Molecular Cloning and Characterization of Chondroitin Polymerase from *Escherichia coli* Strain K4*

Received for publication, February 20, 2002, and in revised form, April 9, 2002
Published, JBC Papers in Press, April 9, 2002, DOI 10.1074/jbc.M201719200

Toshio Ninomiya‡, Nobuo Sugiiura‡, Akira Tawada‡, Kazunori Sugimoto‡, and Koji Kimata‡

From the Institute for Molecular Science of Medicine, Aichi Medical University, Yazucho, Nagakute, Aichi 480-1195, Japan

Central Research Laboratories, Seikagaku Corporation, Tateno, Higashiyamato-shi, Tokyo 207-0021, and Department of Chemistry, Graduate School of Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan

*Escherichia coli* strain K4 produces the K4 antigen, a capsule polysaccharide consisting of a chondroitin backbone (GlcUA \( \beta(1 \rightarrow 3) \)-GalNAc \( \beta(1 \rightarrow 4) \)) to which \( \beta \)-fructose is linked at position C-3 of the GlcUA residue. We molecularly cloned region 2 of the K4 capsular gene cluster essential for biosynthesis of the polysaccharide, and we further identified a gene encoding a bifunctional glycosyltransferase that polymerizes the chondroitin backbone. The enzyme, containing two conserved glycosyltransferase sites, showed 59 and 61% identity at the amino acid level to class 2 hyaluronan synthase and chondroitin synthase from *Pasteurella multocida*, respectively. The soluble enzyme expressed in a bacterial expression system transferred GalNAc and GlcUA residues alternately, and polymerized the chondroitin chain up to a molecular mass of 20 kDa when chondroitin sulfate hexasaccharide was used as an acceptor. The enzyme exhibited apparent \( K_m \) values for UDP-GlcUA and UDP-GalNAc of 3.44 and 31.6 \( \mu \)M, respectively, and absolutely required acceptors of chondroitin sulfate polymers and oligosaccharides at least longer than a tetrasaccharide. In addition, chondroitin polymers and oligosaccharides and hyaluronan polymers and oligosaccharides served as acceptors for chondroitin polymerization, but dermatan sulfate and heparin did not. These results may lead to elucidation of the mechanism for chondroitin chain synthesis in both microorganisms and mammals.

Chondroitin sulfate (CS)\(^1\) is a glycosaminoglycan having a repeated disaccharide unit consisting of \( \alpha \)-glucuronic acid (GlcUA) and N-acetyl-D-galactosamine (GalNAc), with sulfate residues at various positions. Proteoglycans having CS chains are abundant in cartilage (1), aorta, skeletal muscle, eye (2), lung (3), and brain (4, 5). They are synthesized intracellularly and secreted to form a macromolecular complex in the extracellular matrix or localized on the cell surface.

Biogenesis of the CS chain of CS-proteoglycans occurs in the Golgi apparatus (6). First, the linkage tetrasaccharide, GlcUA-Gal-Gal-Xyl, is synthesized onto a serine residue of the core protein by sequential addition. Then a chondroitin chain is synthesized onto the linkage by the alternating addition of monosaccharide units of GalNAc and GlcUA. During polymerization, the chain undergoes sulfation at various positions with a variety of sulfotransferases (7). Many animal species produce CS chains, including *Drosophila* (8), squid (9, 10), king crab (11), sea cucumber (12, 13), and *Caenorhabditis elegans* (8). Although bacteria do not produce CS chains, some Gram-negative bacteria such as *Escherichia coli* K4 contain polysaccharides similar to CS chains in their capsule. The K4 antigen capsule contains a polysaccharide of chondroitin (GlcUA \( \beta(1 \rightarrow 3) \)-GalNAc \( \beta(1 \rightarrow 4) \)) to which fructose is \( \beta \)-linked at position C-3 of the GlcUA residue (14). The addition of fructose branches occurs after the chondroitin elongation (15). However, the glycosyltransferases needed to achieve chain polymerization and modification have not been identified.

