and a 3'-primer (5'-GAGATCTCATCTGCGTCGCCCTCC-3') containing a BglIII site located 72 bp downstream of the stop codon. PCR was carried out with KOD-Plus DNA polymerase (TOYOBO, Tokyo) for 30 cycles at 94°C for 30 s, 58°C for 30 s, and 68°C for 150 s in 5% (v/v) dimethyl sulfoxide. The PCR fragment was subcloned into the BamHI site of pGIR201protA (20), resulting in the fusion of the insulin signal sequence and the protein A sequence present in the vector, as described previously (8, 14, 16). The nucleotide sequence of the amplified cDNA was determined in a 377 DNA sequencer (PE Applied Biosystems). Soluble forms of ChSy-1, ChSy-2, and ChPF were constructed previously (8, 14, 16).

**Site-directed Mutagenesis—**A two-stage PCR mutagenesis method was used to construct CSGlcA-T (ChSy-3) mutant. Two separate PCRs were performed to generate two overlapping gene fragments using the soluble form of CSGlcA-T (ChSy-3) cDNA as a template. In the first PCR, the sense 5'-primer described above and the antisense internal mutagenic primer listed below were used: D184A 5'-GCACATATGTTGGCATCTGCGATGAT-3' (the mutated nucleotide is underlined). In the second round of PCR, the sense internal mutagenic primer (complementary to the antisense internal mutagenic primer) and the antisense 3'-primer described above were used. These two PCR products were gel-purified and then used as a template for a third PCR containing the sense 5'-primer and the antisense 3'-primer described above. The final PCR fragment was subcloned into the BamHI site of pGIR201protA (20). The nucleotide sequence of the amplified cDNA was determined in a 377 DNA sequencer (PE Applied Biosystems).

**Expression of a Soluble Form of CSGlcA-T (ChSy-3) and Enzyme Assays**—The expression plasmid (6.0 μg) was transfected into COS-1 cells on 100-mm plates using FuGENETM 6 (Roche Applied Science) according to the manufacturer's instructions. For co-transfection experiments, the CSGlcA-T (ChSy-3) and ChSy-1, ChSy-2, or ChPF expression plasmids (3.0 μg each) were co-transfected into COS-1 cells on 100-mm plates using FuGENE 6, as above. Two days after transfection, 1 ml of the culture medium was collected and incubated with 10 μl of IgG-Sepharose (Amersham Biosciences) for 1 h at 4°C. The beads recovered by centrifugation were washed with and then resuspended in the assay buffer and tested for GalNAcT and GlcUA transferase activities, as described below. To quantify the protein absorbed onto IgG-Sepharose beads, the bound protein was eluted with 1 M acetic acid and then quantified using the BCA Protein Assay Reagent (enhanced protocol; Pierce). Assays for GalNAcT-II and GlcAT-II were carried out using chondroitin as an acceptor and UDP-GalNAc or UDP-GlcUA as a sugar donor, respectively, as described previously (21, 22). Polymerization reactions using α-TM, GlcUAβ1-3Galβ1-O-NM, or GlcUAβ1-3Galβ1-O-C2H4NH-Cbz as acceptors were co-incubated in reaction mixtures containing the following constituents in a total volume of 20 μl: 1 nmol of α-TM, 100 nmol of GlcUAβ1-3Galβ1-O-NM or 100 nmol of GlcUAβ1-3Galβ1-O-C2H4NH-Cbz, 0.25 mm UDP-[3H]GalNAc (5.28 × 10⁵ dpm), 0.25 mm UDP-GlcUA, 100 mM MES buffer, pH 6.5 or 5.8, 10 mM MnCl₂, and 10 μl of the resuspended beads. The mixtures were incubated at 37°C overnight.

**Characterization of the Enzyme Reaction Products—**Products of polymerization reactions on α-TM were isolated by gel
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filtration on a Superdex peptide column with 0.2 M NH₄HCO₃ as the eluent. The [³H]GalNAc-labeled oligosaccharide chains were released from α-TM by alkaline reduction treatment using 1.0 M NaBH₄, 0.05 M NaOH and then exhaustively digested with chondroitinase ABC using 50 µlU of the enzyme for 1 h, as described previously (23). An aliquot of the enzyme digest was subjected to gel filtration on a Superdex peptide column, as described above. To determine the size of reaction products, the remaining aliquot was subjected to gel filtration on a Superdex 200 column with 0.2 M NH₄HCO₃ as the eluent. Calibration of the Superdex 200 column was performed using a series of commercial polysaccharides of known size.

Pull-down Assays—The cDNA fragment of a truncated form of CSGlcA-T (ChSy-3), lacking the first 57 N-terminal amino acids of CSGlcA-T (ChSy-3), was amplified using a 5’-primer (5’-CGGAATTCAGCTGGCTGACTCCTCTGGC-3’) containing an XhoI site and a 3’-primer (5’-CGGAATTCCATTCGTCCTGCCCCTCC-3’) containing an EcoRI site. The cDNA fragment of a truncated form of ChSy-2, lacking the first 129 N-terminal amino acids of ChSy-2, was also amplified using a 5’-primer (5’-CGCTCTAGAGCCCGTCCGCGGAGCGTCC-3’) containing an in-frame XbaI site. Each DNA fragment was inserted into a polymerization vector (Invitrogen). The nucleotide sequence of the ampli-
fication vector (Clontech). The Golgi marker virus (pEGFP-Golgi) was constructed using the pECFP-Golgi vector (Clontech) that harbors a sequence encoding the N-terminal 81 amino acids of human B1–4-galactosyltransferase (24). This region of human B1–4-galactosyltransferase contains the membrane-anchoring signal peptide that targets the fusion protein to the trans-medial region of the Golgi apparatus (25–27). The region from pEGFP-Golgi was digested with Nhel and BamHI and subcloned into pEGFP-N1. In addition, the ER marker vector (pDsRed2-ER) was obtained from Clontech. Combinations of GFP-tagged and DsRed-Monomer-tagged expression vectors (3.0 µg each) were transfected into HeLa cells on glass bottom dishes (Matsunami Glass) using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Fluorescent images were obtained using a laser-scanning confocal microscope, FLUOVIEW (Olympus, Tokyo, Japan).

