Nematode Chondroitin Polymerizing Factor Showing Cell/Organ-specific Expression Is Indispensable for Chondroitin Synthesis and Embryonic Cell Division*§

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Chondroitin polymerization was first demonstrated in vitro when human chondroitin synthase (ChSy) was coexpressed with human chondroitin polymerizing factor (ChPF), which is homologous to ChSy but has little glycosyltransferase activity. To analyze the biological function of chondroitin, the Caenorhabditis elegans ortholog of human ChSy (sqv-5) was recently cloned, and the expression of its product was depleted by RNA-mediated interference (RNAi) and deletion mutagenesis. Blocking of chondroitin synthesis resulted in defects of cytokinesis in early embryogenesis, and eventually, cell division stopped. Here, we cloned the ortholog of human ChPF in C. elegans, PAR2.4. Despite little glycosyltransferase activity of the gene product, chondroitin polymerization was demonstrated as in the case of mammals when PAR2.4 was coexpressed with chSy in vitro. The worm phenotypes including the reversion of cytokinesis, observed after the depletion of PAR2.4 by RNAi, were very similar to the ChSy (sqv-5)-RNAi phenotypes. Thus, PAR2.4 in addition to chSy is indispensable for the biosynthesis of chondroitin in C. elegans, and the two cooperate to synthesize chondroitin in vivo. The expression of the PAR2.4 protein was observed in seam cells, which can act as neural stem cells in early embryonic lineages. The expression was also detected in vulva and distal tip cells of the growing gonad arms from L3 to the young adult stage. These findings are consistent with the notion that chondroitin is involved in the organogenesis of the vulva and maturation of the gonad and also indicative of an involvement in distal tip cell migration and neural development.

Chondroitin sulfate proteoglycans (CS-PGs)¹ are universally distributed glycoproteins consisting of CS chains substituted on core proteins and are located in the extracellular matrices and on cell surfaces in various kinds of human tissues. Some CS-PGs modulate cell adhesion, cell proliferation, and morphogenesis (for reviews, see Refs. 1 and 2). We have revealed that chondroitin is required for normal cell division and cytokinesis at an early developmental stage in Caenorhabditis elegans (3). In addition, recent studies have demonstrated that CS chains are major inhibitory molecules affecting axon growth after spinal cord injury in the central nervous system of adult mammals (4), but that they can also stimulate neuronal differentiation in a structure-dependent fashion (2). Thus, it is imperative to elucidate the mechanism of the biosynthesis of chondroitin and CS for a better understanding of various developmental processes in lower organisms through mammals.

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. Then, chondroitin polymerization with alternating GalNAc and GlcUA takes place by the action of a complex consisting of chondroitin synthase (ChSy) (5) and chondroitin polymerizing factor (ChPF), a unique protein factor required for the polymerization (6). Also, the functionally redundant, multiple glycosyltransferases involved in chondroitin biosynthesis have been cloned (7–11). This redundancy makes it difficult to investigate the mechanism of chondroitin biosynthesis by gene knockout or characterization of individual glycosyltransferases.

To clarify the mechanism of chondroitin biosynthesis in vivo,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank and EBI Data Bank with accession number(s) AB110823 and AB110824

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¹ The abbreviations used are: CS-PG, chondroitin sulfate proteoglycan; Chz, benzylxoycarbonyl; dsRNA, double-stranded RNA; GFP, green fluorescent protein; EGF, enhanced GFP; GalNAcT, N-acetylgalactosaminyltransferase; GlcAT, glucuronoyltransferase; RNAi, RNA-mediated interference; ΔHexUA, unsaturated hexuronic acid; MES, 2-(N-morpholino)ethanesulfonic acid; ChSy, chondroitin synthase; ChPF, chondroitin polymerizing factor; DIC, differential interference contrast; DTC, distal tip cells; HS, heparan sulfate; HPLC, high pressure liquid chromatography.
C. elegans Chondroitin Polymerizing Factor

we have been using lower organisms such as C. elegans because they are predicted to have few glycosyltransferases and a simple mechanism for production of the chondroitin chain. C. elegans is one of the most tractable model animals because all genomic DNA sequences and all cell lineages are known. In addition, it is easy to knock down specific genes using the reverse genetic method, RNA-mediated interference (RNAi) (12). Furthermore, C. elegans produces a large amount of nonsulfated chondroitin but not CS (13, 14). Recently, we cloned a ChSy ortholog (cChSy or sqv-5) in C. elegans and depleted expression of its product using RNAi methods (3). cChSy-RNAi-treated worms showed defects of cytokinesis in early embryogenesis, and cell division eventually stopped as a result of depletion of chondroitin (3, 15).