Studies on gene clusters of group 2 K antigens including K1, K5, K7, K12, and K92 have revealed a genomic organization of three functional regions conserved in a number of *E. coli* strains (16–18). Regions 1 and 3 at both ends are common among the group 2 K antigen gene clusters and express various proteins such as capsule-specific CMP-2-keto-3-deoxymanno-octonic acid synthetase, a stabilizer of the polysaccharide biosynthetic complex, and the ATP-binding cassette transporters. In contrast, the central region termed region 2, containing genes that encode enzymes for the synthesis of the specific K antigen, determines the polysaccharide structure. The nucleotide sequence of region 2 of the *E. coli* K5 capsule gene cluster has recently been determined (GenBank\(^TM\) accession number X77617) (19). This region contains four genes termed *kfiA*, *-B*, *-C*, and *-D*. The polypeptides of *KfiA* and *KfiC* show activity of both GlcNAc and GlcUA transferases (20, 21), whereas *KfbB* is a protein associated with *Kfc* and *KfiA* on the cytoplasmic membrane, and *KfdD* is a UDP-glucose dehydrogenase. These proteins, likely forming a complex, may synthesize the K5 polysaccharide (N-acetylheparosan)\((\text{GlcUA } \beta(1 \rightarrow 4)\text{-GlcNAc } \alpha(1 \rightarrow 4) \))\(_n\).

Because the K4 antigen is a member of the group 2 K antigen family (22) and both the K4 and K5 capsules contain glycosaminoglycan backbones, the K4 strain may possess a similar gene cluster to K5. Actually, regions 1 and 3 of the K4 gene show high homology to those of K5. Thus, we hypothesized that region 2 of the K4 gene cluster contains enzymes responsible for chondroitin polymerization. In this study, we found that the K4 antigen gene cluster exhibits a genomic structure similar to
K5. Then we cloned region 2 of the K4 capsular gene cluster and identified a bifunctional glycosyltransferase (chondroitin polymerase) gene within the region. The enzyme was further characterized as a soluble recombinant protein expressed in a bacterial expression system.

**EXPERIMENTAL PROCEDURES**

**Materials—** E. coli strain K4, serotype O5:K4:L:H4 (ATCC 23502) (14), and strain K5, serotype O10:K5:L:H4 (ATCC 23506) (23), were obtained from American Type Culture Collection. UDP-[14C]GlcNAc (0.125 Ci/mmol), UDP-33H[GlcNAc (1.0 Ci/mmol), and UDP-[14C]Glc-NAc (0.125 Ci/mmol) were purchased from PerkinElmer (C002). UDP-Glc-NAc, UDP-GlcNAc, and UDP-GlcNAc were from Sigma. CS from shark cartilage (M, 20,000), chemically desulfated chondroitin (M, 10,000) from CS (24), dermatan sulfate from pig skin (M, 15,000), heparin from porcine intestine (M, 10,000), and hyaluronan from chick comb (M, 800,000) were obtained from Seikagaku Corp. Oligosaccharides of CS (tetra- and hexaxaccharides), chondroitin (tetra- and hexaxaccharides), and hyaluronan (tetra-, hexa-, octa-, deca-, and tetradeoxasaccharides) and short chains of hyaluronan (M, 5,000, 10,000, and 20,000) were prepared by partial digestion of CS, chondroitin, or hyaluronan with heparidase from sheep testis (Sigma) and separation by gel filtration and ion exchange chromatography (25). CS heparidase was chemically prepared by adding GalNAc to the hexaxaccharide using K4 chondroitin polymerase (K4CP) obtained in pI-strain. Briefly, 350 nmol of CS hexaxaccharide and 1.5 μmol of UDP-GlcNAc were incubated in the reaction buffer (50 μl) containing purified recombinant K4 chondroitin polymerase (16 μg of protein) at 30 °C for 1 h. The heparidase product was separated with a HiLoad 16/60 Superdex 30-100 column (Amersham Biosciences) column.

**Cloning of Region 2 of K4 Gene Cluster—** Genomic DNA of E. coli cells was prepared by the method described (26) with Stu3AI and the library was constructed from a EMBL3 (Stratagene) and the digests, using Gigapack III Gold Packaging kit (Stratagene) according to the manufacturer’s instructions. Probes were used for hybridization with the probes from those of defined from those of K5 (GenBank™ accession numbers X74567 and X53819) and K4 genomic DNA as template. The sets of primers were CS-S (5′-CGAGAAATACGAACGCTTT-3′) and CS-N (5′-GCGTCTTCACCAATAAAT-TACAACCTATATCGG-3′) and TM-S (5′-CGAGAAATACGAACGCTTT-GGTA-3′) and TM-A (5′-ACTCAATTTCTTCTTACGCTTCTTGTG-3′) the PCR profile was 94 °C for 1 min, 30 cycles of 94 °C for 45 s, 47 °C for 30 s, 72 °C for 5 min, and 72 °C for 10 min for region 1, and 94 °C for 30 s, 50 °C for 45 s, 50 °C for 30 s, 72 °C for 5 min, and 72 °C for 10 min for region 2. The PCR products (K4RI, 1.3 kbp in region 1, and K4RII), 1.0 kbp in region 3, see Fig. 1) were confirmed by direct DNA sequencing using an ABI PRISM 310 Genetic analyzer (PerkinElmer Life Sciences).

