Human Homolog of \textit{Caenorhabditis elegans sqv-3} Gene Is Galactosyltransferase I Involved in the Biosynthesis of the Glycosaminoglycan-Protein Linkage Region of Proteoglycans

(Received for publication, April 20, 1999, and in revised form, June 7, 1999)

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A cDNA encoding a novel galactosyltransferase was identified based on BLAST analysis of expressed sequence tags, and the cDNA clones were isolated from a human melanoma line library. The new cDNA sequence encoded a type II membrane protein with 327 amino acid sequence and showed 38% homology to the \textit{Caenorhabditis elegans sqv-3} gene involved in the vulval invagination and oocyte development. Extracts from L cells transfected with the galactosyltransferase cDNA in an expression vector and a fusion protein with protein A exhibited marked galactosyltransferase activity specific for \(p\)-nitrophenyl-\(\beta\)-xlylopyranoside. Moreover, transfection with the cloned cDNA restored glycosaminoglycan synthesis of galactosyltransferase I-deficient Chinese hamster ovary mutant pgsB-761 cells. Analysis of the enzyme product by \(\beta\)-galactosidase digestion, mass spectroscopy, and NMR spectroscopy revealed that the reaction product was formed via \(\beta\)-1,4 linkage, indicating that the enzyme is galactosyltransferase I (UDP-galactose: \(\beta\)-\(\beta\)-xlyosylprotein 4-\(\beta\)-\(\beta\)-galactosyltransferase, EC 2.4.1.133) involved in the synthesis of the glycosaminoglycan-protein linkage region of proteoglycans.

Proteoglycans are polyamionic molecules consisting of different core proteins and different types, numbers, and length of glycosaminoglycans (GAGs) and are present not only on the cell surface but also in the extracellular matrices of various tissues. A wide variety of proteoglycans containing characteristic sulfated GAG chains are generated in a cell type-specific manner, and their strictly regulated expression patterns have suggested their roles in the regulation of cell proliferation/differentiation, tissue development and organogenesis, and infections.

The biosynthesis of the sulfated GAGs on proteoglycans is initiated by the addition of Xyl to Ser residues in the core proteins; and then the addition of two Gal residues and a GlcA residue subsequently takes place. Alternatively, the addition of GlcNAc or GalNAc residues to the common linkage structure leads to the formation of heparin/heparan sulfate or that of chondroitin sulfate/dermatan sulfate, respectively. The sequential transfer of individual sugars has been considered to be catalyzed by specific glycosyltransferases, and some glycosyltransferases genes responsible for individual steps have recently been isolated, i.e., glucuronolactosyltransferase I, GlcA/GlcNAc transferases to elongate the heparan sulfate chain, and a GalNAc/GalNAc transferase to initiate this process.

In the present study, we isolated cDNA clones encoding human galactosyltransferase I (XGalT-1), which is involved in the biosynthesis of the common carbohydrate-protein linkage structure GlcA\(\beta\)1,3Gal\(\beta\)1,3Gal\(\beta\)1,4Xyl\(\beta\)-O-Ser, based on the BLAST analysis of expressed sequence tags (EST) using the cDNA sequence of the \textit{Caenorhabditis elegans sqv-3} gene. We demonstrate here the substrate specificities of the cloned cDNA product and the restoration of GAG expression on the mutant CHO cells deficient in galactosyltransferase I after the introduction of the cloned cDNA. These results, as well as the structure analyses of the enzyme product, indicated that the cloned gene encodes human galactosyltransferase I.

\textbf{EXPERIMENTAL PROCEDURES}

Materials—UDP-Gal, \(p\)-nitrophenyl-\(\beta\)-xlylopyranoside (\(p\)-Nph-\(\beta\)-Xyl), \(p\)-nitrophenyl-\(\beta\)-galactopyranoside (\(p\)-Nph-\(\beta\)-Gal), \(p\)-nitrophenyl-\(\beta\)-N-acetyl-\(\beta\)-galactosaminide (\(p\)-Nph-\(\beta\)-GalNAc), \(p\)-nitrophenyl-\(\beta\)-N-acetyl-\(\beta\)-glucosaminide (\(p\)-Nph-\(\beta\)-GlcNAc), and \(p\)-nitrophenyl-\(\beta\)-xylopyranoside (\(p\)-Nph-\(\beta\)-Xyl) were purchased from Sigma. \(^32\text{P}\)-labeled with a Megaprime™ DNA labeling system (Amersham Pharmacia Biotech) and used to screen the SK-MEL-57 cDNA library. Approximately, \(4 \times 10^8\) recombinant colonies were obtained by colony hybridization. The nucleotide sequence was determined by the dideoxy termination method using an ABI PRISM™ 310 genetic analyzer (Applied Biosystems). The newly cloned gene was designated \(XGalT-1\) for the reasons described below.

