rib-2, a Caenorhabditis elegans Homolog of the Human Tumor Suppressor EXT Genes Encodes a Novel α,1,4-N-Acetylglucosaminyltransferase Involved in the Biosynthetic Initiation and Elongation of Heparan Sulfate*

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The proteins encoded by the EXT1, EXT2, and EXTL2 genes, members of the hereditary multiple exostoses gene family of tumor suppressors, are glycosyltransferases required for the heparan sulfate biosynthesis. Only two homologous genes, rib-1 and rib-2, of the mammalian EXT genes were identified in the Caenorhabditis elegans genome. Although heparan sulfate is found in every tissue. They consist of a protein core to which heparan sulfate glycosaminoglycan (GAG)1 chains are attached. Heparan sulfate proteoglycans are distributed on the surfaces of most cells and in the extracellular matrices of virtually every tissue. They consist of a protein core to which heparan sulfate glycosaminoglycan (GAG)1 chains are attached. Heparan sulfate GAGs show a tremendous diversity of structures that are known to play important roles in many biological recognition events of vertebrates and invertebrates, such as cell adhesion, growth factor/cytokine action, and regulation of important signaling pathways (for reviews see Refs. 1 and 2). Their biological activities are believed to be expressed through interactions with various proteins through specific saccharide sequences. These GAGs are synthesized on the so-called GAG-protein linkage region, GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1–O-Ser, attached to specific Ser residues of core proteins, which is common to the GAGs including heparin/heparan sulfate and chondroitin sulfates/dermatan sulfate (for reviews see Refs. 3 and 4). The linkage region synthesis is initiated by the addition of Xyl to Ser followed by the addition of two Gal residues and is completed by the addition of GlcUA, each reaction being catalyzed by the respective specific glycosyltransferase (3, 4). The GAGs are built up on this linkage region by the alternate addition of N-acetylgalactosamine and GlcUA residues. Heparin/heparan sulfate is synthesized once GalNAc is transferred to the common linkage region, whereas chondroitin sulfate/dermatan sulfate is formed if GalNAc is first added. However, the biosynthetic sorting mechanisms for different GAG chains remain enigmatic.

Recent cDNA cloning of the glycosyltransferases involved in the GAG biosynthesis revealed that the heparan/heparan sulfate biosynthesis in mammals is associated with the EXT gene family, the hereditary multiple exostoses (HME) gene family of tumor suppressors (for a review see Ref. 5). HME is an autosomal dominant disorder characterized by cartilage-capped skeletal excretions, which may lead to skeletal abnormalities and short stature (6). Although the exostoses represent osteochondromas that are benign bone tumors, malignant transformation into chondrosarcomas or osteosarcomas occurs in ~2% of HME patients (6, 7). Genetic linkage of this disorder has been ascribed to three independent loci on chromosomes 8q24.1 (EXT1), 11p11–13 (EXT2), and 19p (EXT3) (8–10). This family of EXT genes has recently been extended by the identification of three additional EXT-like genes, EXT1L, EXT2L, and EXT3L (11–14). Exostoses-derived and sporadic chondrosarcomas are attributable to the loss of heterozygosity for the markers in EXT1 and EXT2 loci (15, 16), indicating that the EXT genes may encode tumor suppressors. It has been demonstrated that both mammalian EXT1 and EXT2 proteins are the
heparan sulfate copolymerases that polymerize GlcUA and GlcNAc alternately (17–19), and the human EXT2 protein is GlcNAc transferase I (20), which determines and initiates the heparan sulfate synthesis on the common GAG-protein linkage region (21).

