We previously demonstrated a unique α-N-acetyl-
galactosaminyltransferase that transferred N-acetyl-
galactosamine (GalNAc) to the tetrasaccharide-serine,
GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–O-Ser (GlcA represents
glucuronic acid), derived from the common glycosamin-
glycan-protein linkage region, through an α,4-linkage.
In this study, we purified the enzyme from the serum-
free culture medium of a human sarcoma cell line. Pept-
tide sequence analysis of the purified enzyme revealed
100% identity to the multiple exostoses-like gene
EXTL2/EXTR2, a member of the hereditary multiple exostoses
(EXT) gene family of tumor suppressors. The expression of
a soluble recombinant form of the protein produced
an active enzyme, which transferred α-GalNAc from
UDP-[3H]GalNAc to various acceptor substrates includ-
ing GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–O-Ser. Interestingly,
the enzyme also catalyzed the transfer of N-acetylglu-
cosamine (GlcNAc) from UDP-[3H]GlcNAc to GlcAβ1–
3Galβ1–O-naphthalenemethanol, which was the accep-
tor substrate for the previously described GlcNAc
transferase I involved in the biosynthetic initiation of
heparan sulfate. The GlcNAc transferase reaction prod-
uct was sensitive to the action of heparitinase I, estab-
lishing the identity of the enzyme to be α1,4-GlcNAc
transferase. These results altogether indicate that
EXTL2/EXTR2 encodes the α1,4-N-acetylgalactosaminyl-
transferase that transfers GalNAc/GlcNAc to the tet-
rasaccharide representing the common glycosaminogly-
can-protein linkage region and that is most likely
the critical enzyme that determines and initiates the hepa-
rin/heparan sulfate synthesis, separating it from the
chondroitin sulfate/dermatan sulfate synthesis.

Sulfated glycosaminoglycans (GAGs), including heparin/
heparan sulfate and chondroitin sulfate/dermatan sulfate, are
distributed on the surfaces of most cells and in the extracellu-
lar matrices of virtually every tissue. They are implicated in
the regulation and maintenance of cell proliferation, cytodi-
fferentiation, and tissue morphogenesis, exhibiting their biological
activities by interacting with various proteins through specific
saccharide sequences. They are synthesized as proteoglycans,
on specific Ser residues in the so-called GAG-protein linkage
region, GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–O-Ser, which is com-
mon to the GAGs (for reviews, see Refs. 1 and 2). The linkage
region synthesis is initiated by the addition of Xyl to Ser fol-
lowed by the addition of two Gal residues and is completed by
the addition of GlcA, each reaction being catalyzed by a specific
glycosyltransferase (1, 2). The GAGs are built up on this link-
age region by the alternating addition of N-acetylgalactosamine
and GlcA residues. Heparin/heparan sulfate is synthesized
once GlcNAc is transferred to the common linkage region,
whereas chondroitin sulfate/dermatan sulfate is formed if Gal-
NAc is first added. However, biosynthetic sorting mechanisms
of different GAG chains remain enigmatic. Although at least
eight different kinds of glycosyltransferase reactions are re-
quired to synthesize these GAGs, only the GlcA transferase
that completes the tetrasaccharide linkage region and the
heparan sulfate-polymerase that polymerizes GlcA and GlcNAc
have been cloned (3, 4).

Recent cDNA cloning of the latter enzyme of bovine origin
revealed its 94% sequence identity to human EXT31, a member
of the hereditary multiple exostoses (EXT) gene family of tumor
suppressors (4). EXT is an autosomal dominant disorder char-
acterized by cartilage-capped skeletal excrences, which may
lead to skeletal abnormalities and short stature (5). Although
the exostoses represent osteochondromas that are benign bone
tumors, malignant transformation into chondrosarcomas or os-
teoarcoma occurs in approximately 2% of EXT patients (5, 6).
Genetic linkage of this disorder has been ascribed to three
independent loci on chromosomes 8q24.1 (EXT1), 11p11–13
(EXT2), and 19p (EXT3) (7–9). This family of EXT genes has
recently been extended by the identification of three additional
EXT-like genes, EXT1L, EXT2L/EXTR2, and EXT3L/EXTR1
(10–13). Sporadic and exostoses-derived chondrosarcomas are
attributable to the loss of heterozygosity for the markers in
EXT1 and EXT2 loci (14, 15), indicating that the genes respon-
sible for EXTs and the EXT-like genes may encode tumor
suppressors.