Speculating that the fundamental mechanism underlying the biosynthesis of chondroitin in C. elegans might be similar to that in humans, we hypothesized that an ortholog of ChPF might exist in C. elegans. Screening of a data base using the amino acid sequence of human ChPF identified a candidate protein, PAR2.4. In addition, it was predicted that only two genes, cChSy and PAR2.4, are critically involved in chondroitin biosynthesis in C. elegans since PAR2.4 and cChSy (sqv-5) RNAi-treated worms showed extremely similar phenotypes (3). Here, we demonstrate that PAR2.4 is indispensable for the biosynthesis of chondroitin in C. elegans in addition to sqv-5 (cChSy).

EXPERIMENTAL PROCEDURES

Strains—All the strains were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN).

Materials—UDP-[U-14C]GlcUA (285.5 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. Arthrobacter azureus chondroitinase AC-II (EC 4.2.2.5) and chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku Corp. (Tokyo, Japan). (GlcUA)5 (H11005), and GalNAc5-4 (H11032) cDNA sequences were obtained from Arthrobacter azureus chondroitinase AC-II (EC 4.2.2.5) and chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku Corp. (Tokyo, Japan). (GlcUA)5 were purchased from PerkinElmer Life Sciences. Unlabeled UDP-GalNAc as a sugar donor and tested for GlcAT-II transferase activity using 5 nmol of the chondroitin-derived hexa- and heptasaccharide as an acceptor and UDP-GlcUA, as described previously (3, 16, 20). A polymerization reaction using 100 nmol of GlcUA3Galβ1-4GalNAcβ1-3Gal was incorporated into the medium after the addition of 10 ml of MeOH and 3.5 ml of H2O was added 2.5 ml of 0.107 M sodium acetate buffer described below, and then tested for GalNAcT-II transferase activity using 5 nmol of the chondroitin-derived heptasaccharide and UDP-GlcUA, as described previously (3, 16, 20).

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on GlcUA3Galβ1-4GalNAcβ1-3Gal were separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.
F(ab')2 against mouse IgG conjugated to alkaline phosphatase (EY Laboratories) as described (3).

Analysis of Glycosaminoglycans—Glycosaminoglycans were prepared from 24 mg of dried homogenates of RNAi-treated nematodes. The unsaturated disaccharides were produced by enzymatic digestion with chondroitinase ABC or a mixture of heparitinases I and II, and the digests were derivatized with 2-aminobenzamide and analyzed with high performance liquid chromatography (HPLC) as described previously (3, 13). It should be noted that the amount of heparan sulfate by high performance liquid chromatography (HPLC) as described previously (3, 13). It should be noted that the amount of heparan sulfate was so small (13) that 100 μg of shark cartilage chondroitin 6-O-sulfate (Seikagaku Corp.), which contained a negligible proportion of non-sulfated disaccharides, was added as a carrier after the borohydride treatment but before the purification steps.

Transgenic Constructs—The expression vector pFX_DsRedXT or pFX_EGFPT is composed of Bluescript (Stratagene, Palo Alto, CA) with an additional multiple cloning site followed by DsRed or enhanced GFP (EGFP) cDNA (Clontech) and about 1 kb of 3′-untranslated region from human ChSy. The predicted amino acid sequences were aligned using the program GENETYX-MAC (version 10). The putative membrane spanning domains are indicated by boxes. Three potential N-glycosylation sites for C. elegans PAR 2.4 are marked with asterisks.

FIG. 1. Comparison of the predicted amino acid sequence among C. elegans PAR 2.4, human ChPF, human chondroitin GlcAT, and human ChSy. The predicted amino acid sequences were aligned using the program GENETYX-MAC (version 10). Closed and shaded boxes indicate that the predicted amino acid in the alignment is identical in all four or any three sequences, respectively. Gaps introduced for maximal alignment are indicated by dashes. The vector containing two XcmI sites in the multiple cloning site was digested with XcmI to make a thymidine overhang in 3′-terminus at both ends for the TA cloning strategy (24), as described below.