The plasmid library of E. coli K4 genomic DNA was screened with the 33p-labeled PCR products (K4RI and K4RII). The plaque-transferred filters were washed three times with 40 °C for 30 min in 0.5 M Church phosphate buffer, pH 7.2, 1 mM EDTA, and 7% SDS, after prehybridization for 1 h at 65 °C. The filters were washed three times with 40 °C for 30 min with 0.1 M NaH2PO4 (pH 8.0) containing 300 mM NaCl and 10 mM imidazole, and 1 mg/ml lysozyme (Sigma) and placed on ice for 30 min. After the suspension was sonicated on ice three times at 10-s intervals and centrifuged at 10,000 × g for 30 min, the supernatant was applied to a nickel-nitrilotriacetic acid-agarose (Qiagen) column. The expression protein was purified according to the manufacturer’s instructions and dried against phosphate-buffered saline containing 20% glycerol for 2 days at 4 °C. Protein content was determined using a micro BCA protein assay kit (Pierce) with bovine serum albumin as standard protein.

**Enzymic Activity and Characterization of Chondroitin Polymerase—** Chondroitin polymerase activity was determined using UDP-[14C]GlcUA and UDP-[3H]GlcNAc as donor substrates and an acceptor substrate such as CS hexaxaccharide as reported previously (28) with a minor modification. Briefly, the reaction was carried out at 30 °C for 30 min in a 50-μl solution containing 50 mM Tris-HCl, pH 7.2, 20 mM MgCl2, 0.1 mM Na2SO4, 1 mM ethylene glycol, the donor and acceptor substrates at the indicated concentrations, and the enzyme preparation. This was followed by boiling for 1 min to stop the reaction. Then 3 volumes of 95% ethanol containing 1.3% potassium acetate was added, and the sample was centrifuged at 10,000 × g for 20 min. The precipitate was dissolved in 50 μl of distilled water, and the radioactivity was determined by a Superdex peptide HR10/30 column, a Superdex 75 HR1/30 column, or a Superdex 30 16/60 column (Amersham Biosciences), equilibrated with 0.2 mM NaCl, and eluted with 0.2 mM NaCl at a flow rate of 0.5–1.0 ml/min. The eluate was fractionated (0.5–1.0 ml/tube), and the radioactivity of each fraction was measured by liquid scintillation counter. The chondroitin polymerase activity was determined by calculating the amount of radioactivity incorporated into fractions with a higher molecular mass than the acceptor substrate. Some of the reaction products were treated with chondroitinase ABC, chondroitinase ACII, Streptomyces hyaluronidase, and heparitinase I (Seikagaku Co.) under the conditions recommended by the manufacturer to determine the substrate specificity of the recombinant enzyme, different combinations of UDP-GalNAc, UDP-GlcNAc, and UDP-GlcUA were used. Double-reciprocal plots of the incorporation activities (v) and the substrate concentrations of one UDP-sugar (S) while holding the other at a constant concentration gave apparent v values of recombinant chondroitin polymerase protein for UDP-GlcUA and UDP-GlcNAc. To determine the acceptor substrate specificity, oligosaccharides (CS tetra- and hexaxaccharides, chondroitin tetra- and hexaxaccharides, and hyaluronan hexaxaccharide), and heparin were used. Effects of pH, temperature, time, and metal ion on the enzyme reaction were also examined.

**SDS-PAGE and Western Blotting—** SDS-PAGE of proteins was carried out on a 10% gel by the method of Laemmli (29). Proteins were detected by Coomassie Brilliant Blue staining. For Western blotting, proteins on the SDS-PAGE gel were transferred onto a nitrocellulose membrane, and the membrane was treated with anti-tetra-His antibody (Qiagen), after blocking with 5% skim milk in 25 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20 (TBS-T). After several washes with TBS-T, the membrane was treated with a peroxidase-conjugated anti-mouse IgG. After washes with TBS-T, reactive proteins were detected with the ECL detection system (Amersham Biosciences).