\textbf{CONSTRUCTION OF EXPRESSION VECTORS}—A cDNA fragment encoding the open reading frame of \(XGalT-1\) was prepared by PCR using a \(5'\) primer XGalT-1, galactosyltransferase I (UDP-galactose: \(\beta\)-\(\beta\)-xlyosylprotein 4-\(\beta\)-galactosyltransferase); MES, 4-morpholinolinethane sulfonic acid; E, expressed sequence tag; RACE, rapid amplification of cDNA ends; NCBi, National Center for Biotechnology Information; NOE, nuclear Overhauser effect; FAB-MS, fast atom bombardment mass spectroscopy.

This paper is available on line at http://www.jbc.org
containing an XhoI site, 5′-CTCGAGAGCTGATCCCTGCG-GAGG-3′ (nucleotides 38–58), and a 3′ primer containing a SpeI site, 5′-TGATACGTGCAATGTGCA-3′ (nucleotides 1027–1007), and the cloned cDNA as a template. The PCR product was inserted into the XhoI and SpeI sites of the pMIKneo vector (provided by Dr. K. Nomura, Tokyo Medical and Dental University). The truncated form of XGalT-1, lacking 53 amino acids from the amino terminus, was prepared by PCR using a 5′ primer containing an EcoRI site, 5′-CAGCTCAATTCTGAGGGGATCTGCCGG-3′ (nucleotides 200–217), and a 3′ primer containing an XhoI site, 5′-TGTTACCTGACTGAT-CAGCTGAATGTGCACCA-3′ (nucleotides 1007–1024) and the cloned cDNA as a template. The product was subcloned into the EcoRI and XhoI sites of the pCD-SA vector XGalT-1-proA.

**Cell Culture**—Mouse fibroblast L cells and CHO-K1 cells were grown in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 7.5% FCS at 37 °C in a 5% CO2 atmosphere. CHO mutant pgsB-761 (11) was obtained from the American Type Culture Collection and grown in F-12K medium (Life Technologies, Inc.) supplemented with 10% FCS.

**Preparation of Membrane Fraction**—L cells were transiently transfected with a expression plasmid (4 μg) by the DEAE-dextran method (11). After 48 h of culture, the cells were harvested, and the membrane fraction was prepared as described (12).

**Preparation of Soluble Forms of XGalT-1**—L cells (10-cm dish) were transfected with pCDS-A-XGalT-1 (4 μg) by the DEAE-dextran method, and the XGalT-1 was purified as described (12). To prepare the sample for NMR spectroscopic analysis, the soluble enzyme was further purified using IgG-Sepharose (Amersham Pharmacia Biotech). The beads-enzyme complex was washed with and then resuspended in 100 mM MES buffer, pH 6.0.

**Galactosyltransferase Assay**—The galactosyltransferase activity was determined according to Lugenwa et al. (14) with modifications. The assay mixture containing 1 μl of MeSO4, 15 mM MnCl2, 50 mM KCl, 1% Triton X-100, 100 mM MES buffer, pH 6.0, 6.0 mM UDP-Gal, 5000 dpm/μl UDP-[14C]Gal (NEN Life Science Products), and 1 μg of the enzyme and substrates in a total volume of 25 μl. At 37 °C for 30 min, the reaction mixture was applied onto a Sep-Pak C18 cartridge (Waters), and the product was eluted with 5 ml of methanol.

**β-Galactosidase Digestion**—One μg of [14C]Galβ-1→4Xylβ1-p-Nph formed using XGalT-1-proA was dissolved in 200 μl of solution containing 50 mM Tris-HCl, pH 7.3, 50 mM NaCl, 20 μg of β-galactosidase of Escherichia coli (Roche Molecular Biochemicals) and incubated for 7 h at 37 °C. One μg of the labeled product was also digested with 20 milliunits of diplococcal β-galactosidase (Roche Molecular Biochemicals) for 23 h at 37 °C in a total volume of 100 μl of 50 mM sodium citrate buffer (pH 6.0), containing 100 mM NaCl and 100 μg/ml bovine serum albumin. The digested product was separated on a Sep-Pak C18 cartridge as described above.

**Purification and Identification of the Enzyme Product**—The enzyme reaction was performed in a mixture consisting of 50 μl of the enzyme-bound IgG-Sepharose, 2.7 mg of pgsB-761, 5000 dpm [14C]Gal using XGalT-1-protA and then subjected to treatment with 7.5% FCS at 37 °C in a 5% CO2 atmosphere. CHO mutant pgsB-761 (10) was obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 10% FCS. The complete conversion of the enzyme reaction products, including 1,2,3,4-di-O-galactosyltransferase (EC 2.4.1.133) (26). Similar results were obtained using a soluble fusion enzyme XGalT-1-proA (data not shown).