The PAR 2.4 reporter gene plasmid was constructed using the vector pFX_DsRedXT. The translational fusion construct contained a 4.4-kb genomic fragment including the 1.0-kb potential promoter region. The fragment was amplified with C. elegans genomic DNA as a template using a 5′-primer (5′-TTTTTCGTGGAATAACAATTTTG-3′) and a 3′-primer (5′-TTTGTCTGGAAATAAACATTGTTT-3′) located just before the stop codon. PCR was carried out with Platinum TaqDNA polymerase High Fidelity (Invitrogen) for 28 cycles of 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 300 s followed by adenine addition with Taq polymerase at 72 °C for 15 min. The PCR fragment was inserted into pFX_DsRedXT using the TA cloning strategy to fuse the coding sequence region of DsRed. The translational fusion construct contained a 6.0-kb genomic fragment including the 5.0-kb potential promoter region. The fragment was amplified with C. elegans genomic DNA as a template using a 5′-primer (5′-ATTTTGGTTTATCGATTGAGCA-3′) and a 3′-primer (5′-TTTTTCGTGGAATAACAATTTTG-3′) located just before the stop codon. PCR was carried out with Platinum TaqDNA polymerase High Fidelity (Invitrogen) for 28 cycles of 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 300 s followed by adenine addition with Taq polymerase at 72 °C for 15 min. The PCR fragment was inserted into the pFX_EGFPT vector using the TA cloning strategy to fuse the coding sequence region of EGFP.

Expression of PAR 2.4—DsRed and ChSy::GFP in the Nematode C. elegans—Germ-line transformation was done as described by Mello et al. (25). The PAR 2.4-DsRed construct was microinjected into the distal gonad of an adult hermaphrodite of Caenorhabditis Genetic Center strain JR687, which expresses GFP in seam cells. The

2 K. Gengyo-Ando and S. Mitani, unpublished results.
PAR2.4::DeRed and cChSy::EGFP constructs were co-injected into the distal gonad of a wild type N2 strain. Transgenic lines containing reporter constructs were isolated. Live transgenic worms were paralyzed with a 50 mM sodium azide solution and placed on an 8-well printed microscope slide glass (Matsunami Glass) and examined by four-dimensional microscopy (a DMRXA full automatic microscope with printed microscope slide glass (Matsunami Glass) and examined by differential interference contrast (DIC) and fluorescent optics, Leica) as four-dimensional microscopy (a DMRXA full automatic microscope with printed microscope slide glass (Matsunami Glass) and examined by differential interference contrast (DIC) and fluorescent optics, Leica) as described (3). The images were processed using MetaMorph software (version 4.6, Universal Imaging).

RESULTS

Molecular Cloning of PAR2.4—Screening of the non-redundant data base at the NCBI, National Institutes of Health (Bethesda, MD), using the deduced amino acid sequence of human ChSy, identified a few homologs in C. elegans. One of them, designated PAR2.4 (WormBase accession PAR2.4), contained a 5′-untranslated region of 133 bp, a single open reading frame of 2,412 bp coding for a protein of 804 amino acids with three potential N-glycosylation sites (Fig. 1), and a 3′-untranslated region of 308 bp. The predicted translation initiation site conformed to the Kozak consensus sequence for initiation (26).

The amino acid sequence displayed 28, 27, and 16% identity to human chondroitin GlcAT (8), ChPF (6), and ChSy (5), respectively (Fig. 1). Thus, the features of the identified protein sequence suggest that the gene product is involved in the biosynthesis of chondroitin in C. elegans but would not possess glycosyltransferase activity.

Coexpression and Characterization of PAR 2.4.—To facilitate the functional analysis of PAR2.4, a soluble form of the protein was generated by replacing the first 52 amino acids 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 at 30 °C as a recombinant protein fused with the protein A IgG-binding domain. The fusion protein secreted in 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. Although the bound fusion protein was assayed for glycosyltransferase activity at 26 °C using chondro-hexasaccharide (GlcUAβ1-3GalNAc3), or chondro-heptasaccharide GalNAcβ1-4(GlcUAβ1-3GalNAc3) as a sugar accepter and either UDP-GalNAc or UDP-GlcUA as a sugar donor substrate, neither GalNAcT-II nor GlcAT-II activity was detected (Table I). However, coexpression of the soluble PAR2.4 with the soluble cChSy augmented the GalNAcT-II activity of cChSy over 20-fold and clearly showed GlcAT-II activity, which had not been detected when only cChSy was expressed. Notably, these effects of coexpression were not due to differences in the expression levels of these proteins, as assessed by Western blot analysis (data not shown). These results are analogous to the findings recently made for human ChPF and ChSy. Thus, we asked whether the coexpression of PAR2.4 and cChSy could result in the expression of polymerization activity. Incubations of the coexpressed proteins with GlcUAβ1-3Galβ1-0-C6H11NHCbz as an acceptor substrate were digested with chondroitinase AC-II as described under “Experimental Procedures.” The chondroitinase AC-II-digested sample (open circles) or the undigested sample (closed circles) was applied to a Superdex peptide column (1.0 × 30 cm), and the radioactivity in the effluent fractions (0.4 ml each) was analyzed as described under “Experimental Procedures.”