Northern Blot Analysis—A commercial human 12-Lane Multiple Tissue Northern blot (Clontech) membrane was used for the analysis. The membrane was probed with a gel-purified, radiolabeled (>1 × 10⁶ cpm/µg), 871-bp CSGlcA-T (ChSy-3)-specific fragment corresponding to nucleotides 3031–3903 of the CSGlcA-T cDNA (GenBank™ accession number AB095612).

Establishment of an Expression Vector for CSGlcA-T (ChSy-3) and Preparation of Cells That Stably Overexpress CSGlcA-T (ChSy-3)—The cDNA fragment encoding CSGlcA-T (ChSy-3) was amplified from KIAA1402 cDNA as a template using a 5’-primer (5’-CGGAATTCAGCTGGCTGACTCCTCTGGC-3’) containing an EcoRI site and a 3’-primer (5’-CGCTCTAGAGCCCGTCCGCGGAGCGTCC-3’) containing an XhoI site. Each DNA fragment was inserted into a pcDNA3Ins-His expression vector, resulting in the fusion of the protein with the insulin signal sequence and His_Tag sequence present in the vector. Combinations of these constructs and the protein A-tagged expression vectors were transfected into COS-1 cells on 100-mm plates using FuGENE™ 6 (Roche Applied Science) according to the manufacturer’s instructions. Two days after transfection, 1 ml of the culture medium was collected and incubated with 10 µl of Ni²⁺-NTA-agarose (Qiagen) overnight at 4 °C. The beads recovered by centrifugation were washed with TBS buffer containing TWEEN 20 three times and subjected to SDS-PAGE (7% gel), and proteins were transferred to a polyvinylidene difluoride membrane. The membrane, after blocking in PBS containing 2% skim milk and 0.1% Tween 20, was incubated with IgG antibody and then treated with anti-mouse IgG conjugated with horseradish peroxidase (Amershams Biosciences). Proteins bound to the antibody were visualized with an ECL advance kit (Amersham Biosciences).

Subcellular Localization—The cDNA fragment encoding CSGlcA-T (ChSy-3) was amplified using a 5’-primer (5’-CGGAATTCGAGCTGGCTGACTCCTCTGGC-3’) containing an EcoRI site and a 3’-primer (5’-CGGAATTCAGCTGGCTGACTCCTCTGGC-3’) containing an EcoRI site. The cDNA fragment encoding ChSy-1 was amplified using a 5’-primer (5’-CCCTCGAGAGCGGCGGAGCGGAGCGGCT-3’) containing an Xhol site and a 3’-primer (5’-CCCTCGAGAGCGGCGGAGCGGAGCGGCT-3’) containing an in-frame XhoI site. The cDNA fragment encoding ChSy-2 was amplified using a 5’-primer (5’-CCCTCGAGAGCGGCGGAGCGGAGCGGCT-3’) containing an Xhol site and a 3’-primer (5’-CCCTCGAGAGCGGCGGAGCGGAGCGGCT-3’) containing an in-frame XhoI site. The cDNA fragment encoding ChPF was amplified using a 5’-primer (5’-CCCTCGAGACTCCTCTGGGCTCTCGG-3’) containing an Xhol site and a 3’-primer (5’-CCCTCGAGACTCCTCTGGGCTCTCGG-3’) containing an in-frame EcoRI site. PCR was carried out with KOD-Plus DNA polymerase (TOYOBO) for 30 cycles at 94 °C for 30 s, 53 °C for 42 s, and 68 °C for 180 s in 5% (v/v) dimethyl sulfoxide. Each PCR fragment was subcloned into the pEGFP-N1 or pDsRed-Monomer-N1 expression vector (Clontech). The Golgi marker virus (pEGFP-Golgi) was constructed using the pECFP-Golgi vector (Clontech) that harbors a sequence encoding the N-terminal 81 amino acids of human B1–4-galactosyltransferase (24). This region of human B1–4-galactosyltransferase contains the membrane-anchoring signal peptide that targets the fusion protein to the trans-medial region of the Golgi apparatus (25–27). The region from pEGFP-Golgi was digested with Nhel and BamHI and subcloned into pEGFP-N1. In addition, the ER marker vector (pDsRed2-ER) was obtained from Clontech. Combinations of GFP-tagged and DsRed-Monomer-tagged expression vectors (3.0 µg each) were transfected into HeLa cells on glass bottom dishes (Matsunami Glass) using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Fluorescent images were obtained using a laser-scanning confocal microscope, FLUOVIEW (Olympus, Tokyo, Japan).

RNA Interference of the CSGlcA-T (ChSy-3) Gene.—A 25-mer double-stranded RNA composed of sense 5’-GGCUUAACAG-UAGAAAUGAACAACUGAG-3’ and antisense 5’-CAGUUGGUUCUAUUACUGUUAGCGAAGCCACAUUAA-3’ sequences and of sense 5’-UCGGCUAGACAAAGAGUAGAGACAG-3’ and antisense 5’-GUCUUAUACUUGUUGGCUCAGGCCAAGAUUAA-3’ sequences for CSGlcA-T (ChSy-3) was designed and purchased from iGENE (Tsukuba, Japan). Silencing, scrambled RNA composed of sequences with no homology to known human sequences (iGENE) was used as a control. The HeLa cells were transfected
with 10 nm small interfering RNA (siRNA) using TransIT-TKO transfection reagent (Takara, Otsu, Japan).

**Quantitative Real Time Reverse Transcription-PCR**—Total RNA was extracted from HeLa cells using a QuickPrep total RNA extraction kit (Amersham Biosciences). The cDNA was synthesized from −1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega) and oligo(dT)20-M4 adaptor primer (Takara). Primer sequences used were as follows: CSGlcA-T (ChSy-3), a forward primer 5′-GCTCGGCTAGACCAAAG-3′ and a reverse primer 5′-TGTAGCTCGGGAGGTCA-3′; and glyceraldehyde-3-phosphate dehydrogenase, a forward primer 5′-ATGGGTGTGAACCATGAGAAGTA-3′ and a reverse primer 5′-GGCAGTGTAGGCGGCTAGAC-3′. Quantitative real time reverse transcription-PCR was performed using a FastStart DNA Master plus SYBR Green I (Roche Applied Science) in a LightCycler ST300 (Roche Applied Science). The expression level of CSGlcA-T (ChSy-3) mRNA was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase transcript.