**Characterization of the Enzyme Product**—To identify the enzyme reaction products, p-Nph-β-d-Xyl was labeled with [14C]Gal using XGalT-1-proA and then subjected to treatment with E. coli β-galactosidase (27). More than 99% of the labeled products were digested (data not shown). Furthermore, the labeled products were also completely cleaved by diplococcal β-galactosidase, which specifically digests terminal β-1,4-galactosyl linkage (data not shown), indicating that XGalT-1-proA catalyzes the galactose transfer from UDP-Gal to the acceptor in β-1,4 linkage.

**Structural Determination of the Enzyme Product**—To characterize the structure of the reaction product, FAB-MS was performed and revealed that the molecular weight of the product is 433 from signals at 434 m/z (M + 1) and 456 m/z (M + Na+), which was attributable to galactosyl products of the
starting Xylb-1-p-Nph. The 1H NMR spectrum showed the p-nitrophenyl moiety and two glycosyl residues. All of the signals of the sugar moieties were assigned by 1D-HOHAHA spectra irradiating at each anomeric signal (δ = 4.49 and δ = 5.25) and COSY (Fig. 2). The structure of the p-nitrophenyl-β-D-xylopyranosyl moiety was confirmed by comparison with the spectrum of the starting molecule. The coupling constant of the galactosyl residue (J1,2 = 7.7 Hz, J2,3 = 9.9 Hz, J3,4 = 3.7 Hz, J4,5 = 0 Hz) indicated that the galactoside is a β-pyranoside. The linkage of the galactoside was determined by NOE difference spectra. By irradiation at Gal H-1, strong NOEs were observed at Xyl H-4, Gal H-3, and Gal H-5, and also, by irradiation at Xyl H-4, Gal H-1 was strongly enhanced. Thus, the structure of the product was identified as Galb1–4Xylb1-p-Nph (28).

Restoration of GAG Synthesis by the Cloned XGalT-1 in a GAG-deficient Mutant Cell—To confirm that this enzyme is involved in the biosynthesis of GAGs in vivo, CHO mutant pgsB-761 (galactosyltransferase I-deficient) cells were transiently transfected with pMIKneo-XGalT-1. As shown in Fig. 3A, about 50% of the transfected cells were stained with mAb HeppSS-1, whereas the mock-transfected cells were negative, indicating that the cloned cDNA actually encodes galactosyltransferase I. This result was also confirmed by 35S-labeled GAGs with CSase or HSase digestion (Fig. 3B).

Expression of the XGalT-1 Gene—Northern blotting with cDNA as a probe revealed that the XGalT-1 gene was expressed in all human tissues examined (including the heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas), and 1.8-kilobase pair transcripts were detected (data not shown).
Molecular Cloning of Human Galactosyltransferase I

DISCUSSION

Newly cloned XGalT-1 was identified as a possible member of the β-galactosyltransferase family in the BLAST analysis of EST using the cDNA sequence of C. elegans sqv-3. sqv-3 was identified as one of the genes possibly encoding the components of a conserved glycosylation pathway and required for vulval invagination (9, 17). Because of the similarity of the amino acid sequence to the cloned mammalian β-galactosyltransferases, sqv-3 seemed to be a β1,4-galactosyltransferase to create galactose β1,4-N-acetylgalcosamine linkage (9). The predicted amino acid sequence of the new gene had a higher homology to sqv-3 than to other mammalian β-galactosyltransferases, suggesting that the gene encodes a β1,4-galactosyltransferase distinct from known β4-galactosyltransferases involved in the synthesis of Galβ1,4GlcNAc (or Glc) structures. As expected, the substrate specificity analysis revealed that the new gene encodes XGalT-1, involved in the biosynthesis of the GAG-protein linkage region of proteoglycans.

XGalT-1 was studied by Rodén’s group (26, 27) and was further characterized in the study of CHO mutants defective in galactosyltransferase I (10) and in the study of clinical cases (29). The substrate specificities and storage stability were similar to the characters of the XGalT-1 enzyme analyzed in this study. The $K_m$ value for p-Nph-β-D-Xyl reported (29) was also in good agreement with our results. All of these data, in addition to the results with the mutant CHO cells and those with FAB-MS and NMR, supported the identity of the new gene as human galactosyltransferase I.

Northern blotting showed a ubiquitous expression pattern, indicating the universal importance of the gene product. Since the activating and inhibitory effects of proteoglycans on cell proliferation have been elucidated (30, 31), important roles of GAGs on proteoglycans have been increasingly recognized. The phenotypes of C. elegans defective of sqv-3 gene strongly suggested the critical roles of its homolog in the morphogenesis and development of mammalian tissues. Thus, the linkage between the genetic study of C. elegans and mammalian glyco-biology research would promote further understanding of the biological significance of GAGs on proteoglycans.

Acknowledgments—We thank Dr. J. D. Esko (University of California at San Diego) for kindly providing CHO mutant pgS-B-761. We also thank Dr. S. Taïju (Riken Research Institute) for providing an expression vector pCDSA for a protein A fusion enzyme.

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