Depletion of chondroitin by PAR2.4-RNAi treatment, A, non-viable eggs produced in a PAR2.4-RNAi treatment worm. PAR2.4-RNAi treatment (>60 h) gave rise to multinucleated embryos due to incomplete cytokinesis, resulting in early embryonic death (scale bar, 10 μm). A QuickTime movie regarding abnormal embryonic cell division (the one-cell embryo directly divided into a four-cell embryo) and reversion of cytokinesis (divided from four cells to eight cells and then reverted to four cells) is available as a supplement in the on-line version of this article. B, Western blot analysis of total protein from mixed-stage wild-type hermaphrodites treated with or without PAR2.4 dsRNA. The presence of chondroitin was examined using anti-chondroitin stub antibody (anti-Ch Ab). After 60 h of PAR2.4-RNAi treatment, chondroitin disappeared completely (right lane), although chondroitin was abundant in untreated worms (left lane).

Table I

| Protein       | GalNAcT-II activitya | GlcAT-II activitya |
|---------------|----------------------|--------------------|
|               | pmol/ml medium/h     | pmol/ml medium/h   |
| cChSy         | 0.03                 | ND                 |
| PAR2.4        | ND                   | ND                 |
| cChSy/PAR2.4  | 0.57                 | 0.55               |

a (GlcUAβ1-3GalNAc3) was used as an acceptor substrate.

b GalNAcβ1-4(GlcUAβ1-3GalNAc3) was used as an acceptor substrate.

* ND, not detected (<0.01 pmol/ml medium/h).

FIG. 2. Identification of polymerization reaction products using GlcUAβ1-3Galβ1-0-C6H11NHCbz as an accepter. 14C-labeled polymerization reaction products obtained using GlcUAβ1-3Galβ1-0-C6H11NHCbz as an acceptor substrate were digested with chondroitinase AC-II as described under “Experimental Procedures.” The chondroitinase AC-II-digested sample (open circles) or the undigested sample (closed circles) was applied to a Superdex peptide column (1.0 × 30 cm), and the radioactivity in the effluent fractions (0.4 ml each) was analyzed as described under “Experimental Procedures.” Numbered arrowsheads 2, 10, and 12 indicate the elution position of chondroitin-derived authentic di-, deca-, and dodecasaccharides, respectively. The total volume was around fraction 60 (not shown).

FIG. 3. Depletion of chondroitin by PAR2.4-RNAi treatment. A, non-viable eggs produced in a PAR2.4-RNAi treatment worm. PAR2.4-RNAi treatment (>60 h) gave rise to multinucleated embryos due to incomplete cytokinesis, resulting in early embryonic death (scale bar, 10 μm). A QuickTime movie regarding abnormal embryonic cell division (the one-cell embryo directly divided into a four-cell embryo) and reversion of cytokinesis (divided from four cells to eight cells and then reverted to four cells) is available as a supplement in the on-line version of this article. B, Western blot analysis of total protein from mixed-stage wild-type hermaphrodites treated with or without PAR2.4 dsRNA. The presence of chondroitin was examined using anti-chondroitin stub antibody (anti-Ch Ab). After 60 h of PAR2.4-RNAi treatment, chondroitin disappeared completely (right lane), although chondroitin was abundant in untreated worms (left lane).
Treated with PAR2.4 RNAi—

In a previous study (3), the sis is severely affected in shown). The results strongly suggest that chondroitin synthesis is impaired in RNAi-treated worms (data not available). Although nucleic division proceeded without cytokinesis, normally so that the early L1 (the first larval stage) worm can hatch. After hatching, the animal passes through four larval stages (L1–L4), and at the end of each larval developmental region located upstream of the PAR2.4 gene, we constructed a transcriptional reporter that fuses the gene (DsRed) encoding DsRed fluorescent protein to the putative promoter/enhancer region located upstream of the PAR2.4 sequence as described under “Experimental Procedures.” Expression of PAR2.4::DsRed proteins was first observed in the late embryonic stages or in the L1 stage and continued to adulthood. Expression of the proteins in the vulva and in distal tip cells (DTC) was observed in the early L3 or young adult stage, when this organ and these cells are formed. In adult worms, expression of the reporter was seen in vulval cells (Fig. 4, e and f), and an especially strong expression was observed in the lateral zone. To identify the cells expressing PAR2.4 in this region, we injected the PAR2.4::DsRed reporter transgene into gonads of the JH67 strain that expresses GFP in the nuclei of seam cells, which are in the cell lineage of neuronal stem cells and are glia-like neuron-supporting cells (29). Strong PAR2.4::DsRed signals were found in the cytoplasm of seam cells expressing strong GFP signals (Fig. 4, a and b), which led us to conclude that PAR2.4 proteins are strongly expressed in these cells. Next, we asked whether the PAR2.4 and Chs genes could be expressed in the same cells or not. To identify cells expressing the Chs gene in the nematode, we constructed a Chs::EGFP transgene with a full-length coding sequence as described in detail in “Experimental Procedures.” Values for the worms treated with Chs::EGFP and PAR2.4::DsRed reporter construct were injected simultaneously into gonads of N2 worms, both Chs::EGFP and PAR2.4 were expressed in the head and body region of adult worms. Both proteins were expressed in the