**Derivatization of Glicosaminoglycans from HeLa Cells Using a Fluorophore, 2-Aminobenzamide**—Cells were homogenized in acetone and air-dried. The dried materials were digested with heat-pretreated (60 °C for 30 min) actinase E in 200 μl of 0.1 M borate-sodium, pH 8.0, containing 10 μM calcium acetate at 60 °C for 24 h. Following incubation, each sample was treated with trichloroacetic acid, and the resultant precipitate was removed by centrifugation. The soluble fraction was extracted with ether. The aqueous phase was neutralized with 1.0 m sodium carbonate and adjusted to contain 80% ethanol. The resultant precipitate was dissolved in 50 μl pyridine acetate and subjected to gel filtration on a PD-10 column using 50 μM pyridine acetate as an eluent. The flow-through fractions were collected and evaporated to dryness. The dried sample was subsequently dissolved in water. Digestion with chondroitinase ABC (5 mIU) was conducted as described previously at 37 °C for 1 h in a total volume of 10 μl (28). Reactions were terminated by boiling for 1 min. Each digest was derivatized with 2-amino-benzamide and then analyzed by HPLC, as reported previously (29).

**RESULTS**

**Glycosyltransferase Activity of CSGlcA-T (ChSy-3)**—Recent studies revealed that co-expression of any two of ChSy-1, ChSy-2 (CSS3), and ChPF augmented glycosyltransferase activities when compared with ChSy-1 or ChSy-2 expressed alone (14, 16). These findings prompted us to investigate whether the co-expression of an additional ChSy family member, CSGlcA-T (ChSy-3), despite having only GlcAT-II activity responsible for the elongation of CS chains (13), with ChSy-1, ChSy-2 (CSS3), or ChPF might augment the glycosyltransferase activities. Hence, co-expression of CSGlcA-T (ChSy-3) with ChSy-1, ChSy-2 (CSS3), or ChPF was carried out. To facilitate the functional analysis of CSGlcA-T (ChSy-3), a soluble form of CSGlcA-T (ChSy-3) was generated by replacing the first 57 amino acids of the protein with a cleavable insulin signal sequence and a protein A IgG-binding domain, as described under “Experimental Procedures.” Then the soluble protein was expressed in COS-1 cells as a recombinant protein fused with the protein A IgG-binding domain. The fusion protein secreted into the medium was adsorbed onto IgG-Sepharose beads for purification to eliminate endogenous glycosyltransferases, and then the protein-bound beads were used as an enzyme source. When CSGlcA-T (ChSy-3) bound to beads was exposed to polymerizing activities for disaccharide-repeating units of CS onto polymer chondroitin as an acceptor substrate, and the values are the mean ± S.E. of three determinations.

### TABLE 1

| Protein                        | GalNAcT-II activity* | GlcAT-II activity* |
|-------------------------------|----------------------|--------------------|
| CSGlcA-T (ChSy-3)             | 1.0 ± 0.03           | 0.4 ± 0.05         |
| ChPF                          | ND                   | ND                 |
| ChSy-1                        | 1.0 ± 0.2            | 3.3 ± 0.5          |
| ChSy-2                        | 0.1 ± 0.01           | 0.1 ± 0.01         |
| ChSy-1/ChPF                   | 14.2 ± 2.3           | 54.8 ± 3.5         |
| ChSy-1/ChSy-2 (CSS3)          | 38.1 ± 2.9           | 34.4 ± 3.2         |
| ChSy-1/CSGlcA-T (ChSy-3)      | 36.2 ± 3.8           | 64.2 ± 5.3         |
| ChSy-2 (CSS3)/ChPF            | 5.2 ± 0.1            | 2.6 ± 0.2          |
| ChSy-2 (CSS3)/CSGlcA-T (ChSy-3) | 7.9 ± 0.2       | 5.2 ± 0.3          |
| CSGlcA-T (ChSy-3)/ChPF        | 2.4 ± 0.2            | 3.4 ± 0.6          |
| CSGlcA-T (ChSy-3)/D184A       | ND                   | ND                 |
| ChSy-1/CSGlcA-T (ChSy-3)/D184A | 29.0 ± 2.1          | 33.8 ± 3.0         |
| ChSy-2 (CSS3)/CSGlcA-T (ChSy-3) | 0.9 ± 0.07       | ND                 |
| CSGlcA-T (ChSy-3)/D184A/ChPF  | 0.6 ± 0.05           | ND                 |

* Polymer chondroitin was used as an acceptor substrate, and the values are the mean ± S.E. of three determinations.

ND, not detected (<0.01 mmol/mg/h).

ChSy-1/ChPF, ChSy-1/ChSy-2 (CSS3), ChSy-1/CSGlcA-T (ChSy-3), ChSy-2 (CSS3)/ChPF, ChSy-2 (CSS3)/CSGlcA-T (ChSy-3), ChPF/ChSy-1/CSGlcA-T (ChSy-3) D184A, ChSy-2 (CSS3)/CSGlcA-T (ChSy-3) D184A, and CSGlcA-T (ChSy-3) D184A/ChPF represent co-expressed ChSy-1 and ChPF, ChSy-1 and ChSy-2 (CSS3), ChSy-1 and CSGlcA-T (ChSy-3), ChSy-2 (CSS3) and ChPF, CSGlcA-T (ChSy-3) D184A, CSGlcA-T (ChSy-3)/ChPF (31), CSGlcA-T (ChSy-3)/D184A, and CSGlcA-T (ChSy-3)/ChPF (31, 32, 33) and CSGlcA-T (ChSy-3)/D184A, and CSGlcA-T (ChSy-3)/D184A and ChPF, respectively. In addition, the efficiency of the complex formation seems to be similar among the co-expressed proteins in various combinations (see supplemental Fig. 2).
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![Graph showing the comparison of chondroitin polymerizing activity using various combinations of CSGlca-T (ChSy-3), ChSy-1, ChSy-2, and ChPF.](image)

ChSy-2 (CSS3), or ChPF. When α-TM was used as an acceptor substrate for the polymerization assay in the presence of UDP-[3H]GalNAc and UDP-GlcUA, incubation of both of the co-expressed putative enzyme complexes yielded radiolabeled saccharide chains on α-TM.