Interestingly, the EXT gene family has also been identified in invertebrate genomes such as the nematode worm Caenorhabditis elegans (22) or the fruit fly Drosophila melanogaster (23), suggesting the presence of heparan sulfate in these organisms. In fact, heparan sulfate is found in invertebrates, including C. elegans and Drosophila. Recent studies have shown that both of these genetically tractable organisms make heparan sulfate and chondroitin or chondroitin 4-sulfate (24–26). Although analysis of mutants defective in the Drosophila EXT1 homolog, ttt, has provided some evidence that Ttv might also be a heparan sulfate polymerase (25), glycosyltransferase activities have not yet been reported for these invertebrate EXT family proteins. A data base search revealed only the two homologous genes, rib-1 and rib-2, of the mammalian EXT genes in the C. elegans genome (22). Notably, however, no C. elegans homolog of the mammalian EXT2 gene encoding GlcNAc transferase I required for the chain initiation of heparan sulfate was found. These observations, therefore, suggested that the biosynthetic mechanism of heparan sulfate in C. elegans might be distinct from that reported for mammals. In the present study, to clarify the involvement of the rib-2 protein in heparan sulfate biosynthesis, the substrate specificity of a soluble recombinant form of the rib-2 protein was determined and compared with those of the recombinant forms of the mammalian EXT1, EXT2, and EXT2I proteins. The findings demonstrated that the rib-2 protein was a novel and unique α,1,4-N-acetylgalcosaminyltransferase involved in the biosynthetic initiation and elongation of heparan sulfate.

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-[U-14C]GlcUA (285.2 mCi/mmol), UDP-[3H]GlcNAc (60 Ci/mmol), and UDP-[3H]GalNAc (10 Ci/mmol) were purchased from PerkinElmer Life Sciences. Unlabeled UDP-GlcUA, UDP-GlcNAc, and UDP-GalNAc were obtained from Sigma. *Flavobacterium heparinum* heparitinase I and Jack bean β-N-acetylgalactosaminidase were purchased from Seikagaku Corp. (Tokyo, Japan). The chemically synthesized linkage tetrasaccharide- and trisaccharide-glucosamine (GlcNAc) was purchased from Fujii Biochemicals Co., Ltd. (Osaka, Japan). Linkage tetrasaccharide- and trisaccharide-serine GlcNAc and Ser (27) and linkage pentasaccharide-serine GlcNAc-Ser (27) were provided by T. Ogawa (RIKEN, Institute of Physical and Chemical Research, Saitama, Japan). GlcUAβ1–3Galβ1–4Xylα1–O-Ser (27) and linkage pentasaccharide-serine GlcNAc-Ser (27) were provided by T. Ogawa (RIKEN, Institute of Physical and Chemical Research, Saitama, Japan). GlcUAβ1–3Galβ1–4Xylα1–O-Ser (27) and linkage pentasaccharide-serine GlcNAc-Ser (27) were provided by T. Ogawa (RIKEN, Institute of Physical and Chemical Research, Saitama, Japan). GlcUAβ1–3Galβ1–4Xylα1–O-Ser (27) and linkage pentasaccharide-serine GlcNAc-Ser (27) were provided by T. Ogawa (RIKEN, Institute of Physical and Chemical Research, Saitama, Japan). GlcUAβ1–3Galβ1–4Xylα1–O-Ser (27) and linkage pentasaccharide-serine GlcNAc-Ser (27) were provided by T. Ogawa (RIKEN, Institute of Physical and Chemical Research, Saitama, Japan). GlcUAβ1–3Galβ1–4Xylα1–O-Ser (27) and linkage pentasaccharide-serine GlcNAc-Ser (27) were provided by T. Ogawa (RIKEN, Institute of Physical and Chemical Research, Saitama, Japan).

**Construction of Expression Vectors Encoding Soluble Forms of the Rib-2, EXT-1, EXT-2, and EXT2 Proteins**—The cDNA fragment encoding a truncated form of the rib-2 protein was determined and compared with those of the recombinant forms of the mammalian EXT1, EXT2, and EXT2I proteins. The findings demonstrated that the rib-2 protein was a novel and unique α,1,4-N-acetylgalcosaminyltransferase involved in the biosynthetic initiation and elongation of heparan sulfate.

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acetate buffer, pH 7.0, containing 2 mM calcium acetate at 37 °C overnight. The enzyme digest was analyzed using the same Nova-Pak® C18 column as described above.