While searching for the key enzyme involved in biosynthetic
sorting of chondroitin sulfate/dermatan sulfate from heparin/

GlcA, d-glucuronic acid; HPLC, high-performance liquid chromatogra-
phy; PCR, polymerase chain reaction.
products from the α-GalNacT reaction using N-acetylated chondroside as an acceptor was carried out by gel filtration on a Superdex 30 column (Amersham Pharmacia Biotech) equilibrated with 0.25 M NH₄HCO₃, 7% 1-propanol. The radioactive peak containing the product was pooled and evaporated to dryness. The isolated product (about 50 pmol) was digested with 15 mU of β-N-acetylgalactosaminidase or 39 mU of α-N-acetylgalactosaminidase in a total volume of 20 μl of 50 mM sodium citrate buffer, pH 4.5, respectively, at 37 °C overnight. The enzyme digest was analyzed using the same Superdex 30 column as that noted above.

The isolation of the products from the GlcNAcT-I reaction using GlcAlα1–3Galβ1–O-naphthalenemethanol was performed by HPLC on a Nova-Pak® C₁₈ column (3.9 × 150 mm; Waters, Tokyo, Japan) in an LC-10AS system (Shimadzu Co., Kyoto, Japan). The column was developed isocratically for 15 min with H₂O at a flow rate of 1 ml/min at room temperature; thereafter, a linear gradient was applied to increase the methanol concentration from 0 to 100% over a 5-min period, and the column was then developed isocratically for 40 min with 100% methanol. The radioactive peak containing the product was pooled and evaporated to dryness. The isolated product (about 74 pmol) was incubated with 14 mIU of β-N-acetylgalactosaminidase in a total volume of 20 μl of 50 mM sodium citrate buffer, pH 4.5, or with 3 mIU of heparitinase I for testing the digestability in a total volume of 30 μl of 20 mM sodium acetate buffer, pH 7.0, containing 2 mM Ca(OAc)₂ at 37 °C overnight. The enzyme digest was analyzed using the same Nova-Pak® C₁₈ column as that noted above.

Expression Levels of the Enzyme in Human Tissues—Human Multiple Tissue cDNA Panels (CLONTECH) were used for the analysis. The manufacturer normalizes each cDNA sample against six housekeeping genes. To verify this, we determined the levels of amplification of the glyceraldehyde-3-phosphate dehydrogenase, whose transcript is always present in the tissues at a constant level. The amplification reaction was carried out in a total volume of 50 μl using the 5′ primer, 5′-ACCACTGTCCTAGGCAACTACAG-3′, and the 3′ primer, 5′-TCCACAA-CACGTTTCTGTA-3′, by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s. A 10-μl aliquot of the amplified products was visualized by electrophoresis on a 1.0% agarose gel containing ethidium bromide. Using the normalized cDNA input, we then performed the amplification of a transcript, using a serial number of cycles (25-30-35) to find the conditions for a semiquantitative amplification. The best results were obtained by carrying out 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 75 s using the 5′ and 3′ primers described above, which were designed to span the two introns in the EXT2 gene (10) to discriminate a PCR product amplified from cDNA from, if any, one amplified from contaminating genomic DNA. PCR products were then visualized by electrophoresis on a 1.0% agarose gel containing ethidium bromide. To confirm that the amplified DNAs were derived from the EXT2 mRNA, the amplified fragments were gel-purified, subcloned into the pGEM®-T Easy vector (Promega, Madison, WI), and sequenced. The nucleotide sequences of the amplified DNAs were identical to that of the human EXT2 DNA (10) (data not shown).

RESULTS

The Enzyme Is Encoded by a Tumor Suppressor-like Gene—α-GalNacT was purified from the serum-free culture medium of a human sarcoma (malignant fibrous histiocytoma) cell line to near homogeneity mainly by affinity chromatographies on heparin-Sepharose and UDP-GalNAc-Sepharose. Upon SDS-polyacrylamide gel electrophoresis under reducing conditions, the purified α-GalNacT preparation gave a major protein band of 66 kDa (data not shown), which was purified, subjected to NH₂-terminal amino acid sequence analysis. The obtained sequence of thirty amino acid residues (Fig. 1) was 100% identical to that of residues 54–83 of the protein encoded by the EXT2/EXTR2 except for the unidentified amino acid corresponding to the N-glycosylation site (10, 11), as demonstrated

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* H. Shimakawa, Y. Kano, H. Kitagawa, H. Okabe, and K. Sugahara, manuscript in preparation.
The expression plasmid containing the EXTL2/protein A fusion was expressed in COS-1 cells, as has been observed for several other glycosyltransferases (23).