**RESULTS**

**Molecular Cloning of K4 Region 2 Gene Cluster—** Preliminary Southern blot analysis revealed that E. coli K4 genomic DNA digests obtained with various restriction enzymes hybridized with probes of region 1 and region 3 of the K5 capsule gene (data not shown), suggesting that E. coli K4 possesses a capsule gene cluster similar to K5. Thus, we hypothesized that regions 1 and 3 of K4 could be obtained by PCR, which could in turn be used for the cloning of region 2. Two PCR products, K4RI (1.3 kbp) and K4RII (1.0 kbp), obtained from E. coli K4 exhibited 96 and 95% sequence identity to regions 1 and 3 of the K5 gene, respectively (Fig. 1, A and B). By using these PCR products as probes, we screened an E. coli genomic library and obtained 30 positive clones. By a second screening, four and six plagues were selected using K4RIII and K4RI, respectively (Fig. 1C).

The desired ORF was selected and cultured in LB medium containing 100 μg/ml ampicillin at 37 °C until the A600 was ~0.6. β-lactamase-galactosidase (1 mM final) was then added to the culture, and the bacteria were further cultured for 3 h at 37 °C. The cells were harvested by centrifugation, suspended in 50 mM NaH2PO4 (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, and 1 mg/ml lysozyme (Sigma) and placed on ice for 30 min. After the suspension was sonicated on ice three times at 10-s intervals and centrifuged at 10,000 × g for 30 min, the super-
organized in a single transcriptional unit, and kpsS involved in capsule production.

droitin synthase from class 2 hyaluronan synthase (35) and 61% identity to chon-

conserved glycosyltransferase sites, showed 59% identity to chon-

K5 consists of six genes (kpsF, -E, -D, -U, -C, and -S) organized in a single transcriptional unit, and kpsS is located furthest downstream in region 1. Region 3 contains kpsM and kpsT. The small arrowheads over the K5 gene indicate the primers for the probes. Region 2 flanked by region 1 and region 3 is unique to each K antigen and contains genes encoding proteins that determine the structure and complexity of the capsule polysaccharide. Region 2 of the K5 capsule gene cluster contains four genes (kfoA–D). Region 2 of the K4 capsule gene cluster flanked by region 1 and region 3 contains seven predicted genes (kfpA–G) and a transposable element IS2 between kfoC and kfoD. Predicted genes with their location and direction (arrows) in region 2 of K4 with a simple restriction map are shown (B, BamHI; E, EcoRI; S, SalI) (2). The black bars under the K4 gene show the probes in region 1 (K4RI3) and region 3 (K4RIIS5). Nine λ phage clones are shown (C). One of the selected clones, CS23 (14.5 kbp), hybridized with both probes, indicating that these clones contained a full-length region 2. After construction of the restriction map, several DNA fragments cleaved by appropriate overlapping restriction enzymes were subcloned and sequenced on both strands to determine the complete nucleotide sequence of K4 region 2.

The 5’ and 3’ termini of CS23 insert DNA were identical to the kpsS gene of region 1 and kpsT gene of region 3, respectively. By using the GenBank homology search BLAST system in NCBI, seven ORFs in region 2 of the K4 capsule gene were predicted and named kfo (K, four) A–G like in the K5 gene (19), and one insertion sequence-2 (IS2) (30) was located between kfoC and kfoD (Fig. 1B).

The predicted first polypeptide, KfoA of 339 amino acids with a calculated molecular weight of 38,040, showed high homology to UDP-glucose 4-epimerase from Pasteurella multocida (31) (60% identity) and others (32, 33). KfoB (546 amino acids, calculated Mr, 63,567) exhibited considerable homology to KfiB (38% identity) of the E. coli K5 capsule gene cluster (19) and one insertion sequence-2 (IS2) (30) was located between kfoC and kfoD (Fig. 1B). KfoC (686 amino acids, calculated Mr, 79,256) containing two conserved glycosyltransferase sites, showed 59% identity to class 2 hyaluronan synthase (35) and 61% identity to chondroitin synthase from P. multocida (28). However, this gene was shorter than either of these enzymes, lacking a carboxyl-terminal membrane association domain. Fig. 2 shows aligned sequences of the three polypeptides. Although all of them have two consensus β-glycosyltransferase motifs (36), the motifs in KfoC showed only 14 and 19% amino acid identity to those in KfiA and KfiC, reported as GlcNAc and GlcUA transferases of E. coli K5, respectively (20, 21).

KfoD (488 amino acids, calculated Mr, 56,100) and KfoE (522 amino acids, calculated Mr, 60,805) had significant homology to BcbD (35% identity) and BcbG (36% identity) of P. multocida (37), respectively, both of which are likely involved in bacterial capsule production. KfoF (389 amino acids, calculated Mr, 43,716), with significant homology to a number of UDP-glucose dehydrogenases, showed high homology to KfiD (75% identity) (19). KfoG (345 amino acids, calculated Mr, 39,060) containing a glycosyltransferase motif, showed 44% amino acid identity to P. multocida DcbD glycosyltransferase (34), suggesting that the protein has glycosyltransferase activity. Insertion sequence 2 (IS2) of 1,331 bp, located between kfoC and kfoD, was a transposable DNA element (38, 39). The IS2 sequence also contained an ORF encoding “transposase” (96% identity) (40).