We then examined whether the length of the chondroitin chains formed by the co-expressed proteins in various combinations could be different. For this analysis, an equal amount of the co-expressed proteins in various combinations was used as each of the enzyme sources. Each reaction product obtained with α-TM was subjected to reductive β-elimination using NaBH₄/NaOH, and the released radiolabeled saccharides were analyzed by gel filtration chromatography using a Superdex 200 column, as shown in Fig. 1. The lengths of chondroitin chains formed by co-expressed CSGlca-T (ChSy-3) and ChPF were comparable with those of commercial chondroitin chains (a chemically desulfated derivative of whale cartilage chondroitin sulfate A), although chondroitin chains polymerized by the co-expressed CSGlca-T (ChSy-3) and ChSy-1 were longer than those polymerized by the co-expressed CSGlca-T (ChSy-3) and ChPF and shorter than those formed by the co-expressed ChSy-1 and ChPF. In contrast, co-expressed CSGlca-T (ChSy-3) and ChSy-2 (CSS3) formed shorter chains compared with commercial chondroitin chains. It should be noted that no polymerization was induced on α-TM either through one of the soluble forms of ChSy-1, ChSy-2 (CSS3), CSGlca-T (ChSy-3), or ChPF or via a mixture of separately expressed soluble forms of any two of ChSy-1, ChSy-2 (CSS3), CSGlca-T (ChSy-3), and ChPF (data not shown). These results clearly suggest the critical requirement of the co-expressed proteins in any two combinations of ChSy-1, ChSy-2 (CSS3), CSGlca-T (ChSy-3), and ChPF for chondroitin polymerization.

To further determine substrate specificities of the co-expressed proteins in various combinations, chondroitin polymerizing activities were tested using two authentic synthetic substrates, GlcUAβ1–3Galβ1–O-C₃H₇NH-Cbz and GlcUAβ1–3Galβ1–O-NM, both of which share the disaccharide sequence with the glycosaminoglycan–protein linkage region tetrasaccharide. Each reaction product obtained with GlcUAβ1–3Galβ1–O-C₃H₇NH-Cbz or GlcUAβ1–3Galβ1–O-NM was analyzed by gel filtration chromatography using a Superdex 75 column, as shown in Fig. 2, A and B, respectively. When co-expression of ChPF with ChSy-1 was used as an enzyme source, polymerization activity was detected using GlcUAβ1–3Galβ1–O-C₃H₇NH-Cbz as an acceptor substrate (Fig. 2A). On the other hand, cotransfection of ChSy-1 and CSGlca-T (ChSy-3), ChSy-1 and ChPF, or CSGlca-T (ChSy-3) and ChPF showed polymerization activities using GlcUAβ1–3Galβ1–O-NM as an acceptor substrate (Fig. 2B). Interestingly, co-expression of ChPF with CSGlca-T (ChSy-3) formed the longest chondroitin chains when GlcUAβ1–3Galβ1–O-NM was used as an acceptor (Fig. 2B). These results suggest that the various, co-expressed proteins that share polymerization activity might be critical for the differential assembly of CS chains on different core proteins by discriminating the amino acid sequences. In addition, to examine whether the formation of enzyme complexes might be affected by the pH, chondroitin polymerizing activities were also tested at different pH from the above one (pH 6.5). Interestingly, at pH 5.8, in sharp contrast to the results obtained at pH 6.5 (Fig. 2B), co-expression of ChPF with ChSy-1 formed longer chondroitin chains on GlcUAβ1–3Galβ1–O-NM as an acceptor compared with the chain length synthesized on the same acceptor using the co-expression of CSGlca-T (ChSy-3) with ChSy-1 (Fig. 2C).

**Interactions among ChSy-1, ChSy-2 (CSS3), CSGlca-T (ChSy-3), and ChPF—**Recently, interactions between any two of ChSy-1, ChSy-2 (CSS3), and ChPF have been demonstrated by pull-down assays (16). As shown above, co-expression of CSGlca-T (ChSy-3) with ChSy-1, ChSy-2 (CSS3), or ChPF resulted in a marked augmentation of not only glycosyltransferase activities but also polymerase activity. In view of these results, interactions between CSGlca-T (ChSy-3) and other molecules (ChSy-1, ChSy-2 (CSS3), or ChPF) were also expected. Thus, the interactions of these molecules were evaluated by conducting pull-down assays. For this analysis, soluble forms of ChSy-1, CSGlca-T (ChSy-3), and ChPF fused with protein A at their N termini (ChSy-1-ProA, ChSy-3-ProA, and ChPF-ProA), and soluble forms of ChSy-2 (CSS3) and CSGlca-T (ChSy-3) tagged with the His₆ epitope at their N termini (ChSy-2-His and ChSy-3-His) were generated, as described under “Experimental Procedures.” To evaluate the interactions among ChSy-1, ChSy-2 (CSS3), CSGlca-T (ChSy-3), and ChPF.
ChPF, co-expression of ChSy-1-ProA and ChSy-3-His, co-expressing ChSy-3-ProA and ChSy-2-His, or co-expressing ChSy-3-ProA and ChPF-ProA and ChSy-3-His was carried out. First, to confirm that the co-expression of any two proteins in different combinations yields active enzymes, the culture medium from each transfection experiment was purified with IgG-Sepharose and evaluated for enzyme activities. Glycosyltransferase activities were detected in the medium from all transfectants (data not shown). Next, to evaluate the association among these proteins, pull-down assays were performed. In addition, to ensure specificity, we conducted these assays with human EXT-1 (a heparan sulfate co-polymerase), which is expected to interact with human EXT-2 (a heparan sulfate co-polymerase) (30, 31) but not with ChSy-1, ChSy-2 (CSS3), CSGlcA-T (ChSy-3), or ChPF. Ni$^{2+}$-NTA-agarose was added to the culture medium to pull down His-tagged proteins. Then the proteins were subjected to SDS-PAGE followed by Western blotting using an IgG antibody as a primary antibody and detected with an ECL advance kit. No band was detected for the transfectant of either ChSy-3-ProA alone or for the cotransfectant of ChSy-3-ProA and EXT-1-His (Fig. 3, lane 4). In contrast, proteins with a molecular mass of 120 kDa, corresponding to ChSy-1-ProA, were detected for the cotransfectant of ChSy-1-ProA and ChSy-3-His (Fig. 3, lane 1). In addition, co-expressed ChSy-3-ProA and ChSy-2-His (Fig. 3, lane 2), ChPF-ProA and ChSy-3-His (Fig. 3, lane 3), or, as expected, EXT-2-ProA and EXT-1-His showed similar results (Fig. 3, lane 5). These results, in conjunction with our previous findings (16), indicated that ChSy-1, ChSy-2 (CSS3), CSGlcA-T (ChSy-3), and ChPF interact with each other.