### Table II

| GAG disaccharides                  | N2 (wild type) | cChSy (sqv-5) (RNAi) | PAR2.4 (RNAi) |
|-----------------------------------|----------------|---------------------|--------------|
| Chondroitin                       | 5,874 (100)    | 1,575 (27)          | 2,699 (46)   |
| Heparan sulfate                   | 11.7 (100)     | 24.8 (212)          | 19.6 (188)   |

### Table III

| HS disaccharide composition       | N2              | cChSy (RNAi) | PAR2.4 (RNAi) |
|----------------------------------|-----------------|--------------|--------------|
|                                  | pmol %          |              |              |
| $\Delta$HexUA1-4GlcNAc          | 5.4 (46)        | 14.9 (60)    | 11.2 (57)    |
| $\Delta$HexUA1-4GlcNAc(6S)$a$   | 1.5 (13)        | 3.8 (15)     | 1.5 (8)      |
| $\Delta$HexUA1-4GlcN(NS)        | 2.1 (18)        | 2.5 (10)     | 6.5 (33)     |
| $\Delta$HexUA2(S)1-4GlcN(NS)    | 1.2 (10)        | 2.6 (11)     | 0.4 (2)      |
| $\Delta$HexUA2(S)1-4GlcN(6S,6S)| 1.5 (13)        | 1.0 (4)      | ND$b$        |
| Total                            | 11.7 (100)      | 24.8 (100)   | 19.6 (100)   |

$a$ 6S, NS, and 2S represent 6-O-sulfate, 2-N-sulfate, and 2-O-sulfate, respectively.

$^b$ ND, not detected.

$[^{14}C]$GlcUAβ1-3GalNAc, indicating that the polymerization occurred on GlcUAβ1-3Galβ1-O-C2H,NHCbz when PAR2.4 and cChSy were coexpressed.

**Depletion of Chondroitin by PAR 2.4-RNAi Treatment—Embryogenesis in C. elegans** takes only 14 h from fertilization to hatching at 20 °C. Gastrulation begins at the 28-cell stage, and cell division and organogenesis continue until about 6 h after fertilization. During the next 6 h, the embryo changes from a sphere to an elongated shape, and during the final 2 h, the pharynx begins to pump, and the eggshell is softened enzymatically so that the early L1 (the first larval stage) worm can hatch. After hatching, the animal passes through four larval stages (L1–L4), and at the end of each larval developmental stage, nematodes undergo a molt in which a new cuticle is formed and the old cuticle is shed. The larva takes 2–3 days to grow into an adult.

To understand the roles of the PAR2.4 gene in the nematode C. elegans, we examined phenotypes caused by PAR2.4-RNAi experiments. After 12 h of treatment (feeding) with PAR2.4 dsRNA, most of the F1 progeny of the treated worms showed wild-type phenotypes. About 30% of the worms treated with the dsRNA for 20 h and 80–90% of those treated for 48 h died as embryos. F2 worms treated with the dsRNA for more than 60 h died at a rate of nearly 100%. Early embryonic development of the PAR2.4-RNAi-treated worms (F2 or F3 worms) was monitored with a four-dimensional microscopy (a multifocal time-lapse image recording system). The process of early embryonic cell death was identical to that observed for the Chs::RNAi-treated worms. An apparent inversion of cytokinesis was observed as reported for Chs RNAi (see supplementary movies), and embryos failed to complete cytokinesis, became multinucleated, and died (Fig. 3A). Although nuclear division proceeded without cytokinesis, normal chromosome partition also seemed to be affected by GFP-tagged chromosomes in the RNAi-treated worms (data not shown). The results strongly suggest that chondroitin synthesis is severely affected in PAR2.4-RNAi-treated worms. Western blot analysis of proteins of RNAi-treated worms showed a complete lack of chondroitin PG in the worms treated with the dsRNA for more than 60 h (Fig. 3B) as reported for cChSy (sqv-5)-RNAi in the nematode C. elegans (3).