RESULTS

Rib-2 Expressed in COS-1 Cells Generates GlcNAc Transferase Activities for Heparan Sulfate Synthesis—C. elegans rib-2 encodes a protein with homology to the human EXT family members, especially to the EXT2 and EXTL2 proteins (see “Discussion”). In view of the recent findings that EXT2 is a heparan sulfate-polymerase required for the heparan sulfate biosynthesis (17, 19), it was important to clarify the involvement of rib-2 in the heparan sulfate biosynthesis in C. elegans. The rib-2 sequence indicated an open reading frame of 2442 bp coding for a protein of 814 amino acids, with eight potential N-glycosylation sites (22) and a type II transmembrane protein topology characteristic of many glycosyltransferases cloned to date. A soluble form of the protein encoded by rib-2 cDNA was generated by replacing the first 58 amino acids of rib-2 with the cleavable insulin signal sequence and the IgG-binding domain of protein A as described under “Experimental Procedures.” When the expression plasmid containing the rib-2-protein A fusion was expressed in COS-1 cells, an approximate 130-kDa fusion was expressed in COS-1 cells, an approximate 130-kDa protein was secreted into the culture medium. When the expression plasmid containing the rib-2/protein A fusion was transformed into COS-1 cells, an approximate 130-kDa protein was expressed, which was not detected with the Western blotting using IgG data not shown). The apparent Mr of the fused protein was reduced to about 110-kDa after N-glycosidase treatment (data not shown), indicating that a few potential N-linked glycosylation sites of rib-2 were utilized.

The fused enzyme expressed in the medium was absorbed on IgG-Sepharose beads to eliminate endogenous glycosyltransferases and then the enzyme-bound beads were used as an enzyme source for further studies. The bound fusion protein was assayed for GlcUA transferase or GlcNAc transferase activities involved in the heparan sulfate biosynthesis using a variety of acceptor substrates. There is no known C. elegans homolog of the human EXTL2 gene encoding GlcNAc transferase I required for the chain initiation of heparan sulfate (see “Introduction”), which suggested that rib-2 may also have GlcNAc transferase I activity. Hence, the purified fusion protein was also assayed for GlcNAc transferase I activity, in addition to the GlcUA and GlcNAc transferase activities for the polymerization reactions. GlcUAβ1–3Galβ1–O-C4H4NHCbz used as an acceptor substrate for the GlcNAc transferase I reaction shares the disaccharide sequence with the GAG-protein linkage region tetrasaccharide. It served as a good acceptor for the EXT2 protein (Table I) and was comparable with GlcUAβ1–3Galβ1–O-naphthalenemethanol, an artificial, yet authentic oligosaccharide acceptor substrate for GlcNAc transferase I. As shown in Table I, the marked GlcUA transferase activity was detected with N-acetylated glucosaminoglycans GlcUAβ1–4GlcNAcβ1–4GlcUAβ1–1 and GlcUAβ1–3Galβ1–O-C3H3NHCbz but not with the tetrasaccharide-serine GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1–O-Ser as acceptor substrates, whereas no GlcUA transferase activity was observed using N-acetylated glucosaminoglycans GlcNAcα1–4GlcUAβ1–1 and the pentasaccharide-serine GlcNAcα1–4GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1–O-Ser. No detectable GlcNAc transferase activity was recovered by the affinity purification from the control pEF-BOS transfection sample, and it therefore seems unlikely that the results are due to an artifact or an endogenous activity.

To identify these GlcNAc transferase reaction products, the acceptor substrates, N-acetylated glucosaminoglycans GlcUAβ1–4GlcNAcα1–4GlcUAβ1–1 and GlcUAβ1–3Galβ1–O-C4H4NHCbz, were individually labeled by the respective transferase reaction using UDP-[3H]GlcNAc as a donor substrate and the enzyme-bound beads as an enzyme source. Both labeled products were completely digested by heparitinase I, which cleaves an α,4-N-acetylgalactosaminidic linkage in an eliminative fashion, quantitatively yielding a [3H]-labeled peak at the elution position of free [3H]GlcNAc, as demonstrated by gel filtration (Fig. 1A) or hydrophobic HPLC (Fig. 1B). In contrast, they were inert to the action of β-N-acetylhexasaminidase. These findings clearly indicated that a GlcNAc residue had been transferred exclusively to the nonreducing terminal GlcUA of N-acetylated glucosaminoglycans or GlcUAβ1–3Galβ1–O-C4H4NHCbz through an α,4 linkage. The present findings demonstrated that rib-2 was a novel and unique α,4-GlcNAc transferase involved in the biosynthetic initiation and elongation of heparan sulfate.
DISCUSSION