**Golgi Localization of CSGlcA-T (ChSy-3), ChSy-1, ChSy-2 (CSS3), and ChPF**—Since in vitro interactions of CSGlcA-T (ChSy-3) with other ChSy family members were shown above, co-localization of CSGlcA-T (ChSy-3) with other ChSy family members in the cells was also expected. Thus, the intracellular localization of CSGlcA-T (ChSy-3), ChSy-1, ChSy-2 (CSS3), or ChPF was examined in HeLa cells. For this analysis, full-length and 8 indicate the eluted position of 5-kDa saccharides, tetradecasaccharides, and octasaccharides derived from chondroitin, respectively. The total volume was at fraction 60 (not shown).
forms of ChSy-1, ChSy-2 (CSS3), and CSGlcA-T (ChSy-3) fused with EGFP at their C termini (ChSy-1-EGFP, ChSy-2-EGFP, and ChPF-EGFP), and full-length forms of CSGlcA-T (ChSy-3) and ChPF tagged with DsRed at their C termini (ChSy-3-DsRed and ChPF-DsRed) were generated, as described under “Experimental Procedures.” To first confirm whether the fluorescence-tagged ChSy family enzymes were active as glycosyltransferases, each GFP-tagged ChSy family enzyme was transiently transfected into HeLa cells, and then the amount of CS in the HeLa cells was determined by HPLC. All of the cells transfected with GFP-tagged ChSy family enzymes increased the amount of CS compared with mock-transfected cells (supplemental Table 1). These results suggest that these fluorescence-tagged ChSy family enzymes were active in the cells. Next, to determine the intracellular localization of CSGlcA-T (ChSy-3), ChSy-3-DsRed or ChSy-3-EGFP was co-expressed with a Golgi marker (Golgi-EGFP) or an ER marker (ER-DsRed) in the HeLa cells and analyzed by confocal microscopy. ChSy-3-DsRed (Fig. 4B) was co-localized with the Golgi-EGFP marker (Fig. 4C), whereas ChSy-3-EGFP (Fig. 4D) was not completely co-localized with the ER-DsRed marker (Fig. 4F). In addition, co-expressed ChSy-1-EGFP (Fig. 4G) and ChSy-3-3DsRed (Fig. 4H), ChSy-2-EGFP (Fig. 4) and ChSy-3-DsRed (Fig. 4K), or ChSy-3-EGFP (Fig. 4M) and ChPF-DsRed (Fig. 4N) were colocalized in the Golgi apparatus (Fig. 4, I, L, and O), suggesting that CSGlcA-T (ChSy-3) acts as a chondroitin polymerase through its interaction with other ChSy family members in the Golgi apparatus.

Expression Pattern of CSGlcA-T (ChSy-3)—Northern blot analysis of CSGlcA-T (ChSy-3) mRNA showed a single band of ~4.4 kb for all human tissues examined (Fig. 5). Notably, the expression level of CSGlcA-T (ChSy-3) mRNA was largely constant among the tissues examined, in sharp contrast to the dramatic differential expression of ChSy-1, ChSy-2, and ChPF (Fig. 5) (8, 14, 16).

Involvement of CSGlcA-T (ChSy-3) in CS Biosynthesis—Next, we determined the physiological relevance of CSGlcA-T (ChSy-3) in HeLa cells that express ChSy-1, ChSy-2 (CSS3), CSGlcA-T (ChSy-3), and ChPF endogenously. When the expression levels of ChSy-1, ChSy-2 (CSS3), CSGlcA-T (ChSy-3), and ChPF mRNAs were normalized to those of the glyceraldehyde-3-phosphate dehydrogenase transcript, the values of ChSy-1, ChSy-2 (CSS3), CSGlcA-T (ChSy-3), and ChPF/glyceraldehyde-3-phosphate dehydrogenase (× 10^-3 copies) were 13.3, 0.8, 6.0, and 3.7, respectively. To examine the physiological relevance of CSGlcA-T (ChSy-3) in HeLa cells, we first investigated whether overexpression of CSGlcA-T (ChSy-3) increased the amount of CS. A pCMV-Script expression vector, which possesses the cytomegalovirus promoter and the neomycin resistance gene and harbors the open reading frame of human CSGlcA-T (ChSy-3), was transfected into HeLa cells, followed by positive selection in the presence of a neomycin analog, G418. Each of the resultant colonies was picked up and propagated for experiments, and the expression of CSGlcA-T (ChSy-3) was measured by quantitative real time reverse transcription-PCR. As shown in Table 2, the disaccharide composition and the amount of CS isolated from the three stable clones with the different expression levels of introduced CSGlcA-T (ChSy-3), designated ChSy-3-1, ChSy-3-2, and ChSy-3-3 cells, were analyzed by HPLC, as described under “Experimental Procedures.” The results showed that although the disaccharide compositions in ChSy-3-1, ChSy-3-2, and ChSy-3-3 cells were similar to that in the control HeLa cells, the amount of CS was increased in the three stable clones, corresponding to the expression level of CSGlcA-T (ChSy-3) (Table 2). These results indicated that overexpression of CSGlcA-T (ChSy-3) increased the amount of CS in HeLa cells.

Prompted by these observations, we next examined whether knockdown of CSGlcA-T (ChSy-3) expression by RNA interference decreases the amount of CS, as described under “Experimental Procedures.” The efficiency of gene silencing was deter-
mined by quantitative real time reverse transcription-PCR. As shown in Table 2, transfection of the CSGlcA-T (ChSy-3) siRNA (ChSy-3 siRNA cells) resulted in a 50% knockdown of the CSGlcA-T (ChSy-3) mRNA and a 46% reduction of the amount of CS when compared with that of the control siRNA. Overall, these findings suggest that CSGlcA-T (ChSy-3) plays an important role in the biosynthesis of CS through its interaction with ChSy-1, ChSy-2 (CSS3), or ChPF.