**Analysis of the Disaccharide Composition of C. elegans**—In a previous study (3), the

ChS-RNAi F1 worm showed a 73% decrease in chondroitin and a 112% increase in HS. Thus, the glycosaminoglycan content of PAR2.4-RNAi-treated F1 worms was determined by chondroitinase or heparin lyase digestion followed by HPLC as described under “Experimental Procedures.” PAR2.4-RNAi-treated F1 worms showed a 54% decrease in chondroitin and a 68% increase in HS (Table II). In addition, the disaccharides of the HS from the RNAi-treated F1 worms were less sulfated than those of the wild-type worms (Table III). These results were similar to those obtained for the cChS-RNAi F1 worms (3).

**Coexpression of EGFP-cChSy and DsRed-PAR2.4 Fusion Protein in Wild-type Worms**—To characterize the potential expression of the nematode PAR2.4 gene, we constructed a transcriptional reporter that fuses the gene (DsRed) encoding DsRed fluorescent protein to the putative promoter/enhancer region located upstream of the PAR2.4 sequence as described under “Experimental Procedures.” Expression of PAR2.4::DsRed proteins was first observed in the late embryonic stages or in the L1 stage and continued to adulthood. Expression of the proteins in the vulva and in distal tip cells (DTC) was observed in the late L3 or young adult stage, when this organ and these cells are formed. In adult worms, expression of the reporter was seen in vulval cells (Fig. 4, e and f), and an especially strong expression was observed in the lateral zone. To identify the cells expressing PAR2.4 in this region, we injected the PAR2.4::DsRed reporter transgene into gonads of the JH67 strain that expresses GFP in the nuclei of seam cells, which are in the cell lineage of neuronal stem cells and are glia-like neuron-supporting cells (29). Strong PAR2.4::DsRed signals were found in the cytoplasm of seam cells expressing strong GFP signals (Fig. 4, a and b), which led us to conclude that PAR2.4 proteins are strongly expressed in these cells. Next, we asked whether the PAR2.4 and Chs genes could be expressed in the same cells or not. To identify cells expressing the Chs gene in the nematode, we constructed a Chs::EGFP transgene with a full-length coding sequence and the putative promoter/enhancer region located 5 kb upstream of the chs::EGFP sequence. When the Chs::EGFP reporter construct and the PAR2.4::DsRed reporter construct were injected simultaneously into gonads of N2 worms, both Chs::EGFP and PAR2.4 were expressed in the head and body region of adult worms. Both proteins were expressed in the...
co-injected with adult hermaphrodite (head region). The expression of PAR 2.4 proteins (image (a)) and an accompanying fluorescence image (f) are perfectly colocalized (j, arrows). Scale bar, 10 μm.

Fig. 4. Expression patterns of chondroitin polymerizing factor PAR2.4 and ChSy in the nematode C. elegans. a and b, PAR 2.4 proteins (red) are expressed in seam cells (arrows) in the head-region of an adult hermaphrodite worm. GFP-tagged seam cell marker strain J8667 was used in a–f, and the nucleus of the seam cell was highlighted with the gfp marker (green in b, d, and f). The nucleus of the seam cell shows green fluorescence. A DIC image (a) and an accompanying fluorescence image (b) are shown. c and d, PAR 2.4 proteins (red) are expressed in vulva (arrow) of the nematode with green fluorescent seam cells. A DIC image (c) and an accompanying fluorescence image (d) are shown. The inset shows a magnified view of vulva. e and f, PAR 2.4 proteins (red) are expressed in distal tip cells (arrowhead) of the nematode with green fluorescent seam cells. A DIC image (e) and an accompanying fluorescence image (f) are shown. The inset shows a magnified view of distal tip cell. g–j, colocalization of PAR 2.4 proteins (red) and chondroitin synthase (green) in an adult hermaphrodite (head region). g, a DIC image of the nematode co-injected with PAR 2.4:DSRed and ChSy::EGFP. The bright red fluorescence of PAR 2.4 proteins (h) and bright green fluorescence of ChSy proteins (i) are perfectly colocalized (j, arrows). Scale bar, 10 μm.

cytoplasm of the same cells at least in some cells as shown in Fig. 4, g–j, although coexpression of these two genes was not obvious in other cells and a more detailed study of expression patterns is necessary to investigate whether only the PAR2.4 or ChSy gene is expressed alone in some cells.