The Expressed Enzyme Is an Active α-GalNac Transferase—A soluble form of the protein encoded by EXT2/EXTR2 cDNA was generated by replacing the first 57 amino acids of EXT2/EXTR2 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 EXTL2/protein A fusion was expressed in COS-1 cells, an approximately 66-kDa protein was secreted (data not shown). The apparent molecular mass of the fused protein was reduced to 60-kDa after N-glycosidase treatment (data not shown), indicating that the one potential N-linked glycosylation site of EXT2/EXTR2 is being 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 α-GalNacT activity using a variety of acceptor substrates. As shown in Table I, marked glycosyltransferase activity was detected with N-acetylgalactosamine (GlcAβ1–3GlcNAc), GlcAβ1–3Galβ1–O-naphthalenemethanol, and the tetrasaccharide-serine representing the GAG-protein linkage region as the acceptor substrates. In addition, no detectable α-GalNacT activity was recovered by the affinity purification from the control pSVL transfection sample. To identify the α-GalNacT reaction products, N-acetylgalactosamine was labeled by an enzyme reaction using UDP-[3H]GlcNAc as a donor substrate and the enzyme-bound beads as an enzyme source. The labeled products were completely digested by heparitinase I that cleaves an α-1,4-glycosaminide 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. 2A). These results indicated that a GalNAc residue had been transferred to N-acetylgalactosamine through an α-linkage and that the expressed protein was α,1,4-GalNAc transferase.

**TABLE I**

| Acceptor                      | α-GalNAcT activity | GlcNAcT-I activity |
|-------------------------------|-------------------|-------------------|
| GlcAβ1–3Galβ1–O-naphthalenemethanol | 109               | 31                |
| GlcAβ1–3Galβ1–3Galβ1–4Xyβ1–O-Ser | 50                | ND                |
| Glcβ1–3GalNAc                 | 117               | —                 |

a The values represent the averages of two independent experiments.

b ND, not detected (<0.1 pmol/ml medium/h).

c —, not determined.

The reaction product has not been found in naturally occurring GAG chains, which prompted us to hypothesize that the enzyme may have an α-GlcNAc transsterase activity toward the linkage region structure taking into account the tetrasaccharide structure of the acceptor substrate for the α-GalNacT activity. Hence, the purified fusion protein was assayed for GlcNAc transferase activity using UDP-[3H]GlcNAc as a sugar donor and two oligosaccharide acceptor substrates whose structures represent the GAG-protein linkage region. As shown in Table I, a significant GlcNAc transferase activity was detected with GlcAβ1–3Galβ1–O-naphthalenemethanol as an acceptor, whereas no activity was detected using the linkage tetrasaccharide-serine. The observed substrate specificity was consistent with that reported for GlcNAcT-I (17, 20), which is involved in the heparan sulfate biosynthesis. No detectable GlcNAc transferase activity was recovered by affinity purification from a control pSVL transfection sample, excluding the possibility of an artifact or of an endogenous origin of the activity. To identify the GlcNAc transferase reaction products, GlcAβ1–3Galβ1–O-naphthalenemethanol was labeled with [3H]GlcNAc using the enzyme bound to beads. The labeled products were completely digested by heparitinase I that cleaves an α,1,4-glycosaminide linkage in an eliminative fashion, quantitatively yielding a [3H]-labeled peak at the elution position of free [3H]GlcNAc, as demonstrated by HPLC (Fig. 2B), whereas they were inert to the action of β-N-acetylgalactosaminidase. These results clearly indicated that a GlcNAc residue had been transferred exclusively to the nonreducing terminal GlcA of GlcAβ1–3Galβ1–O-naphthalenemethanol through an α,1,4 linkage. Taken together, the present findings demonstrated that EXT2/EXTR2 is an α1,4-
N-acetylhexosaminyltransferase that transfers GalNAc/GlcNAc to the artificial yet authentic oligosaccharide acceptor substrate for GlcNAcT-I (20). The failure of the tetrasaccharide-serine derived from the GAG-protein linkage region to serve as an acceptor (Table I) is discussed below.