**Contribution of Glycosyltransferase Activities of CSGlcA-T (ChSy-3) in Chondroitin Polymerization**—When any two of ChSy-1, ChSy-2 (CSS3), and CSGlcA-T (ChSy-3) are coexpressed, the enzyme complex contains two sets of glycosyltransferase domains. Then, to clarify whether two glycosyltransferase domains of the complex equally contribute to polymerase activity or one totally contributes and the other serves as a chaperone-like molecule, such as ChPF, we tried to construct the CSGlcA-T (ChSy-3) mutant, which is expected to lack any glycosyltransferase activity. Based on the sequence alignment of ChSy family members, CSGlcA-T (ChSy-3) has only one putative DXD motif, QDD (Gln<sup>182</sup>–Asp<sup>184</sup>), which is most likely responsible for UDP-sugar binding in many glycosyltransferases (32). It was therefore expected that the CSGlcA-T (ChSy-3) D184A mutant would not possess any glycosyltransferase activity. To confirm the expression and activity of the mutant protein, the soluble mutant was expressed in COS-1 cells, and the culture medium was purified with IgG-Sepharose. The purified mutant proteins were used for Western blotting analysis and evaluated for glycosyltransferase activities using chondroitin as an acceptor. As expected, neither GalNacT-II nor GlcAT-II activity was detected (Table 1), although the mutant proteins were expressed (Fig. 6, lane 1).

Nevertheless, co-expression of the soluble mutant with other wild-type ChSy family members resulted in an augmentation of glycosyltransferase activities, as in the case of that of wild-type CSGlcA-T (ChSy-3) (Table 1). However, when CSGlcA-T (ChSy-3) D184A was coexpressed with ChSy-1, the complex showed a 20% decrease in GalNacT-II and a 47% decrease in GlcAT-II activities when compared with that of wild-type CSGlcA-T (ChSy-3) with ChSy-1 (Table 1). In addition, although the mutant was co-expressed with ChSy-2 (CSS3) or ChPF, each complex showed no GlcAT-II activity (Table 1).

Since co-expression of CSGlcA-T (ChSy-3) mutant with ChSy-1, ChSy-2 (CSS3), or ChPF resulted in an augmentation of glycosyltransferase activities, interactions between the mutant and ChSy-1, ChSy-2 (CSS3), or ChPF were determined by pull-down assays, as described under "Experimental Procedures." To evaluate the interactions between CSGlcA-T (ChSy-3) D184A and ChSy-1, CSGlcA-T (ChSy-3) D184A and ChSy-2 (CSS3), or CSGlcA-T (ChSy-3) D184A and ChPF, co-expression of ChSy-3 D184A-ProA and ChSy-1-His, ChSy-3 D184A-ProA and ChSy-2-His, or ChSy-3 D184A-ProA and ChPF-His was carried out. Ni<sup>2+</sup>-NTA-agarose was added to the culture medium to pull down His-tagged proteins. Then the proteins were subjected to SDS-PAGE followed by Western blotting using a primary antibody to protein A-tagged proteins and detected with an ECL advance kit. As expected, proteins with a molecular mass of ~120 kDa, corresponding to ChSy-3-ProA, were detected for the cotransfected of ChSy-1-His and ChSy-3-ProA (Fig. 6, lane 2). In addition, co-expressed ChSy-3 D184A-ProA and ChSy-1-His (Fig. 6, lane 3), ChSy-3 D184A-ProA and ChSy-2-His (Fig. 6, lane 4), or ChSy-3 D184A-ProA and ChPF-His (Fig. 6, lane 5) showed similar results. In con-

#### TABLE 2

| Disaccharides | Mock* | ChSy-3-1* | ChSy-3-2* | ChSy-3-3* | Control siRNA* | ChSy-3 siRNA* |
|---------------|-------|-----------|-----------|-----------|---------------|---------------|
|               | pmol/mg (mol %) | pmol/mg (mol %) | pmol/mg (mol %) | pmol/mg (mol %) | pmol/mg (mol %) | pmol/mg (mol %) |
| ΔDi-0S        | 4.1 ± 0.1 (8) | 4.1 ± 0.5 (7) | 5.7 ± 0.9 (8) | 4.6 ± 1.8 (8) | 4.0 (8) | 8.4 (10) |
| ΔDi-6S        | 10.7 ± 0.5 (21) | 8.9 ± 1.0 (16) | 6.2 ± 0.1 (9) | 7.1 ± 1.8 (9) | 11.3 (22) | 3.2 (14) |
| ΔDi-4S        | 33.8 ± 0.3 (66) | 37.9 ± 0.6 (68) | 55.1 ± 0.8 (76) | 44.5 ± 0.2 (76) | 33.7 (65) | 16.7 (71) |
| ΔDi-diS<sub>6</sub> | 2.4 ± 0.2 (5) | 3.3 ± 0.1 (6) | 2.9 ± 0.1 (4) | 2.0 ± 0.3 (4) | 2.2 (4) | 0.6 (5) |
| ΔDi-diS<sub>4</sub> | 0.4 ± 0.1 (1) | 1.4 ± 0.5 (3) | 2.9 ± 0.3 (4) | 2.8 ± 0.3 (4) | 0.4 (1) | 0.7 (3) |
| Total (pmol/mg) | 51.4 ± 0.7 | 55.6 ± 3.0 | 72.8 ± 2.2 | 61.0 ± 1.7 | 51.6 | 23.6 |
| Relative expression | 1.0 | 1.5 | 1.5 | 1.5 | 1.0 | 0.5 |

* Values are the means ± S.E. of three determinations.
* Values are the means from two independent experiments.
* Relative amounts of the CSGlcA-T (ChSy-3) transcript were quantified by quantitative real time RT-PCR. Normalization of the data was performed using the glyceraldehyde-3-phosphate dehydrogenase mRNA levels.
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FIGURE 6. Western blot analysis of CSGlcA-T (ChSy-3) D184A and the pull-down assays of co-expressed CSGlcA-T (ChSy-3) D184A and ChSy-1, ChSy-2 (CSS3), or ChPF. A soluble form of CSGlcA-T (ChSy-3) D184A was expressed as a fusion protein tagged with protein A in COS-1 cells. The recombinant proteins secreted in the medium were purified with IgG-Sepharose (lane 1). Culture medium from cells co-expressing ChSy-3-ProA and ChSy-1-His, ChSy-3 D184A-ProA and ChPF-His, ChSy-3 D184A-ProA and EXT-1-His, or ChSy-3 D184A-ProA and EXT-1-His was purified with Ni²⁺-NTA-agarose (lanes 2–6) and subjected to SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane by Western blotting, allowed to react with IgG as a primary antibody, and visualized using the ECL advance kit. Lane 1, ChSy-3 D184A-ProA; lane 2, ChSy-3-ProA and ChSy-1-His; lane 3, ChSy-3 D184A-ProA and ChSy-1-His; lane 4, ChSy-3 D184A-ProA and ChSy-2-His; lane 5, ChSy-3 D184A-ProA and ChPF-His; lane 6, ChSy-3 D184A-ProA and EXT-1-His.

contrast, no band was detected for the co-transfected of ChSy-3 D184A-ProA and EXT-1-His (Fig. 6, lane 6). These results indicate that the mutant possesses no glycosyltransferase activity but still forms the complexes with other ChSy family members.