DISCUSSION

In previous studies, we revealed that the enzyme complex consisting of human ChSy (5) and ChPF (6) could polymerize chondroitin chains in vitro. In this study, we showed that PAR2.4 is the C. elegans ortholog of human ChPF, and only two genes, PAR2.4 and sqv-5 (cChSy), are required for chondroitin biosynthesis. In addition, we demonstrated for the first time that ChSy and PAR2.4 are indispensable for the biosynthesis of chondroitin chains in vivo, whereas PAR2.4 showed little glycosyltransferase activity like ChPF (6). Although the features of the ChSy protein sequence are similar to those of the human ChSy sequence, the catalytic activities of ChSy resemble those of the human chondroitin GalNAcT-1 (7, 8) and -2 (9, 10), which exhibit GalNAcT-I activity for chain initiation and GalNAcT-II activity for chain elongation of CS, respectively, but not GlcAT-II activity. Coexpression of ChSy with PAR2.4 yielded a ~20-fold increase in the GalNAcT-II activity when compared with the expression of ChSy alone, and the GlcAT-II activity, which was not detected when only ChSy was expressed, could be detected here (see Table 1). In addition, the complex showed chondroitin polymerization activity. These results suggested that the mechanism of chondroitin biosynthesis in C. elegans is quite similar to that in humans and that both ChSy and ChPF are indispensable for the biosynthesis of chondroitin chains in the nematode as well as in humans. Thus, PAR2.4 has been identified as a C. elegans ortholog of human ChPF and named here polymerizing factor for chondroitin-1 (pfc-1).

The mechanism for the biosynthesis of chondroitin is reminiscent of that for HS. The repeating disaccharide region of HS is synthesized by glycosyltransferases encoded by EXT1 (30) and EXT2 (31), which form an enzyme complex (HS polymerase) (32, 33). These genes are involved in the hereditary multiple exostoses, which is an autosomal dominant disorder characterized by the formation of a cartilage-capped tumor, caused by mutations in either EXT1 or EXT2 (34). Thus, it has been suggested that EXT1 and EXT2 cannot have redundant roles in the biosynthesis of HS, which is similar to the observation that PAR2.4 (pfc-1)- or ChSy (sqv-5)-RNAi in C. elegans resulted in a reduction of chondroitin and the RNAi-treated worms showed similar phenotypes. Therefore, both PAR2.4 and ChSy have non-redundant functions as EXT1 and EXT2 do. In addition, these observations suggest that the mechanism for the biosynthesis of chondroitin is quite similar to that for HS.

Analytical data on the expression of PAR2.4 and ChSy in C. elegans suggest that both proteins are localized in seam cells, which can act as stem cells to produce neurons in early embryonic lineages, whereas differentiated and fused seam cells are responsible for production of the cuticular structures. PAR2.4 is expressed in such seam cells from the L1 stage through to adulthood (data not shown). In addition, the expression of PAR2.4 was also detected in DTC and vulva from L3 through to the young adult stage. DTC are located at the tip of the growing gonad arms and are required for the formation of the two U-shaped arms of gonads. Various mutant strains defective in the migration of DTC have been isolated and studied extensively. Since the inhibition of chondroitin synthesis by sqv-5 (cChSy) RNAi or deletion mutagenesis of sqv-5 (ChSy) resulted in an abnormal migration of gonad arms (misshapen phenotypes) with low penetrance (3), the synthesis of chondroitin PG with ChPF could be important for the proper migration of DTC. To study the roles of chondroitin PGs in seam cells, distal tip cells, and other unidentified neuronal cells in the nematode, a transgenic overexpression analysis of sqv-5 (cChSy) and ChPF...
with cell-specific promoters is necessary, and it is also important to identify chondroitin PG core proteins in these cells. Studies along these lines are in progress in our laboratory.