Ubiquitous Expression of the Gene in Human Tissues—To screen tissue expression of the α1,4-N-acetylhexosaminyltransferase, we used PCR-based methods with normalized cDNA pools. A single amplified DNA of the expected size (875 base pairs) was obtained from each cDNA preparation of the 18 adult and 8 fetal human tissues examined, although the amounts of the amplified cDNAs varied (Fig. 3), indicating that the gene was ubiquitously expressed.

DISCUSSION

In the present study, we demonstrated that EXTL2/EXTR2 encoded enzyme with a dual catalytic activity of α-GalNAcT and GlcNAcT-I, i.e. an α1,4-N-acetylhexosaminyltransferase that transferred GalNAc/GlcNAc to the core oligosaccharide representing the GAG-protein linkage region. Thus, the enzyme turned out to be identical to the previously described GlcNAcT-I that determines and initiates the biosynthesis of heparan sulfate (20) and most likely heparin as well. The gene, like other EXT gene family members, was found ubiquitously expressed in virtually every human tissue examined (Fig. 3), which is in accordance with the observations that heparan sulfate proteoglycans are distributed on the surfaces of most cells and the extracellular matrices in virtually every tissue. In view of the present findings of the involvement of EXTL2/EXTR2 in the heparan sulfate biosynthesis together with those of Lind et al. (4), who recently reported that EXT1 and EXT2 both encoded a heparan sulfate-polymerase required for the heparan sulfate biosynthesis, the expression of heparan sulfate seems to play an important role in the tumor suppressor function although the precise mechanism remains unclear.

The initial characterization of crude GlcNAcT-I preparations showed that the enzyme exhibited strict specificity toward GlcAβ1–3Galβ1-O-naphthalenemethanol (20), GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-O-naphthalenemethanol, or GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–O-benzyl (24). Neither N-acetylatedarosan (4GlcAβ1–4GlcNAcα1–), nor the tetrasaccharide-serine, GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-O-Ser derived from the linkage region, was utilized as an acceptor substrate (17, 20).

Hence, it was suggested that the transfer of the first GlcNAc residue to the linkage tetrasaccharide primer is mediated by GlcNAcT-I, distinct from the enzyme that has been termed heparan sulfate-polymerase involved in the formation of the repeating disaccharide units of heparan sulfate (20) and that GlcNAcT-I directly recognizes a specific sequence in the core protein or an aglycone structure attached to the linkage tetrasaccharide (24). These hypotheses have now been proven by the molecular identification and characterization of both enzymes in a recent study (4) and in the present study. The molecular similarity of the two enzymes is consistent with the fact that both enzymes have α1,4-GlcNAc transferase activities and recognize the terminal β-GlcA moiety of their acceptor substrates.

The enzyme protein of the α1,4-N-acetylhexosaminyltransferase composed of 330 amino acids is about half the size of the other EXT family members that have 676–919 amino acids. The variation in size is due to differences on the amino-terminal side of the protein. The protein shows significant homology with the carboxyl termini of the other members of the family.
recently implicated in the Hedgehog diffusion and the formation of embryonic dorsal-ventral polarity, respectively (28, 29). Thus, considering the probability that deletion of the gene would cause the complete elimination of heparan sulfate and heparin unless functional redundancy with other genes exists, it is likely that germ line mutations inactivating the enzymatic activity result in embryonic lethality and that somatic mutations cause much more serious defects than those caused by EXT1 and EXT2, leading to the progression of various tumors or to lethal disorders. In fact, congenital deficiency in heparan sulfate even only in enterocytes results in severe clinical problems and eventually death (30).