The results obtained by sequence analyses indicated that region 2 of K4 consists of genes for the synthesis of a specific capsular polysaccharide similar to that of K5.

Expression and Characterization of K4 Chondroitin Polymerase—As KfoC was expected to have chondroitin polymerase
**FIG. 2.** Sequence alignment of K4 chondroitin polymerase and two *P. multocida* glycosaminoglycan synthases. Sequences of K4 chondroitin polymerase (KfoC), *P. multocida* chondroitin synthase (pmCS), and *P. multocida* hyaluronan synthase (pmHAS) are shown. The boxes indicate identical amino acid residues. The dashes denote the positions skipped for alignment. Two conserved glycosyltransferase domains (broken lines), residues 153–258 (A1) and 435–539 (A2) in K4 chondroitin polymerase amino acid sequences correspond to regions important for hexosamine transferase or for glucuronic acid transferase activity, respectively. The black bars under the two glycosyltransferase domains indicate the conserved UDP-sugar-binding motif (DxD) and domains important for double glycosyltransferase activities (DGS). K4 chondroitin polymerase is truncated at the carboxyl-terminal membrane association domain, in comparison with two enzymes of *P. multocida*.
activity, we expressed KfoC using a bacterial expression system. An ORF of the kfoC gene was amplified by PCR and subcloned into an expression plasmid (pTrcHis-kfoC). TOP10 cells were transformed with the expression construct, and the recombinant polypeptide was purified as described under "Experimental Procedures," yielding 0.2 mg of protein per 50 ml of culture. As predicted, the expressed protein showed an 80-kDa band by SDS-PAGE and Western blotting analyses using anti-tetra-His antibody (Fig. 3). In contrast, the culture extracts from the empty vector transformant showed no immunologically reactive band.

Chondroitin polymerase activity was assayed using a purified recombinant KfoC tagged with His6. First, we used CS hexasaccharide as an acceptor (60 pmol) and UDP-GlcUA and UDP-GalNAc as donors (1 nmol each) either of which was labeled. When the sample after the reaction was applied to a high pressure liquid chromatography gel filtration column, a product with a peak of ~5,000 Da was separated from nucleotide monosaccharides or the degraded saccharides (Fig. 4). No incorporated product was found in the absence of the acceptor (data not shown). All of these incorporated products were completely digested by chondroitinase ABC, indicating that they were chondroitin chains (Fig. 4). When these digests were analyzed by high pressure liquid chromatography for glycosaminoglycan disaccharide component assay (41), only unsaturated nonsulfated chondroitin disaccharide was detected (data not shown). Chondroitinase ACII also completely digested the reaction products, whereas Streptomyces hyaluronidase and heparitinase I did not (data not shown).

Next we performed a time course analysis. The enzyme rapidly incorporated [3H]GalNAc in 3 h as shown in Fig. 5B, and after 6 h it slowly incorporated the radioactivity for 18 h under the described conditions. Fig. 5A shows the gel filtration pattern of [3H]GalNAc incorporation at various incubation times (10 min to 18 h). With a longer incubation, the incorporation increased, and products with a higher molecular mass were obtained. At lower concentrations of the acceptor hexasaccharide, products with a higher molecular mass were rapidly obtained. In contrast, products with a lower molecular mass were obtained with higher concentrations of the acceptor (data not shown).

When UDP-GalNAc alone was used as a donor substrate, heptasaccharide was produced from a CS hexasaccharide whose nonreduced end is GlcUA. When CS heptasaccharide was used as an acceptor whose nonreduced end is GalNAc, GalNAc was not transferred to this donor (Fig. 6 and Table I). In contrast, when UDP-GlcUA alone was used, no product was obtained from the CS hexasaccharide, but an octasaccharide was obtained from the CS heptasaccharide. When UDP-GlcNAc alone was used as a donor and the CS hexasaccharide was used as an acceptor, the enzyme showed slight incorporation (~6%) of GlcNAc and made a heptasaccharide (Table I). However, an octasaccharide or longer saccharide was not obtained even in the presence of both UDP-GlcNAc and UDP-GlcUA (Table I and Fig. 4). No incorporation occurred in the absence of an acceptor, indicating that an acceptor substrate is essential for chondroitin polymerization (data not shown).

Next we tested various acceptor substrates for chondroitin polymerization. As summarized in Table II, CS hexasaccharide served as one of the best acceptor substrates. The chondroitin hexasaccharide prepared from desulfated CS achieved polymerization, but the activity was lower (37%) than that of CS hexasaccharide.
chondroitin sulfate hexasaccharide and heptasaccharide acceptor substrates by K4 chondroitin polymerase. CS hexasaccharide or heptasaccharide was incubated with UDP-[3H]GalNAc alone (●), hexasaccharide, or (■) heptasaccharide acceptor) or UDP-[3H]GlcUA alone (●), hexasaccharide, or (■) heptasaccharide acceptor) in the K4 chondroitin polymerase reaction solution. The sample after the reaction was applied to a Superdex 30 HiLoad 16/60 column, and the radioactivity in the fractions was measured. The arrows denote the eluted positions of 8, octasaccharide, and 6, hexasaccharide of hyaluronan standards.

FIG. 6. Incorporation of single donor substrate into chondroitin sulfate hexasaccharide and heptasaccharide acceptor substrates by K4 chondroitin polymerase. CS hexasaccharide or heptasaccharide was incubated with UDP-[3H]GalNAc alone (●), hexasaccharide, or (■) heptasaccharide acceptor) in the K4 chondroitin polymerase reaction solution. The sample after the reaction was applied to a Superdex 30 HiLoad 16/60 column, and the radioactivity in the fractions was measured. The arrows denote the eluted positions of 8, octasaccharide, and 6, hexasaccharide of hyaluronan standards.

DISCUSSION

In this study, we have succeeded for the first time in cloning the full length of region 2 of the K4 capsule gene cluster essential for biosynthesis of fructose-branched chondroitin polysaccharide (K4 antigen). This region, spanning over 14 kb, included seven predicted genes (kfoA–G) and one insertion sequence 2 (IS2). We further identified kfoC as a gene encoding a bifunctional glycosyltransferase that synthesizes the chondroitin backbone, transferring GlcUA and GalNAc alternately to non-reduced terminals of a chondroitin saccharide chain and related oligosaccharides. KfoC protein, thereby termed (E. coli) K4 chondroitin polymerase, synthesized chondroitin polysaccharide up to a molecular size of 20 kDa when CS hexasaccharide was used as an acceptor substrate. Thus, we demonstrated that the capsular gene cluster of E. coli strain K4 contains a gene for chondroitin polymerase that can produce the chondroitin backbone of the K4 antigen polysaccharide.

The generation of segments of the K4 gene homologous to regions 1 and 3 of K5 by PCR and following molecular cloning of K4 region 2 demonstrated that the K4 gene cluster similarly consists of three regions and that regions 1 and 3 are highly...
The purified enzyme was incubated in 50 μl of assay buffer containing 60 pmol of CS hexasaccharide or heptasaccharide as acceptor substrate either with radiolabeled sugar nucleotide (1 nmol, 0.1 μCi, UDP-[14C]GaINAc, UDP-[14C]GlcNAc, and UDP-[14C]GlcUA) or with unlabeled sugar nucleotide (1 nmol, UDP-GaINAc, UDP-GlcNAc, and UDP-GlcUA) as donor substrates at 30 °C for 30 min. The incorporated products were separated by gel filtration high pressure liquid chromatography and the radioactivity of the collected fractions was measured.

| Radio-labeled sugar nucleotide | Unlabeled sugar nucleotide | Chondroitin polymerase-specific activity nmol/min/mg protein | Acceptor substrates |
|-------------------------------|----------------------------|-------------------------------------------------------------|--------------------|
| UDP-[14C]GaINAc               | None                       | 0.09 ± 0.01 (hepta)                                         | CS hexasaccharide  |
| UDP-[14C]GlcNAc               | None                       | 0.04 ± 0.02 (hepta)                                         | CS heptasaccharide |
| UDP-[14C]GlcUA                | None                       | 0.0 ± 0.0 (−)                                               | ND                 |
| UDP-GaINAc                    | UDP-GlcUA                  | 3.25 ± 0.64 (poly)                                          | ND                 |
| UDP-GlcNAc                    | UDP-GaINAc                 | 2.75 ± 0.28 (poly)                                          | ND                 |
| UDP-GlcUA                     | UDP-GlcNAc                 | 0.05 ± 0.02 (hepta)                                         | ND                 |
| UDPA-GlcNAc                   | UDP-GlcUA                  | 0.0 ± 0.0 (−)                                               | ND                 |

The values given are the mean ± S.D. (n = 3–6).

ND: not determined.