Although heparan sulfate is found in C. elegans, it was unknown which enzyme proteins were involved in the heparan sulfate biosynthesis (24, 25). In the present study, we demonstrated that the C. elegans rib-2 protein was a unique α1,4-GlcNAc transferase involved in the biosynthetic initiation and elongation of heparan sulfate. As shown in Table I, rib-2, characterized in the present study, exhibited distinct but overlapp
proteins. More efforts to detect the GlcUA transferase activity for the rib-1 and rib-2 proteins are required.

Nevertheless, the findings from the present study suggest that the biosynthetic mechanism of heparan sulfate in C. elegans is distinct from that in mammals. In mammals, it was demonstrated that EXT1 and EXT2 are both GlcUA/GlcNAC-cotransferases that need to interact with each other to form an active heparan sulfate polymerase (18, 19). Coexpression of the two proteins, but not mixing of separately expressed recombinant EXT1 and EXT2, yields hetero-oligomeric complexes in mammalian cells, with augmented glycosylation activities (18, 19). The available information suggests that neither of the two cotransferases can substitute for the other. For example, t-cell or Chinese hamster ovary cell mutants deficient in EXT1 were unable to synthesize heparan sulfate even after transfection with EXT2 (18, 33). Moreover, mouse embryonic stem cells derived from EXT1 homozygous null embryos markedly reduced the formation of heparan sulfate, even though the cells contained an EXT2 ortholog (35). Thus, although it is obvious that EXT1 has GlcUA/GlcNAC transferase activities and that it functions in vivo in heparan sulfate formation, the role of EXT2 is unclear given its low activity and the lack of mutants. In addition, 1–2% contamination by EXT1 could easily account for the low level of activity seen for EXT2 in mammalian systems (Refs. 17 and 19; also see Table I) given that the enzymes interact. We have demonstrated that EXT2 encodes GlcNAC transferase I that determines and initiates the heparan sulfate synthesis (20) but have found no EXT homolog that encodes only GlcNAC transferase I or GlcUA/GlcNAC-cotransferase in C. elegans as shown in the present study. Moreover, coexpression of the rib-1 and rib-2 proteins did not result in the promotion of the GlcNAC transferase activities or the expression of the GlcUA transferase activity required for heparan sulfate polymerization (data not shown). Although the possibility cannot be ruled out that nonhomologous genes to the EXT gene family may encode GlcUA/GlcNAC-cotransferases, these findings suggest that the biosynthetic mechanism of heparan sulfate in C. elegans is distinct from that in mammals.

Recent studies have demonstrated a critical role for heparan sulfate in growth factor signaling mediated by Wingless proteins during Drosophila development (2). In addition, a Drosophila homolog of EXT1 (encoded by itt) was recently implicated in the Hedgehog diffusion (2). C. elegans is also a genetically tractable organism ideally suited for the analysis of glycans function during tissue assembly and morphogenesis. In fact, three proteins required for wild-type vulval invagination in C. elegans, which are encoded by sqv-3, -7, and -8 (squashed vulva), respectively, have been reported to be similar to components of a glycosylation pathway (36). sqv-3, -7, and -8 are three of the eight genes in the sqv class, identified on the basis of a common vulval invagination defect. Recently, Bulik et al. (26) showed that sqv-3, -7, and -8 mutants all affected the biosynthesis of GAGs and that sqv-3 and -8 genes encoded enzymes with Gal transferase I and GlcUA transferase I activity, respectively. Because both enzymes are required for the synthesis of the GAG-protein linkage tetrasaccharide common to all GAGs, including chondroitin sulfate and heparan sulfate (37–39), it is reasonable that sqv-3 and -8 mutants showed significantly reduced levels of both chondroitin and heparan sulfate (26). Thus, if rib-2 mutants become available, they would be useful tools for determining which GAG, heparan sulfate or chondroitin, is required for C. elegans vulval invagination.

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