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REFERENCES
1. Schwartz, N. B., and Domowicz, M. (2002) Glycobiology 12, 578–688.
2. Sugahara, K., Mikami, T., Uyama, T., Mizuguchi, S., Nomura, K., and Kitagawa, H. (2003) Curr. Opin. Struct. Biol. 13, 612–620.
3. Mizuguchi, S., Uyama, T., Kitagawa, H., Nomura, K. H., Dejima, K., Gengyo-Ando, K., Mitiu, S., Sugahara, K., and Nomura, K. (2003) Nature 423, 443–448.
4. Bradbury, E. J., Moon, L. D. F., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., Fawcett, J. W., and McMahon, S. B. (2002) J. Biol. Chem. 277, 38189–38196.
5. Kitagawa, H., Izumikawa, T., Uyama, T., and Sugahara, K. (2003) J. Biol. Chem. 278, 3072–3078.
6. Kitagawa, H., Uyama, T., and Sugahara, K. (2003) J. Biol. Chem. 278, 30235–30237.
7. Uyama, T., Kitagawa, H., Tamura, J., and Sugahara, K. (2002) J. Biol. Chem. 277, 8841–8846.
8. Gotoh, M., Sato, T., Akashima, K., 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. (2001) J. Biol. Chem. 277, 38189–38196.
9. Uyama, T., Kitagawa, H., Tanaka, Y., Tsuchida, K., Goto, F., Ogawa, T., Lidholt, K., Lindahl, U., and Sugahara, K. (1995) J. Biol. Chem. 270, 22219–22225.
10. Sato, T., Gotoh, M., Kiyohata, K., Akashima, T., Iwasaki, H., Nomura, K., Kameyama, A., Dejima, K., Gengyo-Ando, K., Mitiu, S., Sugahara, K., and Nomura, K. (2003) Nature 423, 443–448.
11. Yada, T., Gotoh, M., Sato, T., Shionyu, M., Go, M., Kaseyama, A., Iwasaki, H., Kikuchi, N., Kwon, Y.-D., Togayachi, A., Kudo, T., Watanabe, H., Kimata, K., and Narimatsu, H. (2003) J. Biol. Chem. 278, 30235–30237.
12. Fire, A., Xu, S., Montgomery, M. K., Costas, S. A., Driver, S. E., and Mello, C. C. (1998) Nature 391, 806–811.
13. Yamada, S., Van Die, I., Van den Eijnden, D. H., Yokota, A., Kitagawa, H., and Sugahara, K. (1999) FEBS Lett. 459, 327–331.
14. Toyoda, H., Kinoshita-Toyoda, A., and Selleck, S. B. (2000) J. Biol. Chem. 275, 2269–2275.
15. Hwang, H.-Y., Olsen, S. K., Esko, J. D., and Horvitz, H. R. (2003) Nature 423, 439–443.
16. Kitagawa, H., Tanaka, Y., Tsuchida, K., Geto, F., Ogawa, T., Lidholt, K., Lindahl, U., and Sugahara, K. (1995) J. Biol. Chem. 270, 22219–22225.
17. Kitagawa, H., Ujikawa, M., Tsutsumi, K., Tamura, J., Neumann, K. W., Ogawa, T., and Sugahara, K. (1997) Glycobiology 7, 905–911.
18. Tamura, J., Miura, Y., and Freeze, H. H. (1999) J. Carbohydr. Chem. 18, 1–14.
19. Kitagawa, H., and Paulsen, J. C. (1994) J. Biol. Chem. 269, 1394–1491.
20. Kitagawa, H., Tsutsumi, K., Ujikawa, M., Geto, F., Tamura, J., Neumann, K. W., Ogawa, T., and Sugahara, K. (1997) Glycobiology 7, 531–537.
21. Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G., and Ahringer, J. (2001) Genome Biol. 2, research0002.1–research0002.10.
22. Timmons, L., Court, D. L., and Fire, A. (2001) Gen. (Amst.) 263, 103–112.
23. Maeda, I., Kohara, Y., Yamamoto, M., and Sugimoto, A. (2001) Curr. Biol. 11, 171–176.
24. Borovko, A. Y., and Rivikin, M. I. (1997) BioTechniques 22, 812–814.
25. Mello, C. C., Karmer, J. M., Stinchcomb, D., and Ambros, V. (1991) EMBO J. 10, 3959–3970.
26. Kozak, M. (1984) Nucleic Acids Res. 12, 857–872.
27. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132.
28. Wiggins, S., and Munro, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7945–7950.
29. Koff, K., and Rothman, J. H. (2001) Development 128, 2867–2880.
30. McCormick, C., Leduc, Y., Martindale, D., Esford, L. E., Dyer, A. P., and Tufaro, F. (1998) Nat. Genet. 19, 158–161.
31. Lind, T., Tufaro, F., McCormick, C., Lindahl, U., and Lidholt, K. (1998) J. Biol. Chem. 273, 20265–20268.
32. Senay, C., Lind, T., Muguruma, K., Tone, Y., Kitagawa, H., Sugahara, K., Lidholt, K., Lindahl, U., and Kusche-Gullberg, M. (2000) EMBO Rep. 1, 252–256.
33. Kim, B.-T., Kitagawa, H., Tanaka, J., Tamura, J., and Sugahara, K. (2003) J. Biol. Chem. 278, 41618–41623.
34. McCormick, C., and Tufaro, F. (1999) Mol. Med. Today 5, 481–486.