We next measured the polymerizing activity upon the co-expression of soluble CSGlcA-T (ChSy-3) D184A with soluble ChSy-1, ChSy-2 (CSS3), or ChPF. The co-expressed proteins were incubated with α-TM in the presence of UDP-[3H]GalNAc and UDP-GlcUA. The reaction products were subjected to reductive β-elimination using NaBH₄/NaOH, and the released radiolabeled oligosaccharide chains were analyzed by gel chromatography using a column of Superdex 200 (Fig. 7). Although the lengths of chondroitin chains formed by the co-expressed ChSy-1 and CSGlcA-T (ChSy-3) D184A were comparable with those formed by the co-expressed chondroitin sulfate A).

DISCUSSION

In this study, we have demonstrated that CSGlcA-T (ChSy-3) exhibits chondroitin polymerization activity on α-TM bearing the truncated linkage region tetrasaccharide through its interaction with ChSy-1, ChSy-2 (CSS3), or ChPF. Thus, CSGlcA-T (ChSy-3) is the fourth ChSy family member to have been found to be involved in chondroitin polymerization. Previously, Gotoh et al. (13) reported that CSGlcA-T showed only weak GlcAT-II activity but not GalNAC-T-II activity. Hence, the involvement of CSGlcA-T in chondroitin polymerization remained unclear. However, the present study revealed that co-expression of CSGlcA-T (ChSy-3) with ChSy-1, ChSy-2 (CSS3), or ChPF showed polymerizing activities for disaccharide-repeating units of CS onto α-TM, as in the case of co-expression of ChSy-1 and ChPF, ChSy-1 and ChSy-2, or ChSy-2 and ChPF. In addition, interactions of CSGlcA-T (ChSy-3) with ChSy-1, ChSy-2 (CSS3), or ChPF were demonstrated by pull-down assays. Since we previously showed interactions between any two of ChSy-1, ChSy-2 (CSS3), and ChPF (16), these results indicate that ChSy-1, ChSy-2 (CSS3), CSGlcA-T (ChSy-3), and ChPF interact with each other. Moreover, overexpression of CSGlcA-T (ChSy-3) increased the amount of CS in HeLa cells, whereas the RNA interference of CSGlcA-T (ChSy-3) resulted in a reduction in the amount of CS in the cell, as in the case of ChSy-2 (CSS3) (16). These results, in conjunction with our previous findings, indicate that chondroitin polymerization is achieved by multiple enzyme complexes consisting of four ChSy family members.

One explanation for the existence of multiple enzyme complexes consisting of four ChSy family members that share chondroitin polymerization activity is that they initiate and polymerize chondroitin chains on different core proteins by discriminating the amino acid sequences. In fact, using GlcUAβ1–3Galβ1–O-C₄H₄NH-Cbz as an acceptor substrate, chondroitin polymerization activity was only detected by co-expression of ChPF with ChSy-1 (Fig. 3A), whereas chondroitin
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polymerization was demonstrated using α-TM as an acceptor substrate by all of the co-expressed proteins in various combinations (Fig. 1). In addition, using GlcUAβ1–3Galβ1-O-NM as an acceptor substrate, polymerization activity was detected by the co-transfection of ChSy-1 and CSGlcA-T (ChSy-3), ChSy-1 and ChPF, or CSGlcA-T (ChSy-3) and ChPF (Fig. 3B). In this regard, it should be noted that animal cells utilize β-D-xylolides as primers for glycosaminoglycan synthesis, and thus, the core protein can be substituted by the mildly hydrophobic xylosides, such as 4-methylumbelliferyl- or p-nitrophenyl-β-D-xylolides (33). In addition, Fernández and Warren (34) demonstrated CS synthesis using reconstituted Golgi in the presence of 4-methylumbelliferyl-β-D-xylolides. Thus, it appears that hydrophobic aglycones mimic a determinant on core proteins and differentially interact with multiple enzyme complexes consisting of the four ChSy family members. Another explanation is that the chain length of CS is dependent on which enzyme complex is formed in a given tissue expressing the four ChSy family members. Indeed, the chondroitin polymerization reactions were also influenced by the core protein portion. As shown in Fig. 1, the lengths of chondroitin chains formed by co-expressed CSGlcA-T (ChSy-3) and ChPF were comparable with those of commercial chondroitin chains, whereas chondroitin chains polymerized by the co-expressed CSGlcA-T (ChSy-3) and ChSy-1 were longer than those polymerized by the co-expressed CSGlcA-T (ChSy-3) and ChPF and shorter than those formed by the co-expressed ChSy-1 and ChPF. In contrast, co-expressed CSGlcA-T (ChSy-3) and ChSy-2 (CSS3) formed shorter chains compared with commercial chondroitin chains. In addition, when GlcUAβ1–3Galβ1-O-NM was used as an acceptor, co-expression of ChPF with CSGlcA-T (ChSy-3) formed the longest chondroitin chains (Fig. 3B). Together, these results suggest that the enzyme complexes interact with the core proteins, and that the affinity toward the core proteins was different among the enzyme complexes.