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REFERENCES

1. Lindahl, U., and Rodén, L. (1972) in Glycoproteins (Gottschalk, A., ed) pp. 491–517, Elsevier Science Publishing Co., Inc., New York
2. Rodén, L. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed) pp. 267–371, Plenum Publishing, New York
3. Kitagawa, H., Tone, Y., Tamura, J., Neumann, K., Ogawa, T., Oka, S., Kawanishi, T., and Sugahara, K. (1998) J. Biol. Chem. 273, 6615–6618
4. Lind, T., Tufaro, F., McCormick, C., Lindahl, U., and Lidholt, K. (1998) J. Biol. Chem. 273, 26265–26268
5. Solomon, L. (1984) Am. J. Hum. Genet. 16, 351–363
6. Schmale, G. A., Conrad, E. U., and Raskind, W. H. (1994) J. Bone Jt. Surg. 76, 986–992
7. Cook, A., Raskind, W., Blanton, S. H., Pauli, R. M., Gregg, R. G., Francemano, C. A., Puffenberger, E., Conrad, E. U., Schmale, G., Schellenberg, G., Wijman, E., Hecht, J. T., Wells, D., and Wagner, M. J. (1993) Am. J. Hum. Genet. 53, 71–79
8. Wu, Y., Heutink, P., de Vries, B., Sandkuijl, L. A., van den Ouweland, A. M. W., Niermeijer, M. F., Galjaard, H., Reyniers, E., Willems, P. J., and Halley, D. J. J. (1994) Hum. Mol. Genet. 3, 167–171
9. Le Merrer, M., Leggeai-Mallet, L., Jeannin, P. M., Horsthemke, B., Schinzel, A., Plachu, H., Toutain, A., Achard, F., Munnuch, A., and Maroteaux, P. (1994) Hum. Mol. Genet. 3, 717–722
10. Wuyts, W., Van Hul, W., Hendrickx, J., Speleman, F., Wauters, J., De Bouille, K., Van Roy, N., Van Agtmael, T., Bossuyt, P., and Willems, P. J. (1997) Eur. J. Hum. Genet. 5, 382–389
11. Saito, S., Seki, N., Yamauchi, M., Tsuji, S., Hayashi, A., Kozuma, S., and Hori, T. (1998) Biochem. Biophys. Res. Commun. 243, 61–66
12. Wise, C. A., Clines, G. A., Massa, H., Trask, B. J., and Lovett, M. (1997) Genome Res. 7, 10–16
13. Van Hul, W., Wuyts, W., Hendrickx, J., Speleman, F., Wauters, J., De Bouille, K., Van Roy, N., Bossuyt, P., and Willems, P. J. (1998) Genomics 47, 230–237
14. Hecht, J. T., Hogue, D., Strong, L. C., Hansen, M. F., Blanton, S. H., and Wagner, M. (1995) Am. J. Hum. Genet. 56, 1125–1131
15. Raskind, W. H., Conrad, E. U., Chansky, H., and Matsushita, M. (1995) Am. J. Hum. Genet. 56, 1122–1129
16. Kitagawa, H., Tanaka, Y., Tsuchida, K., Goto, F., Ogawa, T., Lidholt, K., Lindahl, U., and Sugahara, K. (1995) J. Biol. Chem. 270, 22190–22195
17. Lidholt, K., Fjedstad, M., Lindahl, U., Goto, F., Ogawa, T., Kitagawa, H., and Sugahara, K. (1997) Glycobiology 14, 737–742
18. Miura, Y., and Freeze, H. H. (1998) Glycobiology 8, 813–819
19. Kitagawa, H., Kano, Y., Shimakawa, H., Goto, F., Ogawa, T., Okabe, H., and Sugahara, K. (1999) Glycobiology in press
20. Fritz, T. A., Gubb, M. M., Wei, G., and Esko, J. D. (1994) J. Biol. Chem. 269, 28809–28814
21. Goto, F., and Ogawa, T. (1993) Pure Appl. Chem. 65, 793–801
22. Kitagawa, H., and Paulson, J. C. (1994) J. Biol. Chem. 269, 1394–1401
23. Paulson, J. C., and Colley, K. J. (1989) J. Biol. Chem. 264, 17615–17618
24. Fritz, T. A., Agrawal, P. K., Esko, J. D., and Krishna, N. R. (1997) Glycobiology 7, 587–595
25. Kitagawa, H., Ujikawa, M., Tsutsumi, K., Tamura, J., Neumann, K. W., Ogawa, T., and Sugahara, K. (1997) Glycobiology 7, 905–911
26. Jayson, G. C., Lyon, M., Paraskeva, C., Turnbull, J. E., Deakin, J. A., and Gallagher, J. T. (1998) J. Biol. Chem. 273, 51–57
27. Binari, R. C., Staveley, B. E., Johnson, W. A., Godavarti, R., Sasisekharan, R., and Manoukian, A. S. (1997) Development (Camb.) 124, 2623–2632
28. Bellaiche, Y., The, I., and Perrimon, N. (1998) Nature 394, 85–88
29. Sen, J., Gottz, J. S., Stevens, L., and Stein, D. (1998) Cell 95, 471–481
30. Murch, S. H., Winyard, P. J. D., Koletzko, S., Wehner, B., Cheema, H. A., Risdon, R. A., Philips, A. D., Meadows, N., Klein, N. J., and Walker-Smith, J. A. (1996) Lancet 347, 1299–1301

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