Homologous between K4 and K5. In contrast, region 2 of K4, containing seven genes and an insertion sequence, exhibits some distinct features from that of K5 containing only four genes. Chondroitin polymerization is achieved by a single protein KfoC in K4, whereas both KfiA and KfiC are likely required for synthesis of N-acetylheparosan in K5 (20, 21). Although region 2 of K4 has some genes comparable with K5, like kfiD and kfiF encoding UDP-glucose dehydrogenase, and kfiB and kfiF encoding a protein interacting with membrane-associated glycosyltransferases, it further contains other genes such as kfoA, kfoD, kfoE, and kfoG. DNA sequence analysis revealed that kfoA encodes UDP-glucose 4-epimerase, which converts glucosamine to galactosamine. Thus, the KfoA and KfoC proteins are likely involved in the synthesis of GalNAc and GlcUA, respectively. In addition, to KfoC, KfoG also has a glycosyltransferase motif in the K4 region 2. As the chondroitin chain of K4 is branched by fructose, this protein may serve as a fructose transferase. Sugar-branched CS are also found in tissues of other organisms; squid cartilage contains a glucose-branched CS (42) and the body wall of sea cucumber contains a fucose-branched CS (43), although the biological significance of these modifications is still unknown. KfoD and KfoE showed homology to bacterial proteins in a capsule production, but their functions remain to be determined. The insertion sequence encodes a transposable DNA element, suggesting that some portion of this region is derived from other microorganisms.

The K4 chondroitin polymerase shows significant homology to both pmHAS (35) and pmCS (28), and all of them contain two glycosyltransferase domains per molecule. In pmCS, an upstream domain termed A1 is likely responsible for the N-acetylgalactosamine β-1-4 glycosyl bond and the downstream domain A2 for the uronic acid β-1-3 glycosyl bond (44). The A1 domain (residues 153–258) of K4 chondroitin polymerase shows a closer resemblance to that of pmCS than pmHAS. As pmHAS transfers GlcNAc instead of GalNAc, the donor specificity of these enzymes may be determined by the amino acid sequence within the A1 domain. In contrast, the downstream A2 domain (residues 435–539) of K4 showed 70% identity to that of pmCS and pmHAS, all of which transfer GlcUA. Both A1 and A2 contain two conserved β-glycosyltransferase motifs: 184DGS and 218PDGS and 218DSD in A2. These motifs provide a UDP-sugar binding site and therefore are essential for glycosyltransferase activity (44). The conserved DXXD motif interacts directly with the ribose of the UDP molecule as well as Mn2+ ion required for the enzymic activity (45). We demonstrated the requirement of metal ions, consistent with the results for pmCS reported by Jing and DeAngelis (44). In contrast to pmCS and pmHAS, K4 chondroitin polymerase lacks a carboxyl-terminal membrane association domain re-
A, chondroitin polymerase-specific activities (v, nmol/ min/mg protein) were measured using 1.3 µg of the affinity purified recombinant enzyme as described under “Experimental Procedures” except that various concentrations (S, 0.6–200 µM) of UDP-[14C]GlcUA (●) and UDP-[3H]GalNAc (□) were incubated with a constant concentration of the other UDP-sugar (240 µM), under conditions in which the incorporation occurred linearly. B, the specific incorporation data from A were plotted as 1/v versus 1/S. ●, UDP-GlcUA; □, UDP-GalNAc. The x axis intercept signifies −1/Km. Values represent means of three independent experiments.

required for interacting with the polysaccharide transport machinery or a membrane-bound partner. In the K4 strain, the chondroitin polymerase may form a complex, which facilitates interaction with the saccharide transporter or membrane-bound partners.

Analyses using various acceptor substrates disclosed several characteristics of the enzyme. First, sulfated chondroitin chains apparently serve as better acceptors than non-sulfated chains in both polymers and oligosaccharides, although K4 bacteria entirely synthesize non-sulfated chondroitin. The increase in charge density and/or solubility caused by sulfated sugar residues may facilitate enzyme-substrate interaction. Alternatively, other factors such as damage of the chondroitin chain by chemical de-sulfation may have hampered enzyme-substrate interaction. Second, the enzyme requires an acceptor at least the size of a tetrasaccharide, which is inconsistent with the observation that pmCS did not require an acceptor for chondroitin polymerization (28). As the activity was assayed using a membrane fraction in the previous study, it is possible that the fraction contained acceptor molecules. It is also possible that the enzyme bound to the membrane indeed requires no acceptors of saccharides, as the K antigen polymerization appears to initiate at a phospholipid-linked saccharide carrier in the cytoplasmic membrane (46). In addition to these findings, we demonstrated that short chain hyaluronan and hyaluronan oligosaccharide also serve as acceptors for chondroitin polymerization. Together with the finding that tetrasaccharide served as an acceptor, the results indicated that the enzyme may recognize GlcUA at the nonreduced end rather than the complicated structure of the chain. In contrast, neither heparin nor dermatan sulfate served as an acceptor, suggesting that iduronic acid epimerized at C-5 of GlcUA hampers recognition of the acceptor by the enzyme.

Analyses on donor selectivity also revealed certain distinctive features of the enzyme. When a single sugar nucleotide (UDP-GalNAc or UDP-GlcUA) was used as a donor substrate in the glycosyltransferase assay reaction, only one sugar molecule was incorporated into the acceptor substrate at the nonreducing terminal. The polymerization reaction occurred only in the presence of both sugar nucleotides as donors. These results indicate that each alternative step of donor saccharide attachment is strictly regulated by the enzyme. This is in contrast to the observation that mammalian hyaluronan synthase synthesizes chitin oligoacaccharide with UDP-GlcNAc substrate only (47). Although we demonstrated a strict donor selectivity during polymerization, we also observed that GlcNAc could be attached to the hexasaccharide, although no further elongation occurred under any conditions. The mechanism of substrate specificity remains to be elucidated.

Recently, a mammalian chondroitin synthase was cloned (48). The enzyme has been shown to attach either GalNAc or GlcUA to the non-reduced end of the chondroitin polymer. Although the authors named “chondroitin synthase” as a single enzyme that exhibits dual glycosyltransferase activity, they have not demonstrated chondroitin polymerization by the enzyme. To date, only pmCS and the K4 enzyme presented here have actually been shown to exhibit chondroitin polymerase activity. In this context, we name this K4 enzyme “chondroitin polymerase.” It is of interest whether the mammalian chondroitin synthase itself has polymerase activity. The mammalian chondroitin synthase shows little homology to pmCS (28). If the mammalian enzyme has polymerase activity, the next question would be how these enzymes distinct in structure share the same polymerase activity. If the mammalian enzyme requires other molecules for polymerization, functional studies on the domains of bacterial chondroitin polymerase would provide insight into the mechanism of mammalian chondroitin synthesis.

Acknowledgments—We thank Dr. T. Saito for bacteria species. We also thank Drs. Y. Kakuta, T. Ogawa, H. Takagi, Y. Kariya, K. Suzuki, M. Ryogoshima, K. Yoshida, and S. Suzuki for helpful comments.

REFERENCES
1. Kimata, K., Okayama, M., Oasa, A., and Suzuki, S. (1974) J. Biol. Chem. 249, 1646–1653
2. Zako, M., Shimomura, T., Miyaishi, O., Iwaki, M., and Kimata, K. (1997) J. Neurochem. 69, 2155–2161
3. David, G., Lories, V., Heremans, A., Van der Schueren, B., Cassiman, J. J., and Van den Berghe, H. (1989) J. Cell Biol. 108, 1165–1173
4. Oohira, A., Katoh-Šemha, R., Watanabe, E., and Matsu, F. (1994) Neurosci. Res. 20, 185–207
5. David, C. L., Orpiszewski, J., Zhu, X. C., Reissner, K. J., and Axsall, D. W. (1998) J. Biol. Chem. 273, 32063–32070
6. Prydz, K., and Dalem, K. T. (2000) J. Cell Sci. 113, 193–205
7. Habuchi, O. (2000) Biochim. Biophys. Acta 1474, 115–127
8. Toyoda, H., Kinosita-Toyoda, A., and Selleck, S. B. (2000) J. Biol. Chem. 275, 2269–2275
9. Vynios, D. H., and Tsiganos, C. P. (1990) Biochim. Biophys. Acta 1033, 139–147
10. Ito, Y., and Habuchi, O. (2000) J. Biol. Chem. 275, 34728–34736
11. Sugahara, K., Tanaka, Y., Yamada, S., Seno, N., Kitagawa, H., Haslam, S. M.,...
Molecular Cloning and Characterization of Chondroitin Polymerase from
Escherichia coli Strain K4
Toshio Ninomiya, Nobuo Sugiura, Akira Tawada, Kazunori Sugimoto, Hideto Watanabe
and Koji Kimata

J. Biol. Chem. 2002, 277:21567-21575.
doi: 10.1074/jbc.M201719200 originally published online April 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201719200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 24 of which can be accessed free at
http://www.jbc.org/content/277/24/21567.full.html#ref-list-1