Although CSGlcA-T (ChSy-3) exhibited a widespread expression, its expression pattern was different from that of ChSy-1, ChSy-2 (CSS3), or ChPF (Fig. 5). The difference in the expression pattern among these proteins suggests that a deficiency of any one of them may be compensated for by the other proteins to some extent. This redundancy makes it difficult to investigate the mechanism of chondroitin biosynthesis by gene knockout or the characterization of individual glycosyltransferases. It was also revealed in this study that CSGlcA-T (ChSy-3) acts as a chondroitin polymerase together with ChSy-1, ChSy-2 (CSS3), and ChPF. The biosynthesis of CS is carried out by ER- and Golgi-residing, membrane-bound glycosyltransferases. In general, the pH decreases down the secretory pathway, and the pH of the trans-Golgi network was found to be about 6.0, ~0.5 pH units more acidic than those of the medial Golgi and trans-Golgi. Therefore, the formation of enzyme complexes might be affected by the pH. In fact, co-expression of CSGlcA-T (ChSy-3) with ChSy-1 formed longer chondroitin chains on GlcUAβ1–3Galβ1-O-NM as an acceptor compared with the chain length synthesized on the same acceptor using the co-expression of ChPF with ChSy-1 at pH 6.5. However, at pH 5.8, co-expression of ChPF with ChSy-1 formed longer chondroitin chains on GlcUAβ1–3Galβ1-O-NM as an acceptor compared with the chain length synthesized on the acceptor using the co-expression of CSGlcA-T (ChSy-3) with ChSy-1 (Fig. 2C). It is also likely that the detail of localization of the Golgi compartment is different among the enzyme complexes. These results suggest that the localization of enzyme complexes might also be one of the important factors regulating chondroitin polymerization.

In the present study, we demonstrated that co-expressed ChSy-1 and ChSy-2 (CSS3), ChSy-1 and CSGlcA-T (ChSy-3), or ChSy-2 (CSS3) and CSGlcA-T (ChSy-3) resulted in a marked augmentation of not only glycosyltransferase activities but also polymerase activity. Since all of ChSy-1, ChSy-2 (CSS3), and CSGlcA-T (ChSy-3) have glycosyltransferase activities, these complexes seem to contain two glycosyltransferase domains. Hence, it had been unclear whether the two glycosyltransferase domains of these complexes equally contribute to polymerase activity or one totally contributes and the other enzyme serves as a chaperone-like molecule. The present analysis using the CSGlcA-T (ChSy-3) mutant that lacks any glycosyltransferase activity but interacts with other ChSy family members showed that the two glycosyltransferase domains of the ChSy-1 and CSGlcA-T (ChSy-3) complex equally contribute to the polymerase activity and that, of the ChSy-2 (CSS3) and CSGlcA-T (ChSy-3) complex, CSGlcA-T (ChSy-3) totally contributes to the polymerase activity, whereas ChSy-2 (CSS3) serves as a chaperone-like molecule (Fig. 7). These findings suggest that the glycosyltransferase activity of CSGlcA-T (ChSy-3) plays an important role in chondroitin polymerization and that ChSy-2 (CSS3) as well as ChPF might mainly serve as chaperone-like molecules for chondroitin polymerization when co-expressed with CSGlcA-T (ChSy-3).

Although this report has focused on the four ChSy family members, it is expected that the regulated expression of sulfotransferases responsible for CS biosynthesis is important in establishing the cell type-specific and developmentally regulated expression of CS structures. Indeed, we recently demonstrated that sog9 cells were deficient in the expression of chondroitin 4-O-sulfotransferase-1 (C4ST-1) and synthesized CS chains with a reduced length and low 4-O-sulfation (35). These results suggested that 4-O-sulfation of CS chains by C4ST-1 facilitates the elongation of CS chains by chondroitin polymerase in vivo. Hence, chondroitin polymerase consisting of any two of ChSy-1, ChSy-2 (CSS3), CSGlcA-T (ChSy-3), and ChPF may interact with C4ST-1 to regulate the length of CS chains (8, 14). Thus, the biosynthesis of CS in mammals may be regulated by not only chondroitin synthase family members but also CS sulfotransferases. As molecular tools become available, it will be of considerable interest to define the degree to which the coordinated expression of groups of CS glycosyltransferases and sulfotransferases is required to affect the elaboration of CS structures important in a number of physiological phenomena.

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REFERENCES

1. Kjellén, L., and Lindahl, U. (1991) Annu. Rev. Biochem. 60, 443–475
2. Esko, J. D., and Selleck, S. B. (2002) Annu. Rev. Biochem. 71, 435–471
3. Prydz, K., and Dalen, K. T. (2000) J. Cell Sci. 113, 193–205
4. Sugahara, K., and Kitagawa, H. (2000) Curr. Opin. Struct. Biol. 10, 518–527
5. Perrimon, N., and Bernfield, M. (2000) Nature 404, 725–728
6. Sugahara, K., Mikami, T., Uyama, T., Mizuguchi, S., Nomura, K., and Kitagawa, H. (2000) Curr. Opin. Struct. Biol. 13, 605–611
7. Kitagawa, H., Uyama, T., and Sugahara, K. (2001) J. Biol. Chem. 276, 38721–38726
8. Uyama, T., Kitagawa, H., Tanaka, J., Tamura, J., Ogawa, T., and Sugahara, K. (2003) J. Biol. Chem. 278, 3072–3078
9. Gotoh, M., Sato, T., Akashima, T., Iwasaki, H., Kameyama, A., Mochizuki, H., Yada, T., Inaba, N., Zhang, Y., Kikuchi, N., Kwon, Y.-D., Togayachi, A., Kudo, T., Nishihara, S., Watanabe, H., Kimata, K., and Narimatsu, H. (2002) J. Biol. Chem. 277, 38179–38188
10. Gotoh, M., Sato, T., Akashima, T., Iwasaki, H., Kameyama, A., Mochizuki, H., Yada, T., Inaba, N., Kikuchi, N., Kwon, Y.-D., Togayachi, A., Kudo, T., Asada, M., Watanabe, H., Kimata, K., and Narimatsu, H. (2000) Glycoconj. J. 11, 381–394
11. Sugahara, K., Ohkita, Y., Shibata, Y., Yoshida, K., and Ikegami, A. (1995) J. Biol. Chem. 270, 7204–7212
12. Sugahara, K., Shigeno, K., Masuda, M., Fujii, N., Kurokawa, A., and Takeda, K. (1994) J. Biol. Chem. 273, 19030–19039
13. Uyama, T., Isida, M., Izumikawa, T., Trybara, E., Tufaro, F., Bergström, T., Sugahara, K., and Kitagawa, H. (2006) J. Biol. Chem. 281, 